Studies on Fibrinolytic Enzyme from Marine *Serratia Marcescens* Subsp. *Sakuensis* (KU296189.1)

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Abstract

Fibrinolytic enzymes belong to the class of proteases that catalyse the breakdown of fibrin clot. Formation of fibrin clot along with the aggregation of platelets result in a hemostatic plug at the site of injury. These fibrinolytic agents are often administered for the treatment of myocardial infarction, strokes, respiratory failure.

The aim of the current research was to examine the effect of sea water and nitrogen sources on the fibrinolytic enzyme activity. Further, an effort was made to purify the enzyme, which was then assayed for fibrinolytic and fibrinogenolytic activities.

Incorporation of sea water in the medium lead to a marginal increase in the enzyme production. Whereas an increase in the NH_4Cl concentration resulted in an increased production of enzyme. A combination of 12% (w/v) yeast extract and 4% (w/v) NH_4Cl, 1.25% (w/v) soya peptone and 0.075% (w/v) $CaCO_3$ resulted in a 4.1 fold increase in the total enzyme activity of 657 U/mL. These results indicate the remarkably high nitrogen demand of the organism *Serratia marcescens* subsp. *sakuensis*.

Purification of fibrinolytic enzyme was accomplished by passing through a Sephadex G-100 column, which resulted in a specific enzyme activity of 250 U/mg with a 5.1-fold purity and 6.25% recovery. Additionally, the fibrinogenolytic activity of the purified enzyme was found to be 4.34 U/mg. The fibrinolytic to fibrinogenolytic ratio was found to be 35:1. This data connotes the specificity of the enzyme towards the substrates fibrin and fibrinogen, with the former being preferred.

Keywords: Fibrinolytic; Fibrinogenolytic; Marine; Purification.

I. INTRODUCTION

Cardiovascular disease is the major cause of deaths and disability worldwide and is a chief contributor to the implication caused by the non-communicable diseases. A study initiated by the world health organisation (WHO) and the world bank, known as the “Global burden of diseases, injuries and risk factors (GBD) study” in 2010, had reported that coronary artery disease (CAD) and strokes together caused one in four deaths worldwide. The three major cardiovascular diseases such as CAD, ischemic stroke and venous thromboembolism (VTE) have thrombosis in common pathologically [1]. Thrombosis is the formation of a clot within the blood vessel, obstructing the blood flow in the affected area or throughout the circulatory system (if it moves to the brain or lungs). A blood clot is formed primarily by the action of platelets and the fibrin, which aggregate at the site of injury forming a mesh and prevent any further blood loss. However, sometimes when this effect is over productive, it forms an embolus that moves around the blood stream to different parts of the body causing more harm. Thrombosis is broadly classified into venous and arterial thrombosis, depending on its location in the body. Myocardial infarction is caused due to a thrombus formed in the coronary artery and is linked to ischemia. As a result of the blockage there is a reduced supply of oxygen to the heart cells, leading to cell death and thereby a heart attack [2].

Naturally occurring anticoagulants within the body are proteins that regulate the clotting once initiated. Once a stable clot is formed, the fibrinolytic system acts on the clot, thereby removing it and restoring normal vascular architecture. Thrombosis could be mainly due to the increased level of procoagulants, decreased level of anticoagulants or defects in the fibrinolytic system [3]. Fibrinolysis is mainly carried out by the enzyme plasmin (EC 3.4.21.7) that circulates as an inactive proenzyme

plasminogen. This plasminogen then attaches to both fibrinogen and fibrin and gets incorporated in the clot. Components such as the tissue plasminogen activator (tPA) and urokinase help in the converting the inactive plasminogen to active plasmin [4].

The lytic action of the fibrinolytic enzyme on the fibrin clot *in vivo* has led to the development of enzyme based therapy. Fibrinolytic enzymes differ in their mechanism of action by two ways, direct and indirect lysis. Direct lysis consists of plasmin like enzymes that act readily on the fibrin clot (eg. Nattokinase), while indirect lysis comprises of enzymes that convert plasminogen to plasmin, thus leading to clot lysis (eg. Urokinase, Streptokinase) [5]. These suffer from several drawbacks such as allergic responses, short half-life span, lesser fibrin specificity, higher therapeutic dose and increased costs. In addition, there are certain commercially used oral anticoagulants (OACs) namely the heparin, warfarin (Coumadin), dabigatran (Pradaxa) and rivaroxaban (Xarelto). The demerits associated with these are delayed onset of action, certain food and drug interactions and varied pharmacokinetics and pharmacodynamics, which in turn requires routine laboratory monitoring and dosage adjustments [6]. Hence, there is a need for the discovery of newer microbial sources capable of producing of a fibrinolytic enzyme that could be employed for the treatment of thrombosis, with increased efficacy and reduced side effects. The current study is aimed at (i) determining the effect of sea water and various nitrogen sources on the fibrinolytic enzyme activity (ii) to purify the enzyme in order to assess the specificity of the enzyme produced by *Serratia marcescens* subsp. *sakuensis*, towards the substrates, fibrin and fibrinogen.

II. MATERIALS AND METHODS

Fibrin and fibrinogen (human plasma origin), bovine serum albumin and sephadex G-100 were procured from Sigma-Aldrich, India. Folin & Ciocalteu's phenol reagent and trichloroacetic acid were from LOBA Chemie, India. All other chemicals and reagents used were of analytical grade.

A. Effect of sea water on the enzyme production:

Table 1 Effect of sea water on enzyme production

Trials	Galactose	Y.E	NaCl	K_2HPO_4	$MgSO_4$	$FeSO_4$	$CaCO_3$	Sea water
	← (% w/v) →							(% v/v)
Control	2	2	0.05	0.01	0.1	0.01	0.1	-
1	2	2	-	-	-	-	-	1
2	2	2	-	-	-	-	-	5
3	2	2	-	-	-	-	-	10
4	2	2	-	-	-	-	-	15
5	2	2	0.05	0.01	0.1	0.01	0.1	5

As the organism was isolated from sea water, its effect on enzyme production was considered as a factor for the study. Experiments were designed with sea water as a medium component. The enzyme activity was estimated by incorporating different concentrations of sea water in the production medium along with the other components as shown in the table (Table 1). A control was maintained without any sea water. The flasks were incubated for 32 h at 37°C, 150 rpm, following which samples were withdrawn and the fibrinolytic enzyme activity was determined.

B. Fibrinolytic assay

Fibrinolytic enzyme activity was determined by the method described by Agrebi et al. [7] using fibrin as substrate. Substrate solution was prepared by dissolving 10 mg of fibrin in 100 mL of 0.1M glycine-NaOH buffer (pH 9). 1 millilitre of enzyme solution was mixed with equal volume of substrate solution, incubated at 55°C for 15 min. Following which, 1 millilitre of 0.2M TCA solution was added and incubated at room temperature for 15 minutes to stop the reaction. The contents were centrifuged at 10,000 × g for 15 min at 4°C to separate the precipitate. Absorbance of the supernatant was measured at 280 nm against a suitable blank. A standard graph was constructed using varying concentrations of tyrosine (0 - 100 µg/mL) and its absorbance being

measured at 280 nm. One unit of fibrinolytic enzyme activity was expressed as 1µg of tyrosine liberated per min at 55°C.

C. Effect of nitrogen on enzyme production:

In our previous trials, the effect of several organic nitrogen sources on the fibrinolytic enzyme production was studied [Krishnamurthy et al., unpublished work]. Hence, in the present study, the effect of various inorganic nitrogen sources on the fibrinolytic enzyme production would be determined. The medium components comprised of the most significant components as observed in our previous trials [Krishnamurthy et al., unpublished work]. Inorganic nitrogen sources such as NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$ and NH_4NO_3 (0.5% to 2% w/v) were incorporated in the medium along with soya peptone (12.5% w/v) and $CaCO_3$ (0.75% w/v). Flasks were incubated for 32 h at 37°C, 150 rpm, samples were then withdrawn and the fibrinolytic enzyme activity was calculated.

Further, attempt was made to study if a combination of yeast extract (12% w/v and 15% w/v) and NH_4Cl (3% w/v and 4% w/v) can augment the fibrinolytic enzyme production, while maintaining the other components same as that in control. The flasks were later incubated for 32 h at 37°C, 150 rpm. Samples were withdrawn and the fibrinolytic enzyme activity was calculated.

Table 2 Summary of steps involved in purification

Purification Steps	Total Protein (mg)	Total Activity (U/mL)	Specific Activity (U/mg)	Recovery (%)	Purification fold
Crude enzyme	3.25	159.8	49.16	100	1
Sephadex G-100	0.04	10	250	6.25	5.1

D. Enzyme Purification:

The partially purified enzyme sample was passed through a Sephadex G-100 gel filtration column, and was eluted 20 mM sodium phosphate buffer of pH 7, flow rate of approximately 0.5 mL/min. The purified samples were collected in separate fractions, were subjected to both fibrinolytic and fibrinogenolytic assays and the specific enzyme activities were determined. Further, the ratio of fibrinolytic enzyme activity to that of fibrinogenolytic activity was calculated.

E. Fibrinogenolytic assay:

The fibrinogenolytic assay was performed as described by Wang et al. [8] with slight modifications. In brief, substrate solution was prepared by mixing 10 mg of fibrinogen in 50 mL of 0.9% (w/v) NaCl solution. Later, 1 millilitre of the substrate solution was added to 0.5 mL of 245 mM phosphate buffer (pH 7) and incubated at 37°C for 5 min. As our aim was to determine the fibrinogenolytic activity, addition of thrombin was avoided, since thrombin converts fibrinogen to fibrin. 1 millilitre of the enzyme solution was added to the mixture, followed by gentle stirring for 5 min at 30°C. The reaction was stopped by adding 1 millilitre of 0.2M TCA solution to the reaction mixture, was centrifuged at 10,000 × g for 10 min at 4°C and the absorbance of the supernatant was measured at 280 nm. One unit of fibrinogenolytic enzyme activity is expressed as 0.01 per min increase in absorbance at 280 nm at 30°C.

III. RESULTS

Effect of sea water on enzyme production:

It was observed that a maximum of 193.75 U/mL was obtained with the addition of 5% (v/v) sea water in the medium (Table 3).

Effect of inorganic nitrogen source on enzyme productivity:

As shown in figure 2, the addition of NH_4Cl (2% w/v) gave rise to a maximum enzyme activity of 356.38 U/mL, while NH_4NO_3 (2% w/v) resulted in a maximum enzyme activity of 398.33 U/mL.

A combination of yeast extract (12% w/v) and NH_4Cl (4% w/v) in the production medium, gave a maximum enzyme activity of 656.88 U/mL (Table 4).

Enzyme purification:

The purified enzyme showed a specific activity of 250 U/mg with 5.1 fold purity and 6.25% recovery, as shown in Table 2, the chromatogram of the purified enzyme is shown in figure 1. The specific fibrinogenolytic activity of the purified enzyme was found to be 4.34 U/mg

(data not shown) and the ratio of fibrinolytic activity to fibrinogenolytic activity was found to be 35:1.

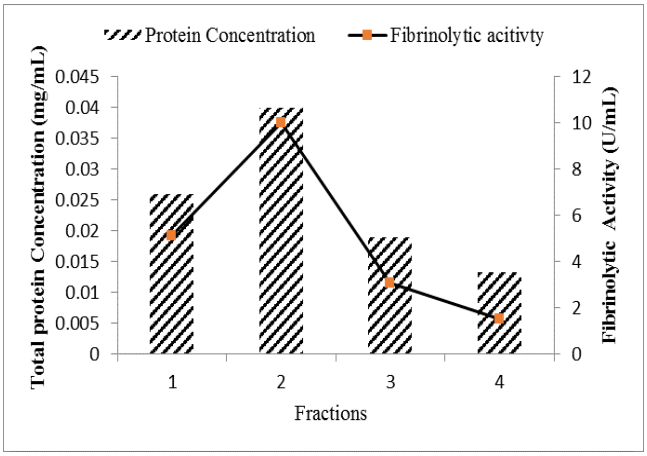

Fig 1. Chromatogram of the fibrinolytic enzyme produced by *Serratia marcescens* subsp. *sakunensis* on Sephadex G-100

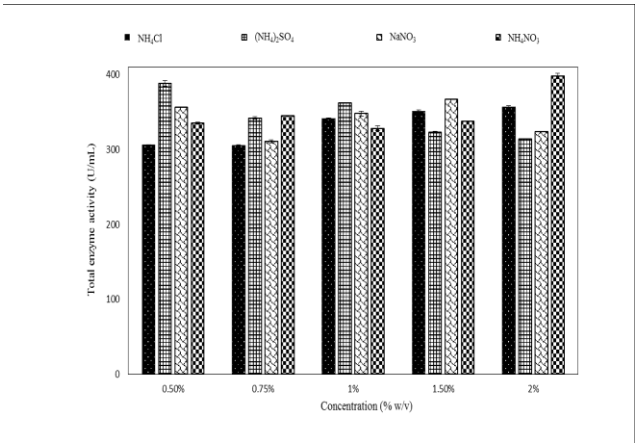

Fig 2. Effect of different inorganic nitrogen sources on total enzyme activity. Bars represent S.D (n=2)

Table 3 Effect of sea water on enzyme production

Trials	Total Enzyme Activity (U/mL)[a]
Control	184.29 ± 1.79
1	193.75 ± 2.21
2	188.32 ± 1.32
3	189.93 ± 2.14
4	182.03 ± 0.56
5	186.51 ± 1.62

[a] average values with S.D (n=2)

Table 4 Total enzyme activity using a combination of yeast extract and NH_4Cl.

Trials	Yeast Extract	Soya Peptone	NH_4Cl	$CaCO_3$	Total Enzyme Activity
←		(% w/v)		→	(U/mL)[a]
Control	9	1.25	2	0.075	492.65 ± 1.58
A	12	1.25	3	0.075	656.55 ± 0.01
B	12	1.25	4	0.075	656.88 ± 0.11
C	15	1.25	3	0.075	648.41 ± 2.03
D	15	1.25	4	0.075	654.86 ± 0.96

[a] average values with S.D (n=2)

IV. DISCUSSION

In view of large scope for finding new fibrinolytic enzymes with higher efficacy, effort was made earlier to isolate, characterize and study marine microorganisms. As a result of which we had isolated a fibrinolytic positive *Serratia marcescens* subsp. *Sakuensis* [Krishnamurthy et al., unpublished work], followed by which attempts were made to improve the fibrinolytic activity and its specificity towards fibrin.

Marginal increase in the enzyme activity was observed in presence of varying concentrations of sea water as a medium component. It was reported that increase in the salt concentration leads to a change in the lipid composition of the cell membrane, thereby decreasing the growth rate, reducing enzyme production [9]. However, Kato et al. [10] had studied the protease production by a marine psychrophilic bacterium using artificial sea water as a medium component. It was observed that both growth and enzyme activity was maximum in the medium containing sea water at the highest concentration.

In industrial microbiology, nitrogen regulation is of utmost significance as it affects the synthesis of enzymes

that are a part of both primary and secondary metabolism. Thus, it is necessary to test the role of various nitrogen sources in the enzyme production. The results obtained from our previous study [Krishnamurthy et al., unpublished work] indicated that the organism has remarkably high nitrogen demand. Hence inorganic nitrogen sources were incorporated in the medium and its effect on the enzyme production was determined by estimating the total enzyme activity. Among the various inorganic nitrogen sources used, a linear increase in enzyme activity was observed with increasing concentration of NH_4Cl and hence was considered for further study. Jayalakshmi et al. [11] had reported a maximum enzyme activity of 1.6 U/mL in the presence of NH_4Cl in comparison to the other inorganic nitrogen sources such as $NaNO_3$, NH_4NO_3 and $(NH_4)_2SO_4$ that were used. However, in our previous study, the addition of yeast extract as a nitrogen source, resulted in a maximum enzyme activity of 487.29 U/mL [Krishnamurthy et al., unpublished work]. Therefore, it can be inferred that, for the production of fibrinolytic enzyme by *Serratia marsescens* subsp. *sakuensis*, organic nitrogen source such as yeast extract is preferred as compared to an inorganic nitrogen source. It is widely known that as we go deeper into the sea, the dissolved or organic nitrogen content keeps increasing and this could account for the preferential use of organic nitrogen by the organism used in our study, since it was isolated from seawater at a depth of 10 m [Krishnamurthy et al., unpublished work]. Combination of yeast extract and NH_4Cl led to a 4.1 fold increase in the fibrinolytic enzyme activity.

The ratio of fibrinolytic activity and fibrinogenolytic activity of the purified enzyme, is an indicator for the relative specificity of the enzyme for the substrate, fibrin and the result obtained in the present study (35:1) suggests that the enzyme is more fibrin specific. Thus, in the present work, a significant increase in the fibrinolytic enzyme activity is achieved with relatively higher specificity for the substrate, fibrin.

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