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Research Article

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Production, purification and Biochemical characterization of alkaline Fibrinolytic enzyme from *Bacillus subtilis*strain-GBRC1

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ABSTRACT

Blood clots are formed from fibrinogen by thrombin and are lysed by plasmin, which is activated from tissue plasminogen activator. It was developed for the treatment of thrombosis because of its efficacy and strong affinity to fibrin. Due to the expense and undesirable side effects such as gastrointestinal bleeding, allergic reaction, and resistance to repercussion. Therefore the search for thrombolytic agents from other source is needed. Production of fibrinolytic enzyme from Bacillus subtilis GBRC1is done by using nutrient broth medium. In addition, a strong fibrinolytic enzyme was purified from the cultivation media. The purified enzyme was almost homogeneous, as examined by SDS−PAGE and sephadex G-75 column chromatography. The enzyme had an optimal pH of 7-12 , an optimal temperature of 50 °C, for fibrin hydrolysis. The molecular mass estimated by gel filtration was 24.6 to 33.0kDa. Phenyl methyl sulphonyl fluoride almost completely inhibited the activity of the enzyme. Futher studies are necessary for the elucidation of their medicinal applications and molecular biological characteristics.

Key words: Plasminogen, SDA-PAGE, Chromatography, enzyme.

INTRODUCTION

Blood clots (fibrin) are formed from fibrinogen by thrombin (EC 3.4.21.5) and are lysed by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator. Although fibrin clot formation and fibrinolysis are maintained in balance by the biological system, thromboses, such as myocardial in fraction, occur when clots are not lysed as a result of a disorder in the balance. Intravenous administration of urokinase and streptokinase was widely used for thrombosis therapy but these enzymes have a low specificity to fibrin. Tissue plasminogen activator was developed for the treatment of thrombosis because of its efficacy and strong affinity to fibrin. However, these enzymes are expensive, and patients may suffer from undesirable side effects such as gastrointestinal bleeding, allergic reaction, and resistance to repercussion. Therefore, the search for thrombolytic agents from other sources is needed.

Over the years, thrombolytic therapies via injecting or orally administrating thrombolytic agents to lyse thrombi in blood vessels have been extensively investigated [4, 5]. Based on their different working mechanisms, the thrombolytic agents are classified into two types. One is plasminogen activators, such as tissue-type plasminogen activator [6, 7] and urokinase [3], which activate plasminogen into active plasmin to degrade fibrin. The other type is plasmin-like proteins,which directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly and completely. Lumbrokinase from earthworm and fibrolase from snake venom are well-known plasmin-like proteins. Oral administration of the fibrinolytic enzyme, nattokinase, can enhance fibrinolytic activity in plasma and the production of tissue type plasminogen activator [1].

Fibrinolytic enzymes have been purified from many plant, animal and microbial sources. Fibrinolytic enzymes were identified and studied among many organisms including snakes, earthworms [8] bacteria: *Streptococcus pyogenes* , [2] *Aeromonashydrophila, Serratia*E15 [9] *Natto* [11] *B. amyloliquefacens, Actinomycetes* [10, 12]

Kim and Choi (2000) also reported a fibrinolytic enzyme (subtilisin DJ-4) secreted by *Bacillus sp*. DJ-4 screened from doen-jang, a traditional Korean fermented food. Chang *et al* (2000) purified a fibrinolytic enzyme from a mutant of *Bacillus subtilis* IMR-NK1, which was isolated from Taiwanese soil.

Fibrinolytic enzyme are one of the largest groups of proteolytic enzymes involved in numerous regulatory processes has the fibrinolytic action. They catalyse the hydrolysis of specific peptide bonds in their substrates and this activity depends on a set of amino acids in the active site of the enzyme. They include both exopeptidases that act on the termini of polypeptide chains and endopeptidases that act in the interior of polypeptide chains and belong to many different protein families that are grouped into clans. A clan is defined as a group of families, the members of which are likely to have a common ancestor. Proteases constitute one of the most important groups of industrial enzymes.

MATERIALS AND METHODS

Bacterial culture collection and maintenance

The bacterial culture *Bacillus subtilis*GBRC1was procured from CAS in Botany University of Madras, Chennai 600 025. The bacterial culture was maintained in Nutrient Agar Medium and also these organisms were maintainedat 4^{o} C in slants as a mother culture.

Growth studies of *Bacillus subtilis* GBRC1

Bacillus subtilis GBRC1was grown in 50ml of nutrient broth at 37 °C in a rotary shaker at 120rpm for 48hrs.The culture was harvested at every 4h interval and its absorbance was read at 540nm using a UV-visible spectrophotometer (Deeepvision 1371, India) and Screening for fibrinolytic enzyme-producing microorganisms were carried out. Optimization of different parameters for fibrinolytic enzymeproduction from *Bacillus subtilis* GBRC1 such as effect of pH, carbon sources and nitrogen sources were done.

Purification of fibrinolytic enzyme from *Bacillus subtilis* GBRC1

Ammonium sulfate precipitation

Solid ammonium sulfate was added to the supernatant containing the fibrinolytic enzyme to make 40% saturation. This mixture stood overnight at 4°C and was centrifuged at 7,000 rpm for 30 min at 4°C to remove particle material. The supernatant was adjusted to 70% ammonium sulfate saturation by further addition of solid ammonium sulfate and stayed overnight at 4°C. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at 4°C for further purification.

Dialysis of fibrinolytic enzyme from cultural filtrate of *Bacillus subtilis* GBRC1

Dialysis was performed with 14KDa dialysis tubing. The protein was resuspended in 50mM sodiumphosphate (pH 7.0) and dialyzed (Memra-cel MD 44-14X100CLR) against the same buffer.

Purification of fibrinolytic enzyme by Sephadex G-75 chromatograph.

The fibrinolytic enzyme protein in fractions was purifiedfurther by chromatography on Sephadex (Sephadex, G-75, SigmaChemical Co.). Dialyzed fraction was passed through a 10 ml Sephadex column (2.5X60) equilibratedwith 10 mM NaC1/5 mM sodium phosphate(pH 7.0) and washed through with an additional20 ml of the same buffer. Contaminatingproteins bound to the column were then removedby elution with 50 mMNaCI/5 mM sodium phosphate (pH 7.0). The fibrinolytic enzymewas theneluted with 200 mM NaC1/5 mM sodium phosphate (pH 7.0). Fractions (3 ml) were collectedand those with an absorbance at 280 nm greaterthan 0.7 were combined and purified further by dialysis against the same buffer.

Analytical methods

Protein estimation

The protein content of the culture filtrate was estimated by the dye binding method of Bradford (1976).

Reagent
Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml of ethanol. To this, 100 ml of 85% (v/v) phosphoric acid was added and made up to one liter. The concentration in the reagent was 0.01 % (w/v), CBB G-250, 4.7 % (v/v), ethanol and 8.5 % (v/v) phosphoric acid.

Procedure
To 1.0 ml of culture filtrate, 5 ml of CBB was added, mixed thoroughly and read at 595 nm in a Beckman DU-50 Spectrophotometer. The reagent with sterile un-inoculated medium served as blank. The amount of protein was calculated using Bovine Serum Albumin Fraction (Sigma Chemicals Co., USA) as the standard.

Assay of Fibrinolytic Enzymes:
Reaction mixtures containing 0.5 ml of 1% of either of casein, haemoglobin or fibrin suspension in 0.1 M tris-HCl (pH 8.0) and 0.5 ml of fungal filtrates were incubated at 37 °C for 30 min. The reaction was terminated by adding 1.0 ml of 0.15 % trichloro acetic acid. Tyrosine was determined in the neat filtrate by measuring the absorbance at 570 nm (Egorov et al., 1982). One fibrinolytic enzyme Unit is that amount of enzyme which liberates 1µmole of tyrosine in one min. under the assay condition

Sodium Dodecyl Sulphate – Polyacrilamide gel (SDS-PAGE)
Sodium Dodecyl Sulphate – Polyacrilamide gel (SDS-PAGE) was performed according to the method of Laemmli (1970) with 10% (w/v) separating and 5% (w/v) stacking gels unless mentioned otherwise. The gel slabs were prepared from stock solutions prepared as detailed below. All stock solutions were prepared in glass distilled water, filtered through Whatman NO.1 filter paper and stored at 4ºCbefore use.

RESULTS

Growth kinetics of *Bacillus subtilis* GBRC1
Bacillus subtilis GBRC1showed a log phase upto 8, 12, and 16 in nutrient broth respectively. The active log phase extended upto 36 h in nutrient broth media.

Agar plate screening for fibrinolytic enzymeactivity
Bacillus subtilis GBRC1, maximum growth and maximum enzyme production was observed at 48 hours. The zone of hydrolysis of *Bacillus subtilis* GBRC1 on milk agar was analysed.

Optimization of different pH**for the production of Fibrinolytic enzymefrom *Bacillus subtilis* GBRC1in liquid state fermentation**
It was evident that the pH significantly influenced the extracellular protein content and fibrinolytic enzyme activity in *Bacillus subtilis* GBRC1. The Bacteria was able to release a maximum protein content of 6.4 mg/ml at pH 7.0 after 36 h and Fibrinolytic enzyme of 1.74 U/ml at pH 7.0 after 36 h.

Optimization of different carbon source for the production of Fibrinolytic enzyme from *Bacillus subtilis* GBRC1in liquid state fermentation
Four different carbon sources such as Glucose, Fructose, maltose, dextrin and sucrose were tested for Fibrinolytic enzymeproduction in *Bacillus subtilis* GBRC1Among the carbon sources; dextrin supported a maximum Fibrinolytic activity of 1.8 U/ml with a highest extracellular protein of 6.5 mg/ml at 36 h.

Production of different nitrogen source for the production of Fibrinolytic enzyme from *Bacillus subtilis* GBRC1 in liquid state fermentation
Five different nitrogen sources such as Sodium Nitrate, Ammonium Nitrate, Ammonium sulphate and Ammonium chloride were tested for extracellular protein and Fibrinolytic enzyme production in *Bacillus subtilis*GBRC1Among them ammonium chloride supported a maximum extracellular protein content of 6.2 mg/ml at 36 h and Fibrinolytic enzymeproduction of 1.6 U/ml at 36 h.

Purification of Fibrinolytic enzyme from *Bacillus subtilis* GBRC1
The cell free extract of *Bacillus subtilis* GBRC1was collected after extraction with sodium phosphate buffer and its proteins were precipitated by salting out with ammonium Sulfate (70%). The crude protein preparation was dialyzed, concentrated by lyophilization and used for further analysis. The flow chart for the purification of

Fibrinolytic enzyme is presented in Fig-5. The fibrinolytic enzymeof *Bacillus subtilis* GBRC1was purified to homogeneity according to the procedure summarized in Table-1.

Table-1. Purification of Fibrinolytic enzymefrom enzyme *Bacillus subtilis* GBRC1

Purification stage	Activity U/ml	Protein mg/ml	Volume Ml	Total activity U	Total protein mg	Specific activity U/ml	Purification fold	Yeild %
Crude sample	1.5	7.2	120	180	864	0.25	1	100
Dialyzed	1	3.6	90	90	325	0.28	1.12	50
Sephadex	0.75	2.1	30	22.5	64.2	0.35	1.4	12.5

Protein concentration was determined by the Bradford method with BSA as the standard.

DISCUSSION

Fibrinolytic enzymes have been isolated and characterizedfrom various organisms, and it is known that snake venomsare the most abundant sources. There were relatively fewer reports on the enzymes from plant. In a survey with the crude extracts, recently found that marine organisms, including a species of marine algae, also had fibrinolytic activity.

The present investigation maximum production of the enzyme by *Bacillus subtilis* GBRC1was at a pH range of 3-8 with an optimum of pH 7.0. Among the different carbon sources used for production of the fibrinolytic enzyme from the *Bacillus subtilis* GBRC1, dextrin was recommended and this strain could utilize sodium citrate, sodium acetate, glycerol–arginine, fructose, mannose and arabinose to produce enzyme in the range of 60-70% of the optimum but utilization of dextrin, rhamnose and lactose could only 40% enzyme.

In this study different nitrogen sources used for production of the fibrinolytic enzyme from the *Bacillus subtilis* GBRC1in which nitrogen sources ammonium chloride best for the fibrinolytic enzyme production.

SUMMARY

- The goal of this study is to produce, purify, and characterize fibrinolytic enzyme from *Bacillus subtilis*-GBRC1.
- Collection of bacterial culture*Bacillussubtilis*-GBRC1was procured from CAS in Department of Botany, University of Madras.
- The enzyme was isolated by centrifugation at every 4hr interval and the supernatant was used for the estimation of fibrinolytic enzyme activity and protein content.
- Isolation of fibrinolytic enzyme from *Bacillus subtilis* GBRC1using Ammonium Sulfate Isolation of fibrinolytic precipitation.
- Purification of fibrinolytic enzyme by sephadex G-75 coloumn chromatography.

CONCLUSION

It could be concluded that the fibirinolytic enzyme fraction 2 with fibrinolytic activity were purified from the *Bacillus subtilis* GBRC1lysates. The molecular weights of each iso-enzyme were from 24.6 to 33.0 kDa. The plasminogen was activated into plasmin by the enzymes. The optimal

temperature of iso-enzymes was 50^{o}C. The optimal pH ranged from pH 7-12. The purified fibirinolytic enzymes were completely inhibited by phenyl metal sulphonyl fluoride. Further studies are necessary for the elucidation of their medicinal applications and molecular biological characteristics.

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