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Evidence that multiple proteases of *Bacillus subtilis* can degrade fibrin and fibrinogen

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Abstract

Fibrinolytic enzyme produced by *Bacillus* spp. are known to possess an unique property to degrade fibrin blood clots. Fibrinolytic enzyme such as nattokinase has commercial applications as a therapeutic agents and functional food formulation. In this study, we report an interesting characteristic feature of *Bacillus subtilis* BR21, a native isolate for its ability to produce multiple proteases that exclusively acts on fibrin and fibrinogen. The crude enzyme preparation was made from the culture grown in soybean powder supplemented Luria Bertani broth. The culture filtrate was found to contain proteases, that could collectively degrade fibrin and fibrinogen effectively. Zymogram indicated presence of six fibrinolytic proteases and these degraded all the four peptide chains of fibrin rapidly. However, only Aα, Bβ chains of fibrinogen were highly susceptible to the enzymes. Activity inhibition by PMSF and EDTA indicated the presence of serine and metallo proteases. Fibrinolytic and fibrinogenolytic activities, specifically the ability to degrade γ-γ′ dimer of fibrin, promises its potential as both therapeutic and prophylactic agents, for thrombosis related disorders as this remains undegraded in such conditions. The fibrinolytic ability of multiple proteases together may help in developing cheaper and effective orally administrable thrombolytic preparations.

Introduction

Among the Cardio Vascular Diseases (CVDs), thrombosis is one of the leading cause of deaths worldwide. Clinically, thrombosis is a condition where undesirable blood clot occurs due to the formation of insoluble fibrin. Under the normal physiological conditions, plasmin or fibrinolysin degrades the fibrin blood clots in blood vessels. However, clots are not lysed under conditions of abnormal homeostasis. This leads to the condition called thrombosis (Previtali *et al*., 2011). The steep increase in the percentage of CVD cases and thrombosis related deaths all over the world has attracted researchers to search for effective thrombolytic agents.

The commonly used thrombolytic drugs like urokinase and tissue-type plasminogen activator (t-PA) activates plasminogen and converts it into plasmin which degrades fibrin. However, these thrombolytic agents are expensive and with shorter half-life. Besides, intravenous administration of these as drugs are known to cause haemorrhage (Blann *et al*., 2002; Turpie *et al.*, 2002). Heparin, aspirin and other antiplatelet agents which are routinely used as prophylactic drugs cause increased bleeding complications for the patient (Fitzmaurice *et al*., 2002). Other thrombolytic agents described are from earthworms (Wang *et al*., 2005), snake venoms (Jia *et al*., 2003), centipede venoms (You *et al.*, 2004), insects and leeches (Chudzinski-Tavassi *et al.*, 1998) etc. Microbial fibrinolytic enzymes that interfere with key factors of the cascade in the formation of undesirable fibrin clots have also been explored in this report (Silverthorn *et al*., 1998).

Bacillus subtilis natto known to produce fibrinolytic enzymes in a medium containing soybean. This blood clot dissolving enzyme known as "Nattokinase" possess fibrinolytic activity (Sumi *et al.*, 1987). Similarly several legume based fermented products are reported for fibrinolytic enzymes. Most of these products have a common substrate soya bean (Mine *et al*., 2005). However, limited information is available on legume based products from India for the isolation and characterization of fibrinolytic enzyme producing *Bacillus* spp. Studies on isolation and characterization of fibrinolytic enzymes from different *Bacillus* spp. followed by using the enzymes for possible use the management of heart diseases are described (Fujita *et al*., 1993; Kim *et al.*, 1996; Peng *et al*., 2003; Seo *et al*., 2004). However, their mechanism of action on fibrin and fibrinogen substrate has not been described. In this study, a *Bacillus subtilis* isolated from an Indian traditional food sample "Dosa batter" has found to produce

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protease with fibrinolytic and fibrinogenolytic activity is reported. The unique activity of the enzymes to degrade γ-γ′ dimer of fibrin indicated its potential for pharmaceutical applications and the details are described in this paper.

Materials and Methods

Bacteriological media and chemicals

Nutrient agar and Luria Bertini broth (LB), Bovine Serum Albumin (BSA) were obtained from Hi-Media laboratories, Mumbai (India). Soy bean powder was purchased from local market, Mysore. Bovine fibrinogen, human plasmin, thrombin from bovine plasma, agarose, Trizma base, and Primers for 16S ribosomal RNA gene and ytc P gene were from Sigma-Aldrich (USA). Laboratory reagents EDTA, CaCl2, NaCl, Triton X-100 and Coomassie Brilliant Blue R-250 used were from SRL Chemicals, Mumbai, (India). Protein molecular weight and prestained protein markers used for SDS-PAGE were from procured from Genei (India) and Fermentas (USA) respectively.

Bacterial cultures and growth conditions

Bacillus isolates in the study were grown in Luria Bertani (LB) medium at 37°C for 24 h under constant shaking condition (100 rpm).

Identification of fibrinolytic enzymes in bacterial culture broth

Bacillus cultures isolated from fermented cereals and legumes were screened for fibrinolytic enzyme production by fibrin plate assay (Wang *et al*., 2006). The fibrin plates were heated at 80°C for 30 min to inactivate inherent fibrinolytic factors. Crude protein of the bacterial cultures grown in LB broth supplemented with soya bean powder (1%) for 72h at 37°C were spotted on the fibrin plates and incubated at 37°C for 12-18 h. The plates were stained with Coomassie Brilliant Blue (CBB) and observed for the zones of clearance. Human Plasmin (0.35U) was used as positive control.

Zymogram assay for fibrinolytic activity

The fibrinolytic activity of the cell free supernatant, grown in LB broth supplemented with 1% soya powder for 72 h, was assessed by SDS-PAGE under non reducing conditions. Culture supernatant mixed with non-reducing 2X zymogram loading buffer (1: 5 v/v) were loaded to10% acrylamide gels containing 0.12% fibrinogen and 10 U of thrombin (Kim *et al*., 1998) were run at 12 mA current at 4°C. The electrophoresed gel was treated with Tris-HCl buffer (pH 7.4) containing 2.5% triton X-100 for 40 min under constant shaking. The gel was washed thrice with distilled water and incubated in zymogram reaction buffer containing 30 mM Tris-HCl (pH7.4) supplemented with 200 mM NaCl, 10 mM $CaCl_2$ for 12-18 h. After destaining the CBB R250 (0.25 g) stained gels, zones of clearance were observed for the activity.

Activity assay by substrate overlay

The culture broth proteins then precipitated at 55-80% ammonium sulphate saturation were dissolved in 20 mM Tris-HCl buffer with pH 7.4 and dialyzed against excess of same buffer to remove ammonium sulphate. The crude protein was electrophoresed in 10% SDS-PAGE gel (Laemmli, 1970) under non reducing conditions as described above. The gel was treated with 2.5% Triton-X 100, washed with distilled water and subsequently over laid with fibrin substrate (0.07%) incorporated in molten agarose in a sterile petri plate and incubated at 37°C for the observance of clear zones. Pre-stained molecular weight marker was run along with the sample.

Assay for fibrinolytic and fibrinogenolytic activity

Fibrin as prepared from plasma clots was subjected to degradation at 37°C using 20µg crude protein (Rajesh *et al*., 2010). After every hour incubation, the reaction was terminated by adding reducing buffer [glycerol (200 µL), sucrose (100 mg), SDS (40 mg), urea (60 mg) and β-mercapto ethanol (60 µL)]. The mixture was boiled for 10 min, centrifuged and 40 µg protein of the supernatant was loaded to SDS-PAGE as described above. The destained gels were observed for degraded products. The banding pattern was compared with that of fibrin (control) electrophoresed, the test sample alongside. Fibrinogenolytic assay was performed with commercial fibrinogen (3 mg/ml) as a substrate.

Identification of fibrinolytic enzyme producing Bacillus *isolates*

Bacillus cultures showing fibrinolytic enzymes was identified by biochemical tests according to Bergeys's manual of Systematic Bacteriology (Holt *et al*.,1994) and for molecular characterization, total DNA was isolated (Mora *et al*., 2000). Identification of the potent fibrinolytic *Bacillus* strain BR21 at species level was carried out by amplification and sequencing of partial 16S rRNA gene. The primers F-5'GAGTTTGATCCTGGCTCAGG3' and R-5'TCATCTGTCCCACCTTCGGC3' were used for 16S rRNA gene amplification (Nithya and Halami, 2013). The gene sequencing was performed at

Xcelris sequencing facility, Ahmedabad, India. DNA sequences thus obtained were subjected to BLAST analysis (Altschul *et al*., 1997), the sequence obtained was deposited in GenBank (Accession no. KC201195). Further confirmation of bacterial culture was carried out using species-specific primer for *Bacillus subtilis* F-5'-GCTTACGGGTTATCCCGC-3' and R- 5'-CCGACCCCATTTCAGACATAT C-3' (Kwon *et al.*, 2009). The strain BR21 has been deposited in CFTRI culture collection centre.

Effect of inhibitors on enzyme activity

The crude enzyme preparation of *Bacillus subtilis* BR21 were assayed for proteolytic activity according to the protocol described by Ambrose *et al.* (1998) using Azocasein (5 mg/ml) as substrate (Hummel *et al.*, 1965). One unit of enzyme activity was defined as increase in 0.01 OD as compared to control. The protein content of cell free supernatant and partially purified enzyme preparation of BR21 was measured by Bradford method (Bradford,1976) using BSA as a standard. The residual activity of the partially purified protein was determined by preincubation with different inhibitors. The assay was carried out as described above.

Results

Zymogram analysis for fibrinolytic activities

The extracellular proteases of strain BR21, which was selected based on larger clear zone on fibrin plate (Figure 1a) and identified as *Bacillus subtilis* was found to hydrolyze fibrin as assessed by zymogram assay producing clear zones. About six prominent clear zones in fibrin zymogram gels revealed that the organism secreted multiple proteases that could degrade fibrin. Of all the enzymes, one of the protease showed very prominent activity as seen from the intensity of clear zone (Figure 1b).

Substrate overlay assay

In order to obtain the approximate molecular mass of the active proteases, electrophoretically separated crude enzyme preparation was overlaid with agarose (1.5%) gel containing 0.07% fibrin. These experiments revealed that the fibrin degrading proteases corresponding to molecular mass 14, 21, 35, and 46 kDa (Figure 2).

Fibrin and fibrinogen degradation by the bacterial proteases

Fibrin is made of four protein chains, usually referred as α polymer, γ-γ′ dimer, α- chain, and β-chain. Electrophoretic analysis revealed that the

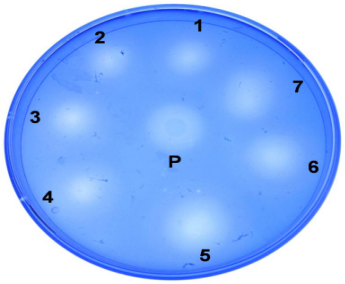

Figure 1a. Plate assay for the detection of fibrinolytic enzyme producing bacilli

Shake flask grown culture filtrate (10 µl) were spotted on agar plate containing 0.07% fibrin. The plates after incubation for 18 h at 37°C were stained with Coomassie Brilliant Blue R250. Fibrinolytic activity were seen as clear zones. Numbers 1 to 7 represents culture filtrates of various bacilli. Positive control used was plasmin (P)

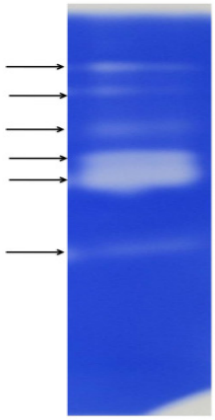

Figure 1b. Fibrin Zymogram

Zymogram pattern of isolate BR21, the arrows indicates the position of enzyme activity

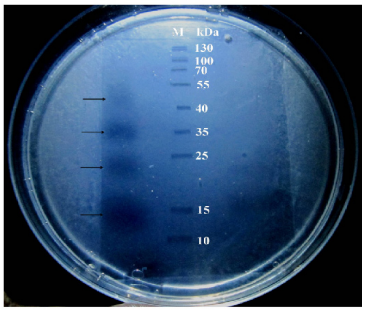

Figure 2. Activity assay fibrinolytic enzymes of *Bacillus subtilis* BR21

Ammonium sulphate precipitated protein (55-70%) from culture supernatant of *B. subtilis* BR21 was run at 4°C in 10% SDS-PAGE under non reducing condition. SDS was removed using 2.5% Triton X-100 and gel was overlaid with fibrin substrate and incubated at 37°C for 12 h, and observed for clear zones. Prestained marker (M) was run along with the enzyme sample. The arrows indicates the position of enzyme activity

α-polymer and α- chain were degraded rapidly by the bacterial proteases as evident by the accumulation of low molecular mass proteins. The β-chain of the fibrin apparently required longer time (two hours) for degradation. The γ-γ′ dimer was resistant to degradation. However, the enzymes could degrade this chain of fibrin when incubation increased to three

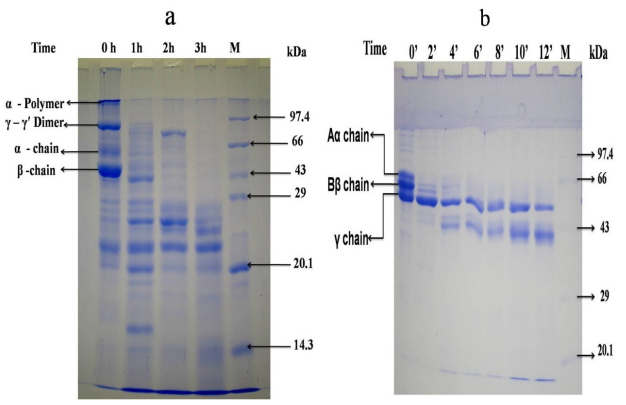

Figure 3a. Fibrinolytic 3b. Fibrinogenolytic activity of multiple proteases of *Bacillus subtilis* BR21.
Each lane indicates enzyme treated sample at different intervals of time a- in hours and b- in minutes. M- Protein Marker

hours (Figure 3a). Despite this activity, degradation of γ-chain of fibrinogen was not found (Figure 3b). However, Aα-chain and Bβ–chain of fibrinogen were rapidly degraded by the bacterial proteases requiring less than eight minutes for degradation. γ -chain degradation was not significant even after an hour of incubation. The activity of the enzymes reduced significantly in presence of PMSF and EDTA compared to other inhibitors (Table 1).

Table 1. Effect of inhibitors on enzyme activity

Inhibitor	Concentration (mM)	Residual activity (%)
Control	0	100
Iodo acetic acid	10	96.42
PMSF	5	42.85
DMSO	10	98.14
EDTA	5	67.85
DTT	10	75

PMSF- Phenyl Methyl Sulfonyl Fluoride, DMSO- Dimethyl sulfoxide, EDTA- Ethylene diammine tetra acetic Acid, DTT -Dithiothreitol

Discussion

Fibrinogen and fibrin are the first and last prime key molecules in the cascade which results in the formation of blood clot. Fibrin is an insoluble protein that gets deposited around the wound forming a mesh. During clotting it dries and hardens. Though important it becomes necessary to avoid clot formation, to manage diseases like thrombosis and arthrosclerosis. Plasmin mediated fibrin degradation occurs naturally in the human body, under such conditions the γ-γ′ dimer remains undegraded (Francis *et al*.,1984) and also results in fibrinogen accumulation (Lassila *et al*., 1993), which is a risk factor for atherosclerosis (Smith *et al*., 1986).

Fibrinolytic activity and plasminogen concentration decreases in cardiovascular diseases (Smith *et al*., 1986). Thus there is a need to identify enzymes that not only act on fibrin but should show the activity on the γ-γ′ dimer and also degrade fibrinogen to prevent atherosclerosis and thrombosis.

The proteases of *Bacillus subtilis* BR21 showed all the above activities, in that it degraded the γ-γ′ dimer of fibrin and also fibrinogen. Thus the proteases characterized in the study appeared to have pharmaceutical applications. *Bacillus subtilis* is classified and considered under GRAS (Generally Regarded As Safe) status organism (Nijland and Kuipers, 2008) and BR21 which is also a *B. subtilis* isolated from food source. The enzyme produced by the culture in food grade medium can be exploited as an enriched fibrinolytic enzyme for application of enzymes in food systems as food supplements.

Most of the *B. subtilis* cultures produce one to three proteases and Production of multiple proteases by a single strain is a rare property (Thuy and Bose, 2011). Zymogram indicated presence of six proteases and interestingly, all were found to be with fibrinolytic activity. However, substrate overlay assay indicated only four clear zones which may be due to potent activity of four enzymes or due to overlapping of clear zones by individual fibrinolytic enzymes. The molecular weights of fibrinolytic enzymes observed based on position of clear zones in gel overlay assay were different from previously reported fibrinolytic enzymes (Peng *et al*., 2005). Reduction in the activity in presence of PMSF and EDTA indicates the serine and metallo-protease nature of enzymes.

Conclusion

This study indicates the huge potential of isolate BR21 to produce variety of fibrinolytic enzymes which need to be further characterized. Combination of multiple proteases with fibrinolytic activity may be effective as orally administrable thrombolytic agents. Further studies on in vivo fibrinolytic activity of *B. subtilis* BR21 enzymes may be helpful in development of orally administrable thrombolytic agent.

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