ABSTRACT

Cardiovascular diseases like ischemic heart disease, acute myocardial infarction, and high blood pressure are the leading causes of mortality and morbidity worldwide [1]. One of the significant factors contributing to the etiology of cardiovascular diseases is the imbalance between fibrin formation and fibrinolysis (haemostatic disorder) that results in intravascular thrombus formation in blood vessels (thrombosis) posing a challenge in its clinical treatment [2]. Furthermore, higher levels of fibrinogen in plasma (hyperfibrinogemia) also stimulate the risk of myocardial infarction by enhancing the blood coagulation and encouraging cardiovascular events [3]. Besides being expensive, currently available thrombolytic drugs, such as streptokinase, urokinase, and tissue plasminogen activator (t-PA) are reported to cause undesirable side effects, such as gastrointestinal bleeding and allergic reactions [4]. To address these issues, several lines of investigation have been pursued to discover superior, cost effective, and safe thrombolytic agents from different natural resources. Based on the above requirements, the objective of the present study was isolation of fibrin(ogen)olytic enzyme(s) producing bacteria through screening of fermented food samples from North-Eastern (NE) India, and taxonomic identification of potential strains. Furthermore, emphasis was placed on purification and characterization of bacterial fibrin(ogen)olytic enzyme(s) produced by isolated potential bacterial strains, and their comparison with commercially available fibrinolytic enzymes. Toxicity as well as thrombolytic activities of these purified fibrin(ogen)olytic enzyme(s) were also investigated both in *in vitro* and *in vivo* conditions.

The thesis is divided into six chapters as outlined below.

Chapter I is the introduction, which describes the background of the investigation and the drawbacks of existing thrombolytic drugs. In this chapter, the previous outcomes of investigations on thrombolytic drug development, including their limitations, and the purpose of the present investigation have also been highlighted.

Chapter II is the review of literature. This chapter deals with a literature survey on fibrin(ogen)olytic enzyme(s) purified and characterized from various natural resources, more particularly bacterial fibrin(ogen)olytic enzyme(s). The available

data on their *in vivo* toxicity and *in vivo* thrombolytic potentials of isolated enzymes have also been discussed.

Chapter III deals with materials and methods and experimental designing related to this study.

Chapter IV describes the results and discussion related to the screening, isolation, and identification of fibrin(ogen)olytic enzyme producing bacterium *Bacillus cereus* strain FF01 isolated from fermented food samples of NE India. This chapter also includes purification, characterization, as well as, assessment of toxicity and *in vivo* thrombolytic activity of an extracellular fibrin(ogen)olytic enzyme (Bacethrombase) purified from *Bacillus cereus* strain FF01.

Chapter V describes the results and discussion related to screening, isolation, and identification of fibrinolytic enzyme producing bacterium *Brevibacillus brevis* strain FF02B isolated from fermented food samples from NE India. Moreover, biochemical and pharmacological characterization and *in vivo* toxicity assay, as well as *in vivo* thrombolytic activity of an extracellular fibrinolytic enzyme (Brevithrombolase) purified from *Brevibacillus brevis* strain FF02B have also been described in this chapter.

Chapter VI presents the overall conclusion of the present work and highlights the future scope of the investigation.

Ten fibrin(ogen)olytic protease(s) producing bacterial strains were isolated by screening of fermented food samples collected from various parts of NE India; out of these ten strains, six strains showed zone of hydrolysis that was ≥ 15.0 mm in fibrin agar plate containing fluconazole (antifungal agent). These bacterial strains were considered as potent bacterial strains for the study and were further subcultured in M9 media. The cell-free culture supernatant was used for subsequent analyses, such as determination of fibrinolytic/caseinolytic (F/C) ratio, substrate specificity and thrombolytic activity. The promising bacterial strains FF01 and FF02B were selected by polyphasic approach. Standard microbiological techniques were obtained. Based on morphological features, biochemical tests and ribotyping data, strains FF01 and FF02B were identified as *Bacillus cereus* strain FF01 and *Brevibacillus brevis* strain FF02B, respectively.

An extracellular fibrin(ogen)olytic protease(s) was isolated and purified from *Bacillus cereus* strain FF01 using multidimensional chromatographic techniques. The biochemical and pharmacological properties of purified protease enzyme were characterized and their *in vivo* toxicity and *in vivo* thrombolytic activities evaluated. Two proteases, namely Bacethrombase [fibrin(ogen)olytic protease] and Brevithrombolase [fibrinolytic protease], were purified from *Bacillus cereus* strain FF01 and *Brevibacillus brevis* strain FF02B. The molecular mass of Bacethrombase and Brevithrombolase was determined by SDS-PAGE and MALDI-TOF-MS analyses and was found to be 39.5 kDa and 38.5 kDa, 55 kDa and 56 kDa, respectively. The N-terminal sequence of Bacethrombase was found to be blocked; however, peptide mass fingerprinting and amino acid composition analyses of Bacethrombase and Brevithrombolase demonstrated their similarity to serine proteases, particularly to subtilisin family of proteases and with serine endopeptidase. The secondary structure of Bacethrombase comprised 14.0% helix, 6.6% beta sheet, and 79.4% random coil, whereas, Brevithrombolase had 30.6% alpha helix and 69.4% random coil. Inhibition study demonstrated that both Bacethrombase and Brevithrombolase were inhibited by PMSF, pBPP and IAA suggesting that Ser-His-Cys residues may function as a catalytic triad during enzymatic reaction. Bacethrombase showed optimum activity at pH 8 and 40 °C; whereas, Brevithrombolase showed optimum activity at pH 7.4 and 37 °C. Fibrinogen followed by fibrin were found to be the most preferred physiological substrates for Bacethrombase suggesting it to be a fibrin(ogen)olytic serine protease. Conversely, Brevithrombolase showed marginal hydrolytic activity towards globulin, casein and fibrinogen, but exhibited highest substrate specificity towards fibrin, suggesting it is a fibrinolytic serine protease. Preferential degradation of α-chain followed by β-chain of fibrinogen as well as fibrin by Bacethrombase suggested it is an α,β-fibrinogenase. Brevithrombolase also showed the above pattern of fibrin-degradation indicating that it belongs to the group of α,β-fibrinogenase. The *Km* and *Vmax* values for the hydrolysis of D-Val-Leu-Lys-p-nitroanilide dihydrochloride (most preferred chromogenic substrate for plasmin) by Bacethrombase at pH 7.4, 37 °C were determined at 0.30 μM and 14 μmol pNA/min, respectively. For Brevithrombolase, *Km* and *Vmax* values towards D-Val-Leu-Lys-p-nitroanilide dihydrochloride was noted at 0.39 μM and 14.3 μmol pNA /min, respectively. By RP-HPLC and SDS-PAGE analyses, Bacethrombase and

plasmin demonstrated almost similar degradation pattern of fibrin and fibrinogen molecules. In a similar way, Brevithrombolase and plasmin also showed identical cleavage pattern of fibrin. Therefore, these two proteases, namely Bacethrombase and Brevithrombolase were considered to be direct acting enzymes, resembling plasmin.

Both Bacethrombase and Brevithrombolase did not degrade Factor Xa to exert anticoagulant activity. Strong anticoagulant activity associated with Bacethrombase correlated to its defibrinogenolytic activity; whereas, Brevithrombolase exercised its anticoagulant action by enzymatic cleavage of thrombin. Nevertheless, anticoagulant activities of Bacethrombase and Brevithrombolase were comparable to the commercial anticoagulants, warfarin and heparin. However, the *in vitro* thrombolytic efficiency of Bacethrombase and Brevithrombolase was found to be superior, as compared to plasmin and streptokinase. Bacethrombase and Brevithrombolase both displayed antiplatelet activity against goat platelet rich plasma (PRP). However, only Bacethrombase showed dose-dependent inhibition of ADP-induced aggregation of PRP with an IC_{50} value of 10.0 nM.

These purified proteases at a dose of 15 μg/ml did not show any hemolytic activity or *in vitro* cell cytotoxicity against HT 29 and HeLa cancer cells, suggesting non-cytotoxic nature of these proteases. Bacethrombase or Brevithrombolase at a dose of 10 mg/kg , did not exhibit lethality toxicity or adverse pharmacological effects on Wistar strain albino rats. The serum biochemical properties and hematological parameters of treated rats were the same ($p > 0.05$) as that of the control group of rats.

The *in vivo* thrombolysis efficiency of Bacethrombase and Brevithrombolase was affirmed by significant inhibition of thrombus formation in carrageenan-induced rat tail, in a dose-dependent manner. At a dose of 600 μg/kg, both the tested proteases showed an efficacy comparable to streptokinase and plasmin, in dissolving thrombus *in vivo*. The *in vivo* and *in vitro* thrombolytic strength of these two enzymes (Bacethrombase and Brevithrombolase) was found to be identical. The *in vivo* anticoagulant property of Bacethrombase and Brevithrombolase was demonstrated by prolongation of activated partial thromboplastin time (APTT) and prothrombin time (PT) in treated rats. Moreover, the effect of Bacethrombase infusion

significantly decreased the fibrinogen level in rat plasma in a dose-dependent manner. The thrombin time (TT) of Bacethrombase treated rats was moderately increased, clearly indicating that the anticoagulant potential of Bacethrombase is associated with its fibrinogenolytic property, rather than inhibition of thrombin or Factor Xa. At least, the *in vivo* anticoagulant potency of Brevithrombolase was found to be its proteolytic action on thrombin, which resulted in an enhanced TT and an insignificant decrease in fibrinogen level of treated rats. These altogether indicate that the future therapeutic application of Bacethrombase and Brevithrombolase to combat thrombosis and hyperfibrogenemia relevant disorders is highly promising.