CHAPTER II

Review of literature

CHAPTER 2
REVIEW OF LITERATURE

2.1 Sources of fibrin(ogen)olytic enzyme(s)

Fibrinolytic enzymes are widely found in nature. Among the variety of fibrinolytic enzyme sources, microorganism and snake venom are considered as natural sources of fibrinolytic enzymes. Besides, a variety of resources such as earthworm [42], algae [43], polychaete [44, 45]and insects [46, 47] have been screen for fibrino(geno)lytic enzymes. Among the sources, microorganisms have been found as most attractive as well as dominant resource for fibrinolytic agents due to its diversity and suitability for industrial production. In recent years, many fibrinolytic enzyme producing microbes have been extracted from both food and non-food sources [6, 20].

2.1.1 Fibrinolytic enzymes producing food borne microbes

The genus *Bacillus* isolated from traditional fermented foods is an important group of microorganisms that has been found as a prominent candidate for fibrinolytic protease producer. For example, nattokinase (NK) produced by *Bacillus natto* was the first screened fibrinolytic enzyme from a traditional Japanese soybean-fermented food named natto [16]. Subsequently, other species of *Bacilli* found in different fermented foods have been characterized and identified as fibrinolytic enzyme producers [20]. Moreover, traditional fermented foods consumed all over the world have been considered as excellent source of fibrinolytic enzyme producing microbes, which are generally regarded as safe (GRAS) category [20]. Different species of fibrinolytic enzyme, producing microbes isolated from traditional foods are summarized in Table 2.1.

Table 2.1: Fibrinolytic enzyme producing microbes isolated from traditional foods

Microorganism	Fibrinolytic enzyme	Food	Reference
Bacillus natto	Nattokinase	Natto (fermented soybean), Japan	Sumi et al. [16]
Bacillus sp.	SMCE	Tofuyo (coagulating fermented soy juice), Japan	Fujita et al. [48]
Pleurotus ostreatus	Metalloprotease	Local culture collection	Choi and Shin [49]
Bacillus sp.	*Bacillus* protease	Kimchi (fermented vegetables with seasonings), Korea	Noh et al. [50]
Bacillus subtilis IMR-NK1	Fibrinolytic enzyme	Natto	Chang et al. [51]
Bacillus sp. *DJ-4*	Subtilisin DJ-4	Doen-jang (fermented soybean), Korea	Kim and Choi [52]
Bacillus amyloliquefaciens DC-4	Subtilisin DFE	Douchi (fermented soybean), China	Peng et al. [53]
Bacillus subtilis QK02	QK-1 and QK-2	Fermented soybean	Ko et al. [54]
Bacillus firmus NA-1	N.A.	Natto	Seo and Lee [55]
Bacillus sp. DJ-2	Subtilisin DJ-2	Doen-jang	Choi et al. [56]
Bacillus subtilis TP-6	TPase	Tempeh (fermented soybean), Indonesia	Kim et al. [57]
Bacillus subtilis DC33	Subtilisin FS33	Ba-bao Douchi	Wang et al. [17]
Bacillus vallismortis Ace02	Ace-02	Chungkook-jang	Kim et al. 2007
Flammulina velutipes	FVP-1b	Local culture collection	Park et al. [58]
Bacillus subtilis LD-8547	LD-8547	Douchi	Wang et al. [59]
Schizophyllum commune	Cultured mycelia of mushroom	Bioresource Collection and Research Center (Taiwan)	Lu et al. [60]

2.1.2 Fibrinolytic enzyme producing non-food borne microbes

Some of the fibrinolytic enzyme producing microorganisms that have been isolated and characterized from non-food origin, including fungi, such as actinomycetes, and bacteria, have been presented in Table 2.2.

Literature survey shows that several fibrinolytic enzyme producing microbes have also been isolated from food and non-food sources of India; however, to the best of our knowledge there is a dearth of report on isolation of fibrinolytic protease producing microbes from traditional fermented foods of North-East India. This has encouraged us to explore the industrially important microbes from fermented food samples / alcohol producing starter cultures from North-Eastern region of India, which is considered as one of the major biodiversity zones of the world [61].

Table 2.2: Fibrinolytic enzymes produced by non-food borne microbes

Microorganism	Enzyme	Source	Reference
Bacteria			
Bacillus subtilis BK-17	BK	Decaying rice plant, Korea	Jeong et al. [62]
Bacillus subtilis A1	BKII	Local soil, Korea	Jeong et al. [63]
Bacillus subtilis QK02	QK-1 and QK-2	Rice leaves	Ko et al. [64]
Bacillus subtilis 168	Vpr and WprA	Culture collection	Kho et al. [65]
Bacillus subtilis A26	Subtilisin BSF1	Marine water in Sfax city (Tunisia)	Agrebi et al. [62]
Bacillus subtilis KCTC 3014	Vpr	Korean collection for type culture	Choi et al. [66]
Bacillus natto NRRL 3666	NA	NRRL culture collection, USA	Mahajan et al. [67]
Paenibacillus polymyxa EJS-3	NA	Root tissue of *Stemona japonica* (Blume)	Lu et al. [68]
Bacillus subtilis K42	Co^{2+} metallo-protease K42	Soybean flour	Hassanein et al. [69]
Staphylococcus sp. *AJ*	Thermoacid-stable AJ	Korean salt-fermented Anchovy-joet	Choi et al. [70]
Actinomycetes			
Actinomyces thermovulgaris T-54	T-54	Culture collection	Egorov et al. [71]
Streptomyces spheroids M8-2	M8-2	Culture collection	Egorov et al. [72]
Streptomyces megasporus SD5	NA	Hot spring water	Chitte and Dey [73]
Streptomyces sp. *CS684*	FP84	Culture collection	Simkhada et al. [74]
Streptomyces sp. *CS624*	Chymotrypsin-like FES624	Soil of Cheonnam province, Korea	Mander et al. [40]

Microorganism	Enzyme	Source	Reference
Fungi			
Penicillium chrysogenum H9	NA	Local soil, Egypt	El-Aassar et al. [75]
Fusarium pallidoroseum	NA	Local soil, Egypt	Samy [76]
Pleurotus ostreatus	Zn2+ metalloprotease	Culture collection, Seoul	Choi and Shin [49]
Aspergillus ochraceus 513	NA	Culture collection	Batomunkueva and Egorov [77]
Rhizopus chinensis 12	NA	Starter for brewing rice wine, China	Xiao-Lan et al. [78]
Fusarium sp. *BLB*	Fusarium protease	Plant leaf (Hibiscus)	Ueda et al.[79]
Fusarium sp. *CPCC 480097*	Chymotrypsin-like Fu-P	Chrysanthemum stem	Wu et al. [80]
Algae			
Codium latum	CIP	Hiroshima marine water, Japan	Matsubara et al.[81]
Codium divaricatum	CDP	The coast of Hiroshima Prefecture, Japan	Matsubara et al. [82]

2.2 Identification of fibrin(ogen)olytic enzyme producing microbes by polyphasic approach

The precise identification of fibrin(ogen)olytic enzyme (s) producing microbes by morphological, physiological and biochemical approaches is one of the important keystones. Classification of microbes through morphological, physiological, and biochemical methods is still largely unexplored; hence, its taxonomic status remains incomplete. Yet, no identification method can clarify the exact picture of bacterial identity, thus requiring a polyphasic approach to identifying bacteria. In the past few years, the overall molecular approach has been adopted for microbial identification. The methods gain its popularity because of their robustness, accuracy, promptness for identifying and classifying microbe's taxonomic position among the closely related genera and species. Table 2.3 shows the methods utilized by various researchers for identification of fibrinolytic enzyme producing bacteria.

Table 2.3: Methods for identification of fibrinolytic enzymes producing bacteria

Organism	Method of identification	Reference
Virgibacillus halodenitrificans SK1-3-7	16S rDNA	Montriwong et al, [83]
Bacillus subtilis ICTF-1	16S rDNA	Mahajan et al ., [19]
Bacillus amyloliquefaciens	16S rDNA	Gad et al., [45, 84, 85]
Bacillus sp. strain AS-S20-I	16S rDNA	Mukherjee and Rai,; Mukherjee et al.,[86, 87]
Bacillus cereus NS-2	16S rDNA	Bajaj el al ., [88]
Brevibacillusbrevis FF02B	16S rDNA; 16S-23S ISR	Majumdar et al.,[89]

2.3 Purification and characterization of bacterial fibrin(ogen)olytic enzyme(s) produced by above bacterial strains

Over a decade, there have been several research publications on bacterial fibrin(ogen)olytic enzymes and characterization of their biochemical as well as biophysical properties. However, various strategies have been applied to purify them up to homogeneity by using multiple chromatographic steps [17]. Usually, a combination of multiple chromatographic strategies is used for this purification. Of these, the most commonly used purification techniques include fractionation of cell-free crude supernatant using ammonium sulfate or chilled organic solvent followed by affinity, ion exchange, hydrophobic interaction and gel-filtration chromatography [17,89]. Till date, many fibrinogenolytic enzymes have been purified and characterized by using various chromatographic techniques and some of the examples are highlighted in Table 2.4.

2.4 Biochemical properties

The biochemical properties, such as optimal temperature, pH, stability, molecular weight, and substrate specificity of purified fibrin(ogen)olytic enzyme(s) have been extensively studied (Table 2.5).

Optimum temperature of microbial fibrinolytic proteases ranges from 30 °C to 70 °C [52, 90], while the CK fibrinolytic enzyme produced by *Bacillus* sp. has been reported

to be highly stable at 70 °C till now. Many other fibrinolytic enzymes have also been found to be stable between 50 °C and 60 °C [53].

The molar mass of the reported fibrinolytic enzymes ranges from 18 to 55 kDa. The microbial fibrinolytic proteases exist in monomeric form and contain four to six isoelectric points [91]. However, several fibrinolytic proteases contain eight isoelectric points [52, 64], while bpDJ-2 contains only 3.5-3.7 pI [92].

2.4.1 Classification of the fibrin(ogen)olytic enzyme(s) based on catalytic mechanisms

Based on the catalytic mechanisms, fibrinolytic enzymes are classified into three types: Serine proteases, metalloproteases, and serine metalloprotease.

2.4.1.1. Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site, for example, NK[48], subtilisin DFE[53], and CK [90].

2.4.1.2. Metalloproteases

These are the most diverse group of proteases characterized by the requirement of a divalent metal ion for their activity [18, 78]. There are also reports showing this category of fibrinolytic enzyme requires divalent metal ion, such as Zn^{2+}, for their catalytic activities in jeot-gal [90], Co^{2+} and Hg^{2+} for enzymes from *Bacillus* sp. KDO-13 [93]. The protease activity of these enzymes is inhibited by chelating agents, such as EDTA and EGTA.

2.4.1.3. Serine and metalloproteases

There are few exceptional examples of fibrinolytic enzymes, which are serine and metalloprotease. For example, *Rhizopus chinens*is 12 [78] and *Streptomyces* sp.Y405 [74] belong to this category.

2.4.2 Active site compositions and structural organization of fibrinolytic enzyme

Almost all serine fibrinolytic enzymes belong to subtilisin of *Bacillus* origin. They own the same catalytic triad made up of Ser221, His64, and Asp32 without any intramolecular disulfide bond reported by many researchers. It has also been

reported that the enzyme activity of several fibrinolytic enzymes are influenced by the presence of DTT and/or Beta mercaptoethanol, which demonstrates the role of –SH groups in the catalytic site and suggests the presence of intramolecular disulfide linkage instead of intermolecular disulfide linkage [41].

Usually, bacterial fibrinolytic enzyme does not conjugate with carbohydrate moiety, but few exceptional examples from *Brevibacillus brevis* [89] and *Pseudomonas* are reported [94]. Mostly all the microbial fibrinolytic enzymes have high substrate specificity towards fibrin, which distinguishes them from other proteases in a broad spectrum. For example, CK enzyme degrades fibrin about eight times higher than that of subtilisin Carlsberg, a common alkaline protease with identical N-terminal sequence [90]; other examples of similar category are NK and subtilisin E [90, 95], subtilisin DJ-4 and subtilisin DFE [52] and subtilisin BPN [53, 96]. Though the common alkaline proteases have highly homologous sequence to their corresponding fibrinolytic enzymes; however, they differ in terms of substrate specificity towards fibrin. The evolutionary changes of critical amino acid residues in the substrate binding sites of these enzymes probably are accountable for this difference [20]. Further, the extended research on structure-function relationship may elucidate this interesting phenomenon reported by many researchers [6]. Structural information on fibrinolytic enzyme has been determined by biophysical techniques, such as fluroscence spectroscopy, circular dichroism, fourier transform infrared spectroscopy, and differential scanning calorimetry [97].

Table 2.4: Purification of fibrinogenolytic enzymes from bacterial sources

Strain used	Chromatographic steps	Purification Fold	Recovery	Reference
Bacillus amyloliquefaciens DC-4	Anion exchange, hydrophobic interaction chromatography and gel filtration chromatography	11.5	2.8%	Peng et al.,[53]
Bacillus megaterium A9542	CM-cellulose column	68.5	48%	Narasaki et al. [39]
Bacillus substilis	Ammonium sulfate fractionation and octyl sepharose and SP sepharose chromatography		26%	Kim et al.,[57]

Strain used	Chromatographic steps	Purification Fold	Recovery	Reference
Bacillus subtilis DC33	Hydrophobic interaction chromatography, anion exchange chromatography and gel filtration chromatography	34.6	13%	Wang et al.,[17]
Bacillus subtilis LD-8547	$(NH_4)_2SO_4$ precipitation DEAE-Sepharose Sephadex G-100	32.4	12.4%	Wang et al.,[59]
Bacillus cereus TKU006	$(NH_4)_2SO_4$ ppt, DEAE-Sepharose CL-6B, CM-Sepharose, Phenyl-Sepharose, Sephacryl S-200, Sephacryl S-100	0.3	0.07%	Wang et al.,[98]
Paenibacillus polymyxa EJS-3	Ammonium sulfate precipitation, hydrophobic chromatography, ion exchange and gel filtration chromatography	14.5	3.3%	Lu et al.,[99]
Streptomyces sp. CS624	Ammonium sulfate precipitation, gel filtration followed by hydrophobic interaction chromatography	5	38%	Mander et al.,[40]
Bacillus subtilis HQS-3	Ammonium sulfate precipitation, alkaline solution treatment, membrane concentration, dialysis, ion exchange, and gel filtration chromatography			Huang et al.,[100]
Bacillus cereus NS	Ammonium sulfate precipitation and diethylaminoethyl sepharose chromatography	2.37	58.3%	Bajaj et al.,[88]
Bacillus amyloliquefaciens FCF-11	Ammonium sulfate precipitation, ion exchange, and gel filtration chromatography	443.5	17%	Kotb [101]
Bacillus pumilus	Ammonium sulfate	16.0	25%	Afifah et

Strain used	Chromatographic steps	Purification Fold	Recovery	Reference
	precipitation, ion-exchange chromatography, and hydrophobic chromatography			al.,[102]
Bacillus amyloliquefaciens An6	Acetone precipitation (0–80%) CM-Sephadex	5.19	71.6%	Agrebi et al.,[62]
Virgibacillus sp. SK 1-3-7	Ammonium sulfate precipitation, hydrophobic chromatography, ion exchange	25.6	10.4%	Montriwong et al., [83]
Bacillus sp. strain AS-S20-I	80% acetone precipitation Gel filtration chromatography High performance liquid chromatography	15.4	19.0%	Mukherjee et al.,[41]
Brevibacillus brevis strain FF02B	Hydrophobic interaction chromatography and gel filtration chromatography	12.4	1.7%	Majumdar et al.,[89]
Bacillus megaterium KSK-07	60 % acetone precipitation, Gel filtration, anion exchange Chromatography	1124	23.0%	Kotb et al., [104]
Lactobacillus plantarum KSK-II	Protein precipitation, Gel filtration, ion exchange chromatography	1,140	33.0%	Kotb el al ., [105]

Table 2.5: Biochemical properties of microbial fibrinolytic enzymes

Enzyme	Microbial origin	Mol. wt, pI,optimal pH and tempera-ture	Substrate specifi-city	Tempera-ture and stability	Protease family /catalytic function	Refer-ence
Nattokinase (NK)	*Bacillus natto*	27.7 kDa, pI 8.6	Synthetic substrate of plasmin or subtilisin	Stable at pH 7-12 and less than 50 °C	Substilisin family serine protease	Sumi et al., [16]

Enzyme	Microbial origin	Mol. wt, pI,optimal pH and tempera-ture	Substrate specifi-city	Tempera-ture and stability	Protease family /catalytic function	Refer-ence
Subtilisin DFE	*B. amyloliquefaciens* DC-4	28 kDa, pI 8.0,pH 10,48 °C	Synthetic substrate of substilisin	Stable at pH 6-10 and less than 50 °C	Substilisin family serine protease	Peng et al [53, 96]
CK	*Bacillus* sp. CK	28.2 kDa,pH 10,48°C	Synthetic substrate for plasmin	Stable at pH 10.5 and less than 50 °C	Thermophilic alkaline serine protease	Kim et al [90]
Substilisin QK-2	*Bacillus substilis* QK02	28 kDa,pH 8.5,55 °C	Synthetic substrate of substilisin	Stable at pH 3-12 and at 40 °C for 30 min	Substilisin family serine protease	Ko et al [64]
Substilisin DJ-4	*Bacillus* sp. DJ-4	29 kDa,pH 10,40 °C	Synthetic substrate of plasmin	Stable at pH 4-11 and at room temperature for 48 °C	plasmin serine protease	Kim and choi [52]
BK-17	*Bacillus substilis* BK-17	31kDa	Synthetic substrate of plasmin		Direct and indirect acting fibrinolytic enzyme	Jeong et al [105]

2.5 *In vitro* and *in vivo* toxicity assessment

In vitro cytotoxicity testing is considered as one of the important methods to rank compounds for consideration in drug discovery [106]. Nevertheless, a specific viability test or cytotoxicity assay method is selected based on the research goals of the study [106]. Several studies have reported *in vitro* cytotoxicity evaluation of fibrin(ogen)olytic enzyme(s) being purified from snake venom [107, 108]; on the other hand, not many reports exist on evaluation of toxicity of microbial fibrinolytic enzymes.

Mukherjee et al. [86] have reported that Bafibrinase (a non-toxic, non-hemorrhagic, direct-acting fibrinolytic serine protease from *Bacillus* sp. strain AS-S20-I) up to a dose of 15 µg/ml does not exhibit any cytotoxicity towards HT29 mammalian cells. Moreover, it does not show any hemolytic effect on washed mammalian erythrocytes. Majumdar et al[89] have demonstrated the non–cytotoxic and non-hemolytic nature of Brevithrombolase at a dose 15 µg/ml (a direct-acting fibrinolytic enzyme isolated from *Brevibacillus brevis* strain FF02B) on Hela and HT 29 cell lines and mammalian erythrocytes.

In vivo toxicological investigation is highly desirable for determination of the safety profile of a new therapeutic agent intended to be used for human and clinical trial registration. Thus, the pre-clinical evaluation of the toxicity for a new compound has to be carried out in rodents and non-human primates before their clinical applications. Rodents and non-human primates are chosen for investigation as they have close biological similarity to humans; therefore, frequently these animal models are especially used as metabolic models for humans in many of the scientific investigations [109].

To meet the market demand as a substitute of currently available commercial drugs, it is necessary to improve clinical complications of fibrinolytic therapeutic agent. Enzymes have sought after for treating different diseases for than a century. Perhaps therapeutics enzymes exert their wide range of application in a multitude of ways. Markland and Swenson (2010) [110] demonstrated safety profile of alifirmeprase and rt-PA at a dose of 0.1 and 1 mg/kg, respectively. Hung and Chiou (2001) [111] also described that at a dose of 5 μg/kg of fibrinolytic enzyme purified from *Bacillus substilis* causes defibrination and not lethal after the intravenous administration. Likewise, Mukherjee et al. (2012) [41] demonstrated non-toxic nature of Bafibrinase at a dose of 2 mg/kg as well as non hemorrhagic activity on BALB/C mouse model [41].

2.6 Pharmacological applications of isolated fibrin(ogen)olytic enzyme(s) in the treatment of thrombosis under *in vivo* conditions

Sumi et al. [112] demonstrated the efficacy of NK in promoting *in vivo* lysis of thrombi. An oral daily dose of as little as 2,000 fibrin units or 50g of natto, when taken intravenously within 12 h of a stroke or heart attack, was found to be more potent in clot dissolving than commercially available recombinant t-PA, UK, and SK, [14] and their observations have been confirmed by many others [14] . Another important finding was also reported by Sumi et al. [16], where they have established the effectiveness of NK capsules to dissolve thrombi in dogs when administered orally. Evidence has been presented to show that NK can be used to dissolve thrombus effectively within 5 h of treatment in dogs in comparison to control dogs treated with placebo; the treatment resulted in restoration of normal blood circulation [20].

The mechanism of clot dissolving property of NK has been studied by Fujita et al. [48]. The authors have observed that NK, being a direct acting plasmin-like enzyme, increases the clot dissolving ability by directly cleaving fibrin and converting the inactive plasminogen into active plasmin when administered orally. Another study by Fujita et al. [48] investigated the thrombolytic action on NK. It was noted NK could recover 62.0% of the arterial blood flow, while those treated with plasmin regained just 15.8%, clearly establishing the particularly strong thrombolytic property of NK *in vivo* in comparison to plasmin. Further, this was also extended up to human trial involving 12 healthy Japanese volunteers [48]. The results showed that oral administration of natto (nattokinase) enhanced the ability of participants to dissolve blood clots. Enzyme therapies are becoming more prevalent in medical practices today displaying their targeting advantages in disease treatment [6].

Now-a-days, several types' animal models are being used to investigate thrombolytic potency of fibrinolytic enzymes. In 1983, Bekemeier first introduced the importance of Carrageenan-induced thrombosis in the rat and mouse as test models for substances influencing thrombosis. Further, the importance of thrombosis model and atherosclerosis was explained by Verbeuren in 2006 [113]. There is a dearth of examples available on *in vivo* thrombolytic study of microbial fibrin(ogen)olytic enzyme(s) in animal model. Table 2.6 highlights recent examples of *in vivo* thrombolytic activity of microbial fibrinolytic enzyme(s) in animal model as explored by various researchers.

Table 2.6: Various methods of thrombus formation in animal models

Microorganism	Fibrino(geno)lytic enzyme(s)	Animal model	Thrombolytic model used	References
B. subtilis QK02	Subtilisin QK	Male mouse	Arterial thrombosis model induced by $Fecl_3$	Yan et al.,[114]
Staphylococcus aureus	Staphylokinase SAK-RGD-K2-Hirul	Rat	Thrombosis was induced secondarily by electrical stimulation	Szemraj et al., [115]
Bacillus subtilis LD-8547	Fibrinolytic enzyme	Rabbits	Carrageenan-induced thrombosis in mice	Yuan et al.,[116]
Streptomyces strain	Streptomyces enzyme	Mice	k-carrageenan-induced mice tail thrombosis	Simkhada et al.,[18]
Bacillus natto	Nattokinase	Rat	Carrageenan-induced mice tail thrombosis	Xu et al., [117]
Brevibacillus brevis FF02B	Brevithrombolase	Wistar strain rats	k-carrageenan-induced rat tail thrombosis	Majumdar et al., [89]