EXTRACTION, PURIFICATION AND CHARACTERIZATION OF *ARTOCARPUS ALTILIS* LATEX PROTEASE AS A FIBRINO(GENO)LYTIC AGENT

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by

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LIST OF SYMBOLS

α	Alpha
β	Beta
γ	Gamma
μ	Micro
±	Plus-minus
%	Percentage
°C	Degree Celsius
ΔG	Change in Gibbs free energy
ΔH	Change in enthalpy
ΔS	Change in entropy
E_a	Energy of activation
E_d	Energy of inactivation
h	Planck's constant
h	Hour
HCl	Hydrochloric acid
K	Kelvin
K_B	Boltzmann's constant
k_d	Inactivation rate constants
K_m	Michaelis-Menten constants
L	Litre
mA	Milli-Ampere
mM	Milli-Molar
min	Minute

N	Avogadro's number
NaCl	Sodium chloride
NaOH	Sodium hydroxide
pI	Isoelectric point
R	Gas constant
$t_{1/2}$	Half-time
U	Unit
V	Voltage
V_{max}	Maximum velocity

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CHCA	α-cyano-4-hydroxycinnamic acid
CID	Collision Induced Dissociation
CV	Column Volume
DB	Database
DTT	Dithiotreitol
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization of the United Nation
FpA	Fibrinopeptide A
FpB	Fibrinopeptide B
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
IUBMB	International Union of Biochemistry and Molecular Biology
kDa	Kilo Dalton
LC	Liquid chromatography
Ln	Natural log
m/z	Mass to charge ratio
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MALDI	Matrix-assisted laser desorption ionization
NTBG	National Tropical Botanical Garden
NIH	National Institutes of Health

PMSF	Phenylmethanesulfonyl fluoride
PVPP	Polyvinylpolypyrolidone
Q	Quadrupole
RG	Rate of gelation
SD	Standard deviation
SDS	Sodium dodecyl-sulphate
STT	Sodium tetrathionate
T	Temperature
TCA	Tricholoroacetic acid
TLE	Thrombin-like enzymes
tPA	Tissue plasminogen activator
TOF/TOF	Tandem time-of-flight

PENGEKSTRAKAN, PENULENAN DAN PENJELASAN PROTEASE LATEKS ARTOCARPUS ALTILIS SEBAGAI AGEN FIBRINO(GENO)LITIK

ABSTRAK

Penuaian buah *Artocarpus altilis* (sukun) di ladang-ladang melibatkan suatu proses pengaliran getah bagi mengelakkan kesan kotor pada kulit buah tersebut. Eksudat dari tumbuhan ini tidak digunakan sepenuhnya dan masih belum mempunyai kegunaan komersil. Kegunaan getah sukun dalam bidang etnofarmakologi sebagai agen penyembuhan luka menunjukkan penglibatannya dalam proses pembekuan darah dan fibrinolisis. Proses penulenan separa getah *A. altilis* menerusi kaedah kromatografi HiTrap™ SP Sepharose telah menulenkan enzim protease yang berberat molekul 72 kDa sebanyak 3.1 kali ganda dengan nilai perolehan semula pada 24% dan aktiviti spesifik sebanyak 4.87 U/mg. Aktiviti enzim protease serina ini direncatkan dengan penggunaan fenilmetanasulfonil fluorida (PMSF) (1 mM and 10 mM) dengan baki aktiviti masing-masing pada 89% dan 27%. Enzim protease ini menunjukkan aktiviti maksimal pada pH 10 apabila menggunakan kasein dan serum albumin bovin (*BSA*) sebagai substrat. Enzim ini optimal pada suhu 80 °C selepas diinkubasi selama 30 minit. Nilai K_m and V_{max} dengan kasein adalah pada 0.453 ± 0.0026 mM dan 0.022 ± 0.001 µmol/min. Enzim protease ini juga stabil pada julat pH yang luas (pH 4-11) apabila tiada pengurangan yang ketara dikesan pada aktivitinya selepas diinkubasi selama 24 jam pada suhu 37°C. Parameter-parameter termodinamik penyahaktifan enzim pada julat suhu 55-75°C seperti perubahan entalpi (ΔH), perubahan tenaga bebas (ΔG) dan perubahan entropi (ΔS) adalah dianggarkan masing-masing di dalam julat 80.10-80.27 kJ/mol,

108.34-109.32 kJ/mol dan 83.97-85.58 J/mol. Tenaga penyahaktifan (E_d) protease *A. altilis* adalah bernilai 82.9 kJ/mol. Enzim protease *A. altilis* (0.125 U) mempunyai kapasiti penggelan yang setara dengan enzim thrombin (0.25 U) pada 75 minit yang pertama tempoh inkubasi. Analisa gel elektroforesis poliakrilamida sodium dodesil sulfat (SDS PAGE) mendapati enzim protease *A. altilis* menghidrolisis subunit fibrinogen mengikut turutan Aα > Bβ > γ. Aktiviti fibrinolitik enzim ini pula didapati telah menunjukkan hidrolisis polimer α yang signifikan selepas diinkubasi selama 15 minit, manakala dimer γ-γ dan subunit Bβ tidak didegradasi sepenuhnya walaupun selepas 24 jam. Jujukan asid amino fibrinopeptida yang dibebaskan dianalisa menerusi kaedah LC-ESI-QTOF MS/MS dan data yang diperoleh diinterpretasi menggunakan perisian PEAKS Studio (Versi 7.5). Analisa menerusi perisian Peptigram mendapati bahawa kebanyakan peptida dibebaskan dari terminal-C subunit-subunit fibrinogen. Selain itu, analisa menerusi perisian iceLogo mendapati asid amino polar mendominasi pada kedudukan P1. Asid amino polar dan bukan polar mempunyai kebarangkalian yang hampir serupa pada kedudukan P2-P6 dan P2'-P6'. Kesimpulannya, penemuan aktiviti fibrino(geno)litik pada enzim protease dari getah *A. altilis* telah membuktikan kepenggunaannya di dalam perubatan tradisional sebagai agen penyembuhan luka. Oleh itu, dapatan hasil kajian diharapkan dapat memberi asas kepada penerokaan potensi eksudat tumbuhan ini sebagai agen hemostatik pada masa hadapan.

EXTRACTION, PURIFICATION AND CHARACTERIZATION OF ARTOCARPUS ALTILIS LATEX PROTEASE AS A FIBRINO(GENO)LYTIC AGENT

ABSTRACT

The harvesting of *Artocarpus altilis* (breadfruit) in plantations involves the process of draining the latex to avoid staining of the epicarp. The exudate is not fully utilized and it has no commercial value. Ethnopharmacological applications of the latex in wound healing imply its action on blood coagulation and fibrinolysis. Partial purification of *A. altilis* latex through HiTrap™ SP Sepharose chromatography had purified a 72 kDa protease by 3.1-fold with 24% recovery and the specific activity of 4.87 U/mg. This serine protease was inhibited by phenylmethanesulfonyl fluoride (PMSF) (1 mM and 10 mM) with 89% and 27% of residual activities respectively. The protease showed maximal activity at pH 10 with casein and bovine serum albumin (BSA) as substrates. The protease's apparent optimal temperature after 30 minutes of incubation was 80°C. The K_m and V_{max} values on casein were 0.453 ± 0.026 mM and 0.022 ± 0.001 µmol/min, respectively. The protease was stable at a broad pH range (pH 4-11) with no significant reductions were detected after 24h. Thermodynamic parameters like the enthalpy (ΔH), free energy change (ΔG) and entropy (ΔS) of the protease's inactivation at the temperature of 55-75°C were estimated to be in the range of 80.10-80.27 kJ/mol, 108.34-109.32 kJ/mol and 83.97-85.58 J/mol respectively. The energy of inactivation (E_d) was 82.9 kJ/mol. *A. altilis* protease (0.125 U) had a comparable fibrinogen clotting capacity with thrombin (0.25 U) during the first 75 min of incubation. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis revealed that *A. altilis*

protease attacks fibrinogen subunits the in order of Aα > Bβ > γ. Fibrinolytic activity on partially-crosslinked fibrin showed that the α polymer was significantly digested after 15 minutes of incubation. The γ-γ dimer and Bβ chain of the fibrin were partially digested after 24h of incubation. Amino acid sequence of fibrinopeptides released was analyzed through LC-ESI-QTOF MS/MS and the data was interpreted using PEAKS Studio (Version 7.5). Peptigram analysis revealed that peptides were preferentially released from the C-terminals of the fibrinogen subunits. The iceLogo analysis showed that the polar amino acid residues dominate the P1 position. Both polar and nonpolar residues have similar probability of occurrence at P2-P6 and P2'-P6'. Overall, the fibrino(geno)lytic activity of *A. altilis* latex protease had substantiated its applications in traditional medicine as wound healing agent. Therefore, this research is hoped to provide a basis in exploring this plant exudate's potential as hemostatic agent in the future.

CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Artocarpus altilis (Parkinson) Fosberg or better known as breadfruit was commonly grown as backyard plant, which is distributed in the tropical and subtropical countries including the Pacific Islands, South America, India, Southeast Asia, Madagascar, the Maldives, Sri Lanka and northern Australia (Ragone, 2006). As a plant that provides satiety, high in carbohydrate and packed with nutritional values, the cultivation of breadfruit is envisioned to mitigate hunger while offering a sustainable source of income to the locals (Turi *et al.*, 2015). Almost every part of the plant is useful, either as food, construction materials or applied as remedies by the traditional healers. With the expansion of commercial plantations, good harvesting practices are essential in producing high quality fruits acceptable to the consumers. Breadfruit however, exudes latex upon harvesting, causing the plant sap to adhere to the surface leading to the staining of the epicarp. This condition is unappealing, causing the fruit to be perceived as low in quality by the end users (Elevitch *et al.*, 2014). According to Stice *et al.* (2007), latex is undesirable, that any circumstances of surface staining must be avoided or at least reduced. In relation to this, proper methods of breadfruit harvesting include the process of latex draining by resting the fruit upside down for approximately an hour until the flow of the sap ceases (Elevitch *et al.*, 2014). The sap will eventually drain to the ground and not fully utilized.

Although it is considered a nuisance in the economic point of view, breadfruit latex is applied as traditional remedy by the native Polynesians healers as a wound healing agent. The natives of Tonga and Tahiti treated wounds, rashes, abscesses,

sores and boils with the plant's exudate (Han, 1998). The white sap of the plant was also proclaimed to exert healing of puncture wounds of the eyes. These ethnopharmacological uses of breadfruit latex as blood hemostatic agent and wound healing remedy imply its action as fibrin(ogen)olytic agent, contributed by the proteolytic components (Rajesh *et al*., 2006). The blood haemostatic effects of the crude *A. altilis* latex was reported by Singh *et al.* (2015) where both clot inducing and clot dissolving properties were observed. However, there is no information regarding the fibrin(ogen)olytic activity of this plant is reported so far.

Upon injuries and infliction of wounds, a cascade of reactions is triggered leading to the cleavage of fibrinogen (Hoffman & Monroe, 2001; Macfarlane, 1964). A serine protease known as thrombin cleaves fibrinogen at specific sites to form fibrin as a mesh along with platelet plugs at the site of injury in the process to arrest bleeding. These temporary fibrin mesh and plugs will be removed by a fibrinolytic enzyme identified as plasmin once recovery is achieved (Deryugina & Quigley, 2012). The striking similarities between the mechanisms of blood coagulation in mammals and latex clotting in plants tempted a lot of researchers to find a common factor between these two systems (Moutim *et al.*, 1999). Given these factors, researches regarding the pro-coagulant and anti-coagulant properties of plant latex are recently expanding. These plant exudates are seen as potential alternatives to the current exogenous hemostatic factors commonly isolated from snake venoms.

Although the application of latex as wound healing agents has been reported throughout different regions of the world, proteases of different classes were reported to exert distinct cleavage specificities and different mode of actions. Shivaprasad *et al.* (2016) proposed that the clot inducing property of plant cysteine proteases are attributed to the enzymes' selective hydrolysis similar to the specificity of human

thrombin. Meanwhile, serine proteases were illustrated to be more general in action and does not form fibrin clot from fibrinogen directly. Despite these notions, the authors admits that the mechanisms of plant serine proteases in blood coagulation and hemostasis is yet to be understood and needed to be explored (Shivaprasad *et al.*, 2016).

1.2 Research scope

This study was designed with the aim to elucidate the proteolytic component of *A. altilis* latex and to investigate its potential as fibrino(geno)lytic agent. Latex samples used in this research were collected from Pulau Aman, located in the district of Seberang Perai Selatan, Penang, Malaysia. Fruits for latex extraction from this study were selected from the phase of 'mature but not ripe', a stage commonly selected by farmers for harvesting. The extracted latex was processed and its proteolytic component was purified to apparent homogeneity as observed through sodium docecyl sulfate (SDS PAGE) analysis. General characterizations of the purified *A. altilis* latex protease were carried out using natural substrates such as casein and bovine serum albumin (BSA). This thesis also covers the stability studies (pH and temperature) in addition to the estimations of its thermodynamic parameters.

The fibrinogen clotting capacity by *A. altilis* protease was investigated based on the spectrophotometric method and the gelation parameters were estimated from the curves obtained from the analysis. This thesis also covers the investigation of the protease's fibrinogenolytic and fibrinolytic activities, studied using pure human fibrinogen solutions and observed through sodium docecyl sulfate (SDS PAGE) and densitometric analysis. Peptides released from the fibrinogenolytic activity were further elucidated through mass spectrometry analyses and the data was interpreted

with the aid of peptide analysis software such as PEAKS Studio, Peptigram and IceLogo. The peptide analysis was carried out with the reference of the sequence of human fibrinogen alpha chain (Uniprot ID: FIBA_P02671), human fibrinogen beta chain (Uniprot ID: FIB_P026715) and human fibrinogen gamma chain (Uniprot ID: FIBG_P02679) as searched against the Universal Protein Resource (UniProt) (The UniProt Consortium, 2017) database.

1.3 Objectives

Given the strong ethnopharmacological background and positive blood hemostatic effects of the crude latex of *A. altilis,* this thesis is aimed to elucidate the proteolytic components of the latex and their mechanisms in hemostasis focusing on its fibrinogenolytic and fibrinolytic activities. Therefore, the specific objectives of this thesis are as outlined below:

i. To extract, purify and characterize protease from latex of *Artocarpus altilis*.
ii. To determine fibrinogen clotting activity of purified *Artocarpus altilis* latex protease.
iii. To investigate the mechanism of fibrinogenolytic and fibrinolytic activity of *Artocarpus altilis* latex protease by SDS PAGE analyses.
iv. To characterize peptides derived from fibrinogenolytic activity of purified *Artocarpus altilis* latex protease by mass spectrometry.

CHAPTER 2

LITERATURE REVIEW

2.1 *Artocarpus altilis* (Fosberg) Parkinson

2.1.1 Description

Artocarpus altilis (Fosberg) Parkinson or commonly known as breadfruit (English) has many local names throughout different regions including *sukun* (Indonesia, Malaysia); *rimas* (Philippines), *beta* (Vanuatu), *kapiak* (Papua New Guinea), *'ulu* (Hawaii, Samoa, Rotuma, Tuvalu) *arbol a pan* (Spanish) (Ragone, 2006). A mature tree of *A. altilis* erects approximately 12 – 15 m from the ground and could even grow up to 21 m tall. *A. altilis* tree is distinguished by its unique leaves, broadly obovate to broadly ovate in shape with the presence of lobes, sinuses and large apical tip (Ragone, 2006). Meanwhile, the shape of the fruit is either round, oval or oblong depending on its variety and the weight of the fruit can ranged between 0.25 – 6 kg (Jones, 2011). The *A. altilis* tree exudes copious amount of milky white latex in almost all parts of the plant. The taxonomic hierarchy of *A. altilis* plant is summarized in **Table 2.1**.

Table 2.1: Taxonomic hierarchy of *Artocarpus altilis* (Integrated Taxonomic Information System (ITIS), 2019)

Rank	Scientific name
Kingdom	Plantae
Division	Tracheophyta
Class	Magnoliospida
Family	Moraceae
Genus	Artocarpus
Species	*Artocarpus altilis* (Parkinson) Fosberg

2.1.2 Distribution

The cultivation of *A. altilis* plant spreads through the pan tropical regions around the world mainly in hot and humid countries which receive annual rainfall of 1500 - 3000 mm. This plant is native to countries such as New Guinea, Indonesia and the Philippines. Some of the varieties are also distributed in the Caribbean, Central America, Africa, India, Southeast Asia, the Maldives and Northern Australia (Ragone 2011; Zerega *et al.*, 2004). The global distribution of *A. altilis* plant is as presented in **Figure 2.1.**

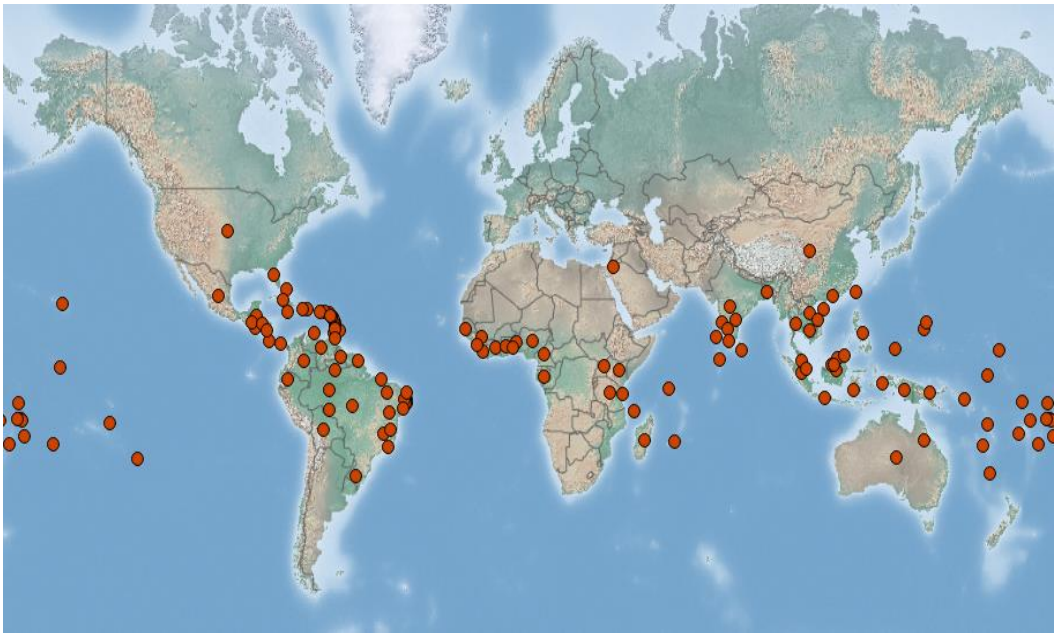


Figure 2.1: The global distribution of *A. altilis* plant. Retrieved from Centre of Agriculture and Bioscience International (CABI). https://www.cabi.org/isc/datasheet.

2.1.3 Economic perspective and agricultural impact

2.1.3(a) Yield

Breadfruit is interesting in terms of its high yields, producing abundant fruits around the year with two peak seasons annually. The tree will begin to bear fruit after 6 - 10 years of planting, whereas vegetatively planted trees will start fruiting sooner within in 3 - 6 years and they are productive for many decades (Liu, 2014).

An average tree produces 100 to 250 fruits per tree per year that makes up approximately 160 to 500 kg of fruits annually with 75 % (dry basis) of its fruit is edible. A study on breadfruit productivity by Liu *et al.* (2014) reported that orchard propagations with the capacity of 50 trees/ha had produced an estimated yield of 5.23 t/ha after 7 years of plantations. This value is comparable to the yield of main staple crops such as rice (4.6 t/ha), maize (5.6 t/ha) and corn (3.4 t /ha) obtained from the FAOstat database (FAO, 2018). In another report by National Tropical Botanical Garden, United States (NTBG, 2009) an average-sized tree with the canopy covers of 25 m^2 is capable to produce approximately 100 kg of fruits, a value far superior compared to the production of other plantains root and tuber crops (10 – 36 kg) on a similar-sized land plot due to the verticality of fruit production of breadfruit.

2.1.3(b) Initiatives on global breadfruit cultivations

As a plant that provides satiety due to its high carbohydrate content and packed with nutrients, breadfruit is envisioned to provide a sustainable solution towards global food crisis mainly in the tropical and sub-tropical regions (Globalbreadfruit, 2018). More importantly, this plant is productive for years and bears abundant of fruit, suitable as a staple crop. The cultivation of breadfruit however, is inferior compared to the major crops such as wheat, maize or rice. They are grown as a backyard plant or ornamental tree, and considered as an underutilized plant in some part of the world (Elevitch & Ragone, 2018). In line with this scenario, aggressive initiatives and campaigns were carried out by non-profit organizations in realizing this mission (**Table 2.2**). The Breadfruit Institute, headed by Dr Diane Ragone which is located at the National Tropical Botanical Garden (NTBG) Hawaii housed approximately 120 breadfruit varieties with the aim to develop competent

methods of propagation and to initiate the distributions of breadfruit plants throughout the tropical regions.

Table 2.2: List of non-governmental organizations, institutes and campaigns in promoting the cultivation of breadfruit as staple crop

Organizations/ Institute/ Initiatives/ Projects	**Objectives**	**Websites**
Breadfruit Institute (2003)	i. To promote conservation, research and use of breadfruits for food and reforestations. ii. To provide economic opportunity iii. To educate publics on the advantages of breadfruit cultivations and consumptions. iv. To educate local communities on improving their economies through farming, retailing and creating value-added products from breadfruit.	https://ntbg.org/breadfruit
Global Hunger Initiatives (2009)	i. An initiative by the Breadfruit Institute as a part of the member of Alliance to End Hunger coalitions. ii. To initiate distributions of high quality breadfruit plantlets throughout tropical and sub-tropical regions. iii. More than 100 000 breadfruits trees have been disseminated to 44 countries.	https://ntbg.org/breadfruit/work/globalhunger
Global Breadfruit	i. Developed micropropagation techniques that enable global distribution of plants.	http://globalbreadfruit.com/
Plant a tree- Grow 'Ulu' Project	i. Increase food security in Hawaii and the Tropics by planting breadfruit. ii. Distributed more than 10 000 trees between 2012 and 2015. iii. Strategic partnership with schools, churches, health centers,	https://growsomegood.org/2015/09/plant-a-tree-of-life-grow-ulu-breadfruit/

2.1.3(c) Commercial scale propagations

Aggressive campaigns in spreading awareness about the benefits of *A. altilis* consumptions and the initiatives taken in distributing *A. altilis* plantlets from plant tissues to various countries are expected to result in the rise of breadfruit cultivations

globally (Murch *et al.*, 2008). In line with this scenario, the expansion of breadfruit cultivations to larger scale plantations requires standardizations of practices in order to maintain the quality and to ensure year-round supply of the fruit. With regard to this matter, the Breadfruit Institute had outlined a compilation of best practices in breadfruit cultivation which encompasses of the pre-harvest, harvest and post-harvest stages (Elevitch *et al.*, 2014). Good practices in cultivations will result to increase in shelf life and qualities of the fruits, minimizes fruit loss and maintain the product value and desirability.

Breadfruit exudes large amounts of latex during fruit harvesting and the appearance of latex on the surface causes the fruits to be perceived as low in quality by the end users. According to Stice *et al.*, (2007), the adherence of the exudate onto the epicarp should be avoided or at least minimized. In relation to this, the Breadfruit Production Guide (Elevitch *et al.*, 2014) had outlined several additional steps during fruit harvesting to tackle this issue. Fruits are suggested to be inverted for at least an hour after harvesting in order to drain the latex until the flow of the sap ceases. The latex however, will be drained to the ground and currently had neither commercial nor agricultural applications.

2.1.4 Scientific research on bioactivities of *A. altilis*

Scientific studies on bioactivities of *A. altilis* were generally focused on the flavonoids with the majority of it were isolated from the leaves. This is in accordance to a review by Sikarwar (2014) who mentioned that approximately 130 flavonoids and flavones compounds were isolated from this plant. There was only one study however, that focused on the bioactivities from the latex. The study by Singh *et al.* (2015) investigated on the comparisons of hemostatic potentials between the crude latex of *A. altilis* and *Tabernaemontana divaricata* on fresh blood coagulation. **Table**

2.3 summarizes recent studies for the past five years (2015 - 2019) on the bioactivities of different parts of *A. altilis* plant as surveyed from the Scopus database.

Table 2.3: Bioactivities of different parts of *A. altilis*

Parts	**Activity**	**Reference**
Leaves	Anti-nephrolithiasis	Putra *et al.* (2018)
Leaves	Anti-malarial	Udonkang *et al.* (2018)
Leaves	Cardio-protective effect	Nwokocha *et al.*, (2017)
Leaves	Anti-oxidant	Mozef *et al.*, (2015)
Leaves	Anti-microbial	Pradhan *et al.*, (2012)
Leaves	Anti-diabetic	Wahyudin *et al.*, (2017)
Leaves	Anti-microbial and anti-oxidant	Ravichandran *et al.*, (2016)
Leaves	Anti-hyperlipidemic	Fajaryanti *et al.*, (2016)
Leaves	Improve renal function	Safithri *et al.*, (2016)
Leaves	Control of cholesterol level	Tugiyanti *et al.*, (2016)
Leaves	Anti-inflammatory	Fakhrudin *et al*., (2016)
Leaves	Anti-cancer (prostate)	Jeon *et al*. (2015)
Leaves and stem bark	Anti-malarial	Achmad *et al*., (2015)
Pulp	Anti-cancer (lung)	Jalal *et al*., (2019)
Pulp	Anti-cancer (cervical)	Jamil *et al.* (2018)
Pulp, peel and whole fruit	Anti-oxidant	Jalal *et al*., (2015)

Table 2.3 (cont.): Bioactivities of different parts of *A. altilis*

Parts	Activity	Reference
Peel	Anti-oxidant	Marjoni *et al*. (2018)
Heartwood	Melanogenesis inhibitory	Kwankaew *et al*., (2017)
Heartwood	Skin protection effect	Tiraravesit *et al*., (2015)
Latex	Haemostatic potential	Singh et al., (2015)

2.2 Plant latex protease

2.2.1 Challenges in extraction and purification of plant proteases

Extraction and purification of enzymes from plants including proteases presented its own challenges mainly attributed to the abundance of the phenolic compounds in their cells and vacuoles (Balbuena *et al.*, 2011; Charmont *et al.*, 2005). The involvement of tissue and cell disruptions during enzyme extraction and purification inevitably ruptures the compartmentalization of these substances, causing the enzymes to be vulnerable towards the attack of phenolic compounds which leads to the inactivations or modifications of the enzyme (Pierpoint, 2004). The phenolic compounds are able to form either reversible or irreversible linkages with the enzymes leading to its precipitation ensuing serious loss of activities (Baxter, 1997). The irreversible covalent linkages occurred due to the oxidation of the vicinal dihydroxy groups of phenolic compounds, forming highly reactive quinones and semi-quinones. These quinones are not only able to polymerize with each other oxidizing more phenols, but they could also react with the functional groups of proteins causing aggregations, precipitations and lost of activities (Gupta

& Vithayathil, 1981; Pierpoint 2004). The occurrence of oxidation in plant extract is often marked by the browning of the solutions (Pierpoint, 2004) where plant enzyme solutions are presumed to be oxidized whenever browning occurs in an extract and considered as a poor source of active enzymes (Loomis, 1974). The auto-oxidation of phenolic compounds is prone to occur in alkaline environments thus, plant protein extractions often takes place in either neutral (Fonseca *et al*., 2010; Patel *et al*., 2012) or acidic conditions (Dubey & Jagannadham, 2003; Liggieri *et al*., 2004; Nallamsetty *et al*., 2003; Prasad & Virupaksha, 1990; Siritapetawee *et al*., 2012), depending on the protein's stability.

2.2.2 Strategies of extraction and purification of plant proteases

In relation to the recalcitrant nature of plants, the selection of strategies is critical in order to ensure successful enzyme extractions and purifications. Procedures adapted are usually with the objective to minimize the eventualities of oxidations through rapid separation of phenolic compounds from the target protein and to create reducing environments during enzyme extractions and purifications (Jervis & Pierpoint, 1989; Pierpoint, 2004).

The extraction and purification of plant proteases from latexes adapted similar strategies through the inclusions of reducing agents, phenol adsorbents and metal ion chelators in its buffer formulations. Reducing agents that are commonly applied in plant proteases extraction buffers include the sodium tetrathionate (STT), 2-mercaptoethanol, dithiothreitol (DTT), ascorbate and ascorbic acid (Dubey & Jagannadham, 2003; Nallamsetty *et al*., 2003; Prasad & Virupaksha, 1990; Siritapetawee *et al*., 2012). These components function to return the oxidized phenolic compounds back into its reductive state, hence maintaining the reducing condition of the environment. Despite being widely used as reducing agents in

protease extractions, STT and other similar thiol reagents such as DTT and 2-mercaptoethanol must be used with caution especially in the isolation of uncharacterized proteases. Instead of reacting with the functional groups of the phenolic compounds, these reducing reagents could reversibly block the active sulfydryl residues of the target protein causing its inactivations (Hermanson, 2013).

In addition, phenol adsorbent polymers such as albumin, hide powders, polyacrylic resins and polyvinylpyrrolidone (PVP) are commonly included in plant protease extraction buffers as they provide rapid separations between the phenolic compounds and the target protein (Pierpoint, 2004). The insoluble form of PVP or better known as polyvinylpolypyrrolidone (PVPP) has great affinity towards tannins and monomeric phenols, often used in plant protein extraction buffers (Charmont *et al*., 2005). This adsorbent effectively captures the contaminating phenols in plant extracts and easily removed from the solutions through physical separations such as centrifugation or filtration.

2.2.3 Purification of plant proteases – ion exchange chromatography

Purifications of active proteins such as enzymes often involved strategies that are designed to preserve the activity through separations from contaminants, mainly of those that are detrimental to its activity (Jervis & Pierpoint, 1989). The number of steps in purification procedures depends on the purity requirements and its intended use. However, increasing the number of steps is often at the expense of decreasing the overall protein recovery due to the longer purification time (GE Healthcare, 2010). High speed procedures are desired especially in the presence of critical contaminants in order to reduce the risk of the enzyme's degradation.

Purification of plant proteases often adapted simple purification procedures that allow rapid separation of the target protein from the contaminants especially the

phenolic compounds (Liggieri *et al*., 2004; Patel *et al*., 2012; Yadav *et al*., 2006). Ion exchange chromatography offers the advantage of isolating while concentrating the target protein simultaneously. The separation which is based on the differences of surface charges produces high resolution separations with high sample loading capacity (Weiss, 2016). In addition, the rigidity of the media allows the application in high flow rates in addition to the rapid binding kinetics of the oppositely charged molecules to the media (GE Healthcare, 2016). Ion exchange chromatograpy separates proteins based on the differences of their net surface charges to the oppositely charged groups immobilized onto the ion exchange matrix. Protein's net surface charge changes with the pH of its surrounding due to its amphoteric nature and each protein has its own specific net charge versus pH relationship (Jungbauer & Hanh, 2009). The net charge of a protein changes with pH up to a point where the number of negative charges equals to the number of positive charges (zero net charge) and this value is marked as the isoelectric point (pI). Protein in a pH solution that is below its pI value will have a positive net surface charge and bind to a negatively charged media (cation exchanger) and vice versa (Jungbauer & Hanh, 2009). Each enzyme is unique in their structures where a protein's binding capacity to ion exchange resin depends on factors such as the overall charge, charge density and the surface charge distribution of its amino acids (GE Healthcare, 2010).

2.2.4 Characterizations of plant latex proteases

Proteases are classified as hydrolase enzyme with the capacity to cleave peptide bonds. These enzymes are further subdivided into four main groups as recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) consisting of serine protease (EC 3.4.21), cysteine protease (EC 3.4.22),

aspartic protease (EC 3.4.23) and metalloprotease (EC 3.4.24), each group is distinguished by their mode of catalytic activities (Hartley, 1959).

Plants proteases have been gaining attentions due to its stability over a wide range of pH and temperatures (Baeyens-Volant *et al.*, 2015; Fonseca *et al.*, 2010; Tripathi *et al.*, 2011). These proteases commonly exist either as cysteine proteases or serine proteases and only a small number were classified under the aspartic and metalloprotease group (Devaraj *et al.*, 2008 ; Kumar *et al.*, 2011). Latex is a rich source of hydrolytic enzymes that exists to regulate various physiological functions of a plant (Konno, 2011). The concentration of protease was reported to be several folds higher in latex compared to other organs of the plant, thus explaining its role in defense mechanism against pathogenic attacks (Konno *et al.*, 2004; Konno 2011). Parallel to the high concentrations of protease in latex and its ease of availability, plant latex is often a preferred source of plant proteases. The characteristics of serine proteases and cysteine proteases from latex of various plant species is summarized in **Table 2.4**.

Cysteine proteases are characterized by the presence of cysteine residue in its catalytic site (Verma *et al.*, 2016). The general mechanism of this protease involves the formation of covalent linkages to form enzyme-substrate complexes which occur through the nucleophilic attack of the sulfhydryl group of the cysteine residue. The molecular weights of cysteine proteases are mostly distributed between 20 - 50 kDa (Rao *et al.*, 1998) with those isolated from latex are usually ranged between 20 - 30 kDa and their optimum pH is usually distributed at the neutral pH range. Cysteine proteases are commonly identified through the inhibition by cysteine class specific inhibitors such as iodoacetate and ρ-chloromercuric benzoate. This class of protease

however, are very prone to oxidation and easily inactivated when exposed to air due to the redox potential of its cysteine residue.

Serine proteases are relatively stable towards oxidation compared to cysteine proteases. This class of protease is characterized by the presence of serine residue in its catalytic center which acted as a nucleophile during enzymatic attack on carbonyl groups of peptide bonds (Hedstrom, 2002). The general characteristics of plant serine proteases were extensively reviewed by several authors such as Antao and Malcata (2005) and Domsalla & Melzig (2008). These authors had summarized that the molecular weights of serine proteases are distributed in a wide range of 19 - 110 kDa but the majority of it lies between 60 - 80 kDa. The optimum pH is usually ranged in the alkaline region between pH 7 - 11. Plant serine proteases are generally more stable in wide pH range and withstand high temperatures. Serine proteases are frequently reported to exist in glycosylated form which contributed to their high molecular weights and confer stabilities in extreme environments.

Table 2.4: Characteristics of serine and cysteine proteases purified from latex of different plants

Protease	**Plant**	**Class**	**Mw (kDa)**	**Optimum pH (substrate)**	**pH stability**	**Optimum temperature (°C)**	**Temp. stability (°C)**	**CHO***	**Ref**
Ficin E	*Ficus carica*	CP	24.3	6.3 *(*Z-Phe-Arg-AMC* and Boc-Gln-Ala-Arg-AMC*)	3 - 10	55	NR	No	Baeyens-Volant *et al.*, **(**2015**)**
Eumiliin	*Euphorbia milii var. hislopii*	CP	29.8	8.0 (casein)	3 - 10	40	NR	NR	Fonseca *et al.*, (2010)
Asclepain c I.	*Asclepias curassavica L.*	CP	23.2	8.5 (casein)	7.5 - 9	NR	< 60	NR	Liggieri *et al.*, (2004)
Funastrain C II	*Funastrum clausum*	CP	23.6	9 - 10 (casein)	6 - 11	NR	< 70	NR	Morcelle *et al.* (2004)
Procerain	*Calotropis procera*	CP	28.8	7 - 9 (azoalbumin)	3 - 12	55 - 60	< 70	No	Dubey & Jagannadham (2003)
Ervatamin A	*Ervatamia coronaria*	CP	27.6	8.0 - 8.5 (casein)	3 - 12	50 - 55	< 70	No	Nallamsetty *et al.*, (2003)

*SP = Serine protease
*CP = Cysteine protease
*NR = Not recorded
*CHO = Carbohydrate
* Z-Phe-Arg-AMC = Benzyloxycarbonyl-phenylalanylarginine-4-methylcoumaryl-7-amide
* Boc-Gln-Ala-Arg-AMC = N(2)-(tert-butoxycarbonyl)-L-glutaminyl-L-alanyl-N-(4-methyl-2-oxo-2H-1-benzopyran-7-yl)-L-argininamide

Table 2.4 (cont.): Characteristics of serine and cysteine proteases purified from latex of different plant species

Protease	**Plant**	**Class**	**Mw (kDa)**	**Optimum pH (substrate)**	**pH stability**	**Optimum temperature (°C)**	**Temp. stability (°C)**	**CHO***	**Ref.**
Eup-82	*Euphorbia cf. lactea*	SP	80	11.0 (fibrinogen)	4 - 12	35	NR	Yes	Siritapetawee *et al.*,(2015)
Hirtin	*E. hirta*	SP	34	7.2 **(**p-tos-GPRNA*)	3 - 9	50	NR	NR	Patel *et al.*, (2012)
Neriifolin S	*E. neriifolia Linn.*	SP	94	9.5 (casein & haemoglobin)	6 - 11	45	< 65	NR	Yadav *et al.*, (2012)
Streblin	*Streblus asper*	SP	64	9.0 (casein & haemoglobin)	3 - 12.5	65	15 - 85	Yes	Tripathi *et al.*, (2011)
Benghalensin	*F. benghalensis*	SP	47	8.0 (casein & haemoglobin)	5.5 - 10	55	20 - 80	Yes	Sharma *et al.*, (2009)
NR	*Synadenium grantii Hook* f.	SP	76	7 (NR)	5 - 10	60	< 65	NR	Menon *et al.*, (2002)

*SP = Serine protease
*CP = Cysteine protease
*NR = Not recorded
*CHO = Carbohydrate
*p-tos-GPRNA= N-4-tosyl-L- Glycyl-L-Prolyl-L-Arginine -4-nitroanilide;

2.3 Blood hemostasis and fibrinolysis

Blood hemostasis is a specialized event which consists of three overlapping phases which include the process of initiation, amplification and propagation as suggested by Hoffman and Monroe (2001) (**Figure 2.2**).

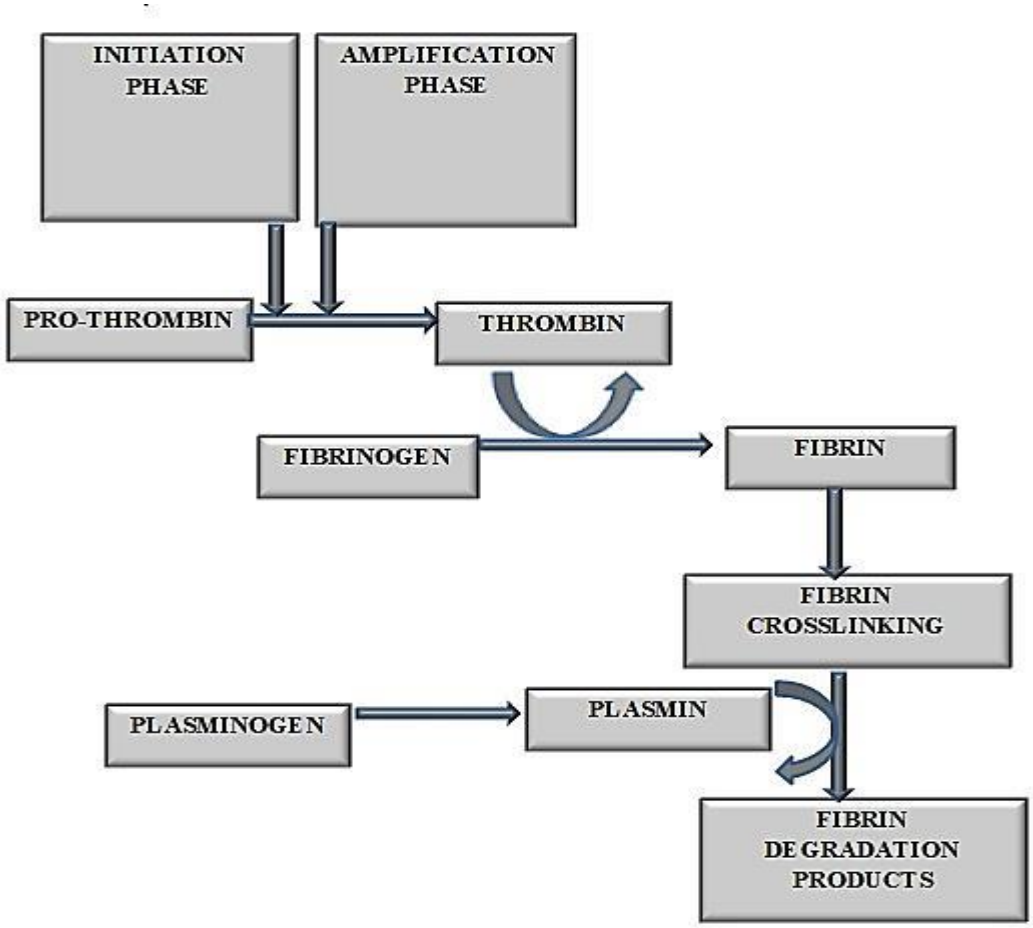


Figure 2.2: Schematic diagram of blood coagulation and fibrinolysis pathways according to the model of Hoffman and Monroe (2001).

Meanwhile, in the classic waterfall cascade model by Macfarlane (1964), the blood coagulation process was described as series of enzyme activations which ultimately results to the activation of thrombin, a serine protease which will convert fibrinogen in circulation into fibrin and subsequently polymerize with each other to form fibrin mesh at the site of injury. The fibrin polymers will be subsequently

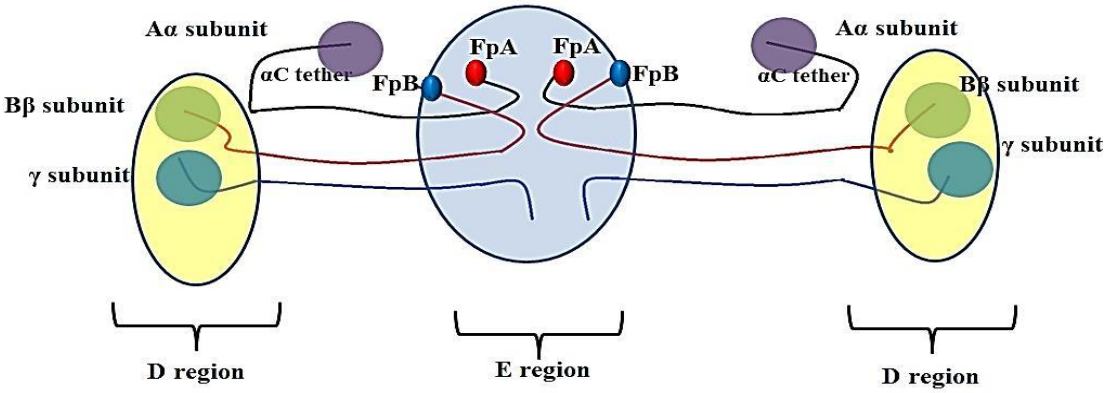
hydrolyzed by another specialized serine protease known as plasmin, once healing is attained.

2.3.1 Fibrinogen

One of the key components in blood hemostasis is fibrinogen, a 340 kDa dimeric glycoprotein which exists in blood circulation at the concentration of 1.5 - 4.0 g/L (Weisel & Litvinov, 2017). Fibrinogen can be converted into fibrin clot when cleaved at specific sites by thrombin. Fibrinogen molecule is composed of three pairs of subunits denoted as the Aα, Bβ and γ chains with the molecular masses of 66.2, 54.2 and 48.3 kDa, respectively (Brennan, 1997).

Fibrinogen has two identical D regions located at the opposite terminals with one E region at the center of the molecule (Medved & Weisel, 2009) (**Figure 2.3**). The N-terminals of all three subunits (Aα, β, and γ) are held together at the central E region by five disulfide bridges. Meanwhile, the C-terminals of all subunits are extended to the opposite sides with each subunit ends up as an individual globular domain (Weisel, 1985). The globular domains (C-terminals) of Bβ and γ are positioned close to each other at the terminals, whereas the C-terminal of Aα chain (known as αC region) folds back to the center of the molecule. Structural elucidations of the αC region was observed to consist of two components, the αC tether (Aα240-410) and the αC nodule (Aα411-629) (Protopopova *et al.*, 2015; Veklich *et al.*, 1993). Both αC regions were reported to be highly susceptible towards proteolytic attacks (Doolittle *et al.*, 1979; Weisel *et al.*, 2017). The αC region plays various important roles with regards to polymerizations, fibrinolysis and crosslinking processes (Gorkun *et al.*, 1998; Veklich *et al.*, 1993; Weisel *et al.*, 2001). Meanwhile, the N-terminals of the Aα chain and Bβ chain denoted as fibrinopeptide A (FpA) and fibrinopeptide B (FpB) will be cleaved off by thrombin during

fibrinogen-fibrin transformation. The schematic diagram of human fibrinogen structure is as depicted in **Figure 2.3**



FpA = Fibrinopeptide A
FpB = Fibrinopeptide B

Figure 2.3: Schematic diagram of human fibrinogen structure based on the model of Medved and Weisel (2009).

2.3.2 Fibrinogen-fibrin transformation

Based on a model proposed by Blomback (1958), the fibrinogen-fibrin transformation can generally be divided into two stages which include the phase of 1) activation of fibrinogen and polymerizations of the activated fibrinogen. The progress of fibrinogen-fibrin polymerization is commonly monitored through spectrophotometric method where the curve obtained usually display a typical gelation pattern which consists of two distinct stages of lag (activation) phase and exponential (polymerization) phase (Blomback & Bark, 2004; Wolberg, 2007) (**Figure 2.4**).

The first stage of fibrinogen activation begins when thrombin cleave FpA and FpB from the N-terminals of their respective subunits. The process of fibrinogen activation is marked by the lag phase at the initial stage of a gelation curve. Thrombin was reported to primarily cleave Aα chain between Arg35-Gly36 and releasing FpA at a faster rate compared to the cleavage of Bβ chain at Arg44-Gly45. The utilizations of other proteases with different specificities however, will result to

different mode of actions. For example, the application of gabonase from the snake venom of *Bitis gabonica* preferentially cleaved Bβ chain compared to the Aα chain (Pirkle, 1986). Proteases of different mechanisms will result to the formation of abnormal clot structures (Matsui *et al.*, 2000).

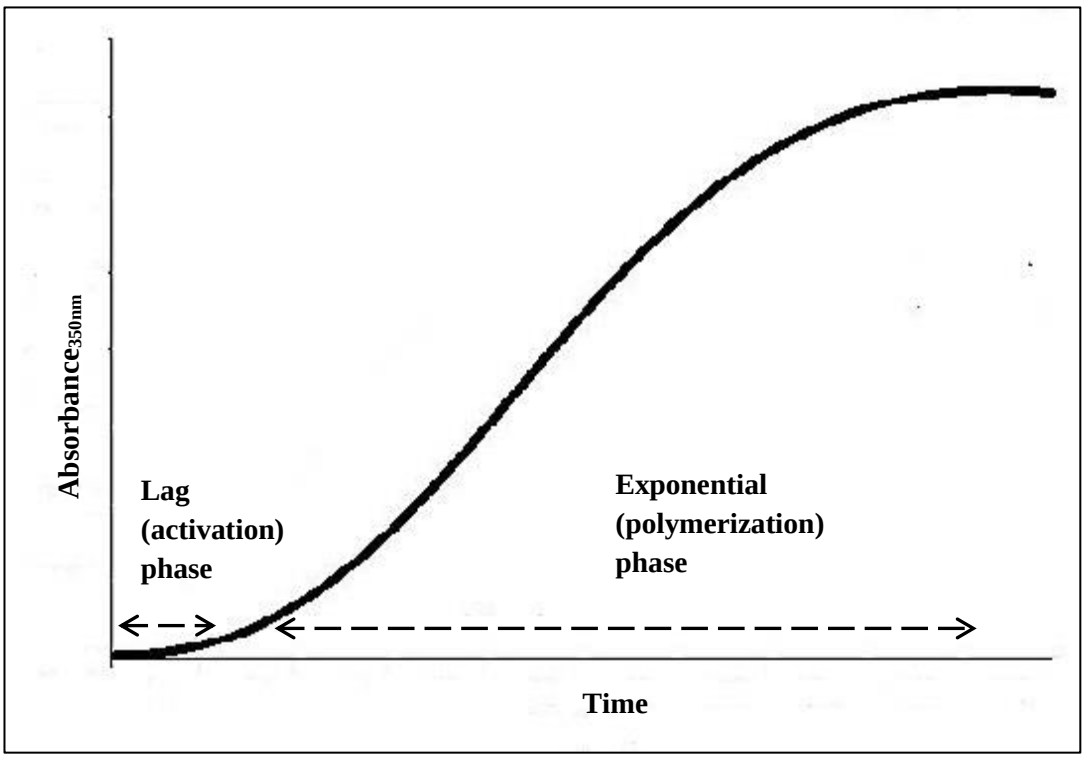


Figure 2.4: A typical fibrinogen gelation profile observed through spectrophotometric method (Blomback & Bark, 2004; Wolberg, 2007).

The subsequent polymerization phase occurs when the newly formed N-terminals after the cleavage of fibrinopeptides termed as 'knobs', interacted with complementary 'holes' located at another fibrinogen molecule. The cleavage of FpA had exposed a new N-terminal ('knob A') with the sequence of Gly-Pro-Arg, complementary to 'hole a' located at the C-terminal of γ chain of another molecule leading to the elongation of the polymers in a half-staggered manner (Litvinov, 2005; Medved & Weisel, 2009). Meanwhile, the cleavage of FpB exposes a sequence of Gly-His-Arg-Pro (knob B) and complementary to 'hole b' located at the β nodule of another molecule. This is turn, will lead to molecule interactions in lateral or side-to-

side aggregations which can be observed through the exponential increase in absorbance in fibrinogen gelation curves (Yang *et al.*, 2000).

2.4 Exogenous hemostatic factors

There are a lot of natural compounds that were discovered to have the capability to interfere with the mammalian's hemostatic system which serve various functionalities to the host, including as defense coordination, incapacitation of prey or as a part of their feeding mechanisms. These compounds termed as exogenous hemostatic factors are isolated from organisms such as insects (Ahn *et al.*, 2003; Czaikoski *et al.*, 2010; Wang *et al.*, 2012), caterpillars (Pinto *et al.*, 2006), fungus (Kim *et al.*, 2006; Moon *et al.*, 2012) bacteria (Lu *et al.*, 2010; Uesugi *et al.*, 2011) plants (Patel *et al.*, 2012; Satish *et al.*, 2012) and animal venoms (Bernades *et al.*, 2008; Samel *et al.*, 2006). Most of these compounds were documented under the Registry of Exogenous Hemostatic Factor of the Scientific and Standardization Committee for the International Society on Thrombosis and Haemostasis. Snake venoms are one of the major sources of the exogenous hemostatic factors and these reptile secretions encompassed various active proteins and peptides (Matsui *et al.*, 2000). Exogenous hemostatic factor generally can be classified into two groups, the pro-coagulants and the anti-coagulants (Chudzinski-Tavassi *et al.*, 2014) as summarized in **Figure 2.5**.

Pro-coagulants are further subdivided into several types including the platelet activators, clotting factor activators and thrombin-like proteases. Meanwhile the anti-coagulant factors can be classified either as plasminogen activators, platelet inhibitor, clotting factor inhibitors or fibrinolytic enzymes. However, this literature review will

focus on only two of these components that are related to fibrinogenolytic and fibrinolytic activities which are the thrombin-like and the fibrinolytic proteases.

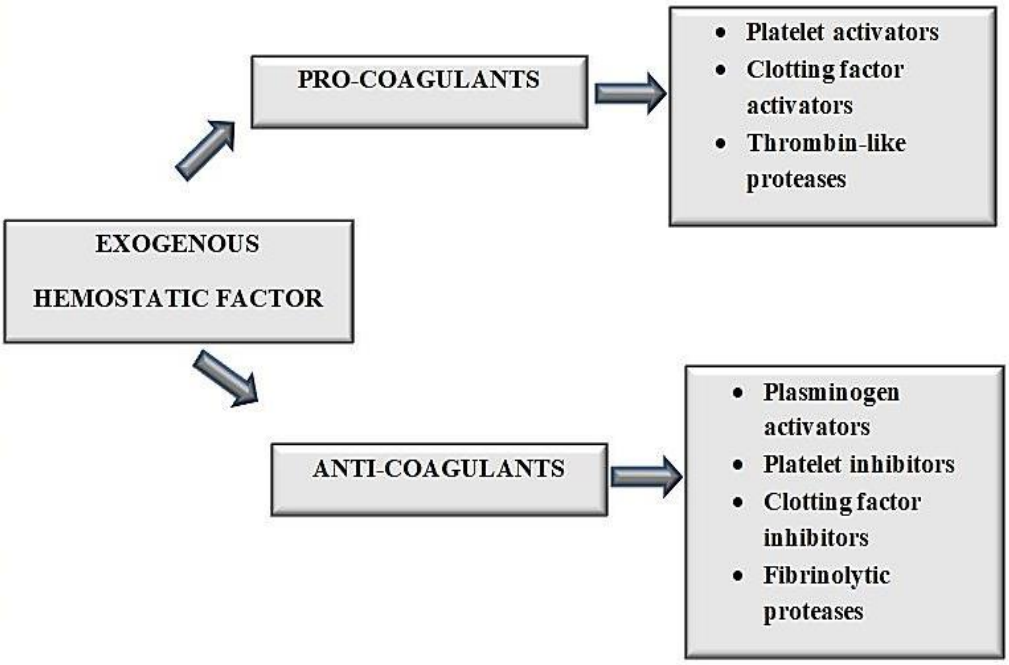


Figure 2.5: Exogenous hemostatic factors and its sub-divisions (Chudzinski-Tavassi *et al.*, 2014).

2.4.1 Thrombin-like enzymes

The term thrombin-like enzyme (TLE) is attributed to protease with the capacity to induce the formation of fibrin clot from fibrinogen and resembles the catalytic actions of thrombin (Mackessey, 2010; Swenson & Markland, 2005). These proteases mimicked the action of thrombin by cleaving at the specific sites of Arg35-Gly36 of the Aα fibrinogen subunit liberating fibrinopeptide A (FpA) or at Arg44-Gly45 of Bβ chain, releasing fibrinopeptide B (FpB). TLEs from venoms thus far were discovered to belong to the serine endoprotease group which is a part of the trypsin-like family (Di Cera, 2009). The snake venom TLEs are currently classified into three categories which are the TLE-AB, TLE-A and TLE-B, each class is distinguished by the type of peptides released from their catalytic action on