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Review Article

Hemostatic Interference of Plant Latex Proteases

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Abstract

Plant latices have been utilized as therapeutic agents to treat various ailments in several traditional systems of medicine. One of the main applications of plant latices is to stop bleeding from minor injuries and to enhance wound healing activity. These activities are associated with hemostatic and fibrinolytic systems. Proteolytic enzymes present in plant latices are found to interfere with hemostatic and fibrinolytic systems. Cysteine proteases of plant latices have been observed for their selectivity towards certain blood coagulation factors and specific cleavage patterns resulting in the induction of blood clot formation. Ficin, a mixture of cysteine proteases from the latex of *Ficus carica* is shown to activate coagulation factor X. Likewise, the purified cysteine proteases, papain from *Carica papaya* latex and pergularain e I from *Pergularia extensa* latex are shown to have thrombin-like activity and directly induce fibrinogen clotting. Plant latex serine proteases including Latex Glycoprotein (LGP) from the latex of *Synadenium grantii* also exhibit procoagulant properties. However, their mechanism of action is not understood. In addition to clot-inducing activity, both the cysteine and serine proteases dissolve blood clot (plasmin-like activity). These properties of plant latex proteases have to be further investigated for their possible utilization in treatment of hemostatic disorders and other clinical applications.

Introduction

Traditional medicine or complementary and alternative medicine (CAM) are implicated in a variety of therapies utilizing indigenous substances to provide health care. Traditional medicine practitioners utilize knowledge and skills that have been used for thousands of years to treat various ailments [1]. These practices vary with geographic distribution and availability of indigenous substances in that region. Traditional medicines become increasingly popular worldwide with the understanding of scientific basis and underlying mechanisms of action. In the modern world, traditional medicine is an integral part of the human healthcare system. Several of the conventionally used pharmacological drugs are derived from traditional medicine [1].

While some are yet to be explored, numerous traditional medicines are under investigation to understand their mechanism of action and for therapeutic utilization. Use of plant latex from several medicinal plants to stop bleeding from minor injuries and to enhance wound healing has been in practice for thousands of years by tribal/rural people of India and other countries [2-5]. These attributes of plant latex have been scientifically investigated for several years. The components of plant latices responsible for these activities have been identified and their biological mechanism of action has been documented. Proteolytically regulated blood coagulation and fibrinolysis are the important events associated with the arrest of bleeding and wound healing process, respectively [6-8]. Plant latices are rich in proteolytic enzymes and are found to have selective actions on blood coagulation factors and the fibrinolytic system. In this review, we have described advancement made in understanding the role of plant latex proteases (PLPs) on hemostasis, and the mechanisms of action of these proteases on blood coagulation and fibrinolytic pathways.

Plant Latex

Plant latex is a viscous fluid exudate produced by the laticiferous tissue found in plants belonging to families; Apocyanaceae, Asclepiadaceae, Caricaceae, Euphorbiaceae, Moraceae etc. [9,10]. More than 35,000 plant species are known to produce latex [1,2]. Plant latex plays important role in plant physiology and the plant self-defense, and is comprised of both inorganic and organic components [11-13]. Importantly, plant latex contains secondary metabolites, proteins and hydrolytic enzymes which have several medicinal values [10,14].

Medicinal Importance of Plant Latex

Among numerous traditional medicines of the plant origin, latices of medicinally important plants are being used to treat various ailments. Most commonly, plant latices are utilized as anthelmintic, analgesic, antinociceptive, to clear skin infections, arrest bleeding from minor injuries and to enhance wound healing [15-17]. In addition, it is known that plant latices have been used in the management of toothache, gum bleeding as well as inducing abortion [3,18,19]. Studies

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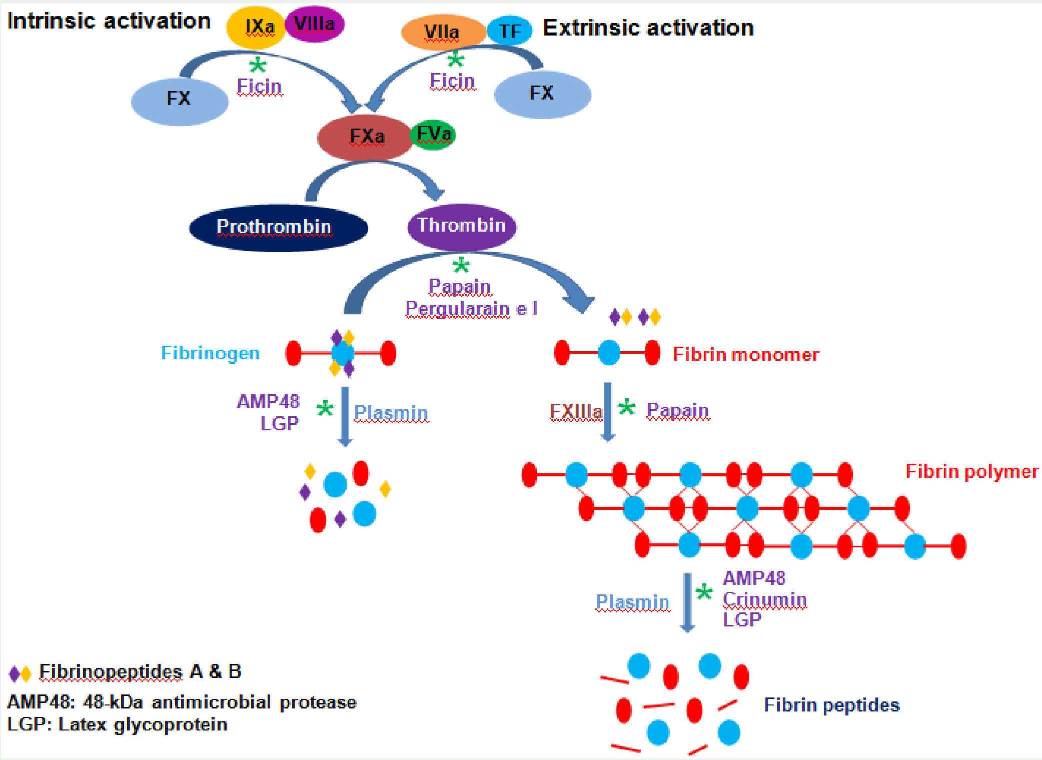

Figure 1: Sites of action of isolated plant latex proteases on blood coagulation cascade and fibrinolysis.
Activation of the intrinsic and the extrinsic pathways of coagulation results in stepwise activation of coagulation factors and results in the generation of thrombin. Thrombin hydrolyses soluble fibrinogen at specific sites releasing fibrinopeptides A and B, and converts into insoluble fibrin. Activation of factor XIII by thrombin cross-links the fibrin assembly to make a meshwork of stable fibrin. Activated fibrinolytic system activates plasmin that degrade fibrin clot. '*' represents examples of plant latex proteases with specific actions on these pathways.

with animal models further elaborated the scope of plant latices as therapeutic agents. For example, plant latices have been shown to prevent ethanol- and aspirin-induced gastric hyperacidity and ulceration in rats [20], suppress autoimmune arthritis by modulating immune mediators in experimental animal models [21,22], anti-hyperglycemic adrenal protective activities, affords protection against complications associated with diabetes in rats [23] and prevents hepatocarcinogenesis in a transgenic mouse model of hepatocellular carcinoma [24]. Interestingly, proteases present in plant latices are involved in several of these observed pharmacological activities [2,10,25].

Plant Latex Proteases (PLPs)

Proteolytic activity in the latex of papaya (*Carica papaya*) was known in the early 1900s [26]. Recent literature survey indicates that latices from hundreds of plants belonging to various families contain at least one proteolytic enzyme [10,27]. More than hundred proteases from plant latices have been isolated and characterized. PLPs are shown to hydrolyze wide variety of protein-substrates including casein, azocasein, gelatin, collagen, fibrinogen, fibrin and several synthetic substrates [2,28,29]. However, specificities or selectivity for their physiological substrates are not clearly known. There is a striking uniqueness in the protease-type present in plant latices. Unlike mammalian system which contain all the four major classes of proteases (serine-, metallo-, cysteine- and aspartate-proteases) [30-33], plant latices contain proteases that belong to either cysteine-or serine-protease class [10,25]. For example, all of the four proteases isolated from *Carica papaya* and *Ficus carica* latex belong to cysteine-protease class [13,34]. Similarly, all of the three proteases isolated in *Euphorbia milii* latex belong to serine-protease class [35,36]. An unusual aspartate-protease isolated from the latex of *Ficus racemosa* and cotinifolin, a metallo protease isolated from *Euphorbia cotinifolia* are two exceptions [37,38]. Furthermore, all the proteases isolated from plant latices are monomers except indicain, a serine protease isolated from latex of *Morus indica* is a dimeric protein [39].

PLPs on Hemostasis

Hemostasis involves a proteolytically regulated system that requires activation of platelets and blood coagulation cascade. Activated platelets create a thrombogenic environment and amplify the coagulation process [40,41]. The coagulation process initiates with the activation of a series of serine proteases which results in the activation of thrombin. Activated thrombin acts on soluble plasma fibrinogen and converts it into an insoluble fibrin network [30,42]. Finally, circulating cells including platelets entrap and enmeshed in the network of fibrin to form a hemostatic plug [40,43]. Fibrinolysis is a process that operates in the opposite of coagulation wherein the formed hemostatic plug is hydrolyzed (Figure 1).The fibrinolytic system comprises of an inactive zymogen form of plasmin, plasminogen. Plasminogen activators, the tissue type plasminogen activator and the urokinase type plasminogen activator, mediate the activation of plasminogen [44]. Thrombin and plasmin are the ultimate enzymes of blood coagulation and fibrinolysis cascades, respectively.

Table 1: List of plant latex proteases (**A**) and crude latex extracts (**B**) with proteolytic activity that are shown to interfere with hemostasis and their target coagulation factors and action. **ND:** not determined.

A

Proteases	Type	Action	Target	References
Eumiilin *(E. milii)*	Cysteine	ND	Fibrinogen	[35]
Ficin *(F. carica)*	Cysteine	Factor X activator	Factor X	[48]
LP_{PII} and LP_{PIII} *(C. procera)*	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[77]
Papain *(C. papaya)*	Cysteine	Thrombin-like and factor XIIIa-like activity	Fibrinogen	[45,56]
Pergularain e I *(P. extensa)*	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[49]
AMP48 *(A. heterophyllus)*	Serine	Fibrino(geno)lytic	Fibrinogen and Fibrin	[58]
Crinumin *(C. asiaticum)*	Serine	Plasmin-like and platelet aggregation inhibition	Fibrin	[78]
Hirtin *(E. hirta)*	Serine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[51]
LGP *(S. grantii)*	Serine	Procoagulant	Fibrinogen and Fibrin	[50]

B

Latex extract	Type	Action	Target	References
Asclepias curassavica	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[25,79]
Calatropis gigantea	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[2,25,80]
Calatropis grandiflora	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[81]
Calatropis puciflorum	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[25]
Euphorbia nivulia	Cysteine	Procoagulant	ND	[82]
Plumeria rubra	Cysteine	Thrombin- and plasmin-like	Fibrinogen and Fibrin	[81]
Synadenium grantii	Serine	Procoagulant	Fibrinogen and Fibrin	[80,82]
Wrightia tinctoria	Serine	Procoagulant	Fibrinogen and Fibrin	[80]
Pedilanthus tithymaloides	ND	Procoagulant	ND	[82]
Ficus domestica	ND	Anticoagulant	ND	[46]
Ficus glabrata	ND	Anticoagulant	ND	[47]

For centuries, latices of several medicinal plants have been used extensively by tribal and rural people of India to arrest bleeding from minor injuries and to enhance wound healing [3]. Blood coagulation and fibrinolysis are the key events associated with the arrest of bleeding and wound healing, respectively. However, there was no scientific study conducted on the involvement of plant latex on these events until 1930s. In 1937, Eagle, et al. reported for the first time the procoagulant (fibrinogen-clotting) activity of papain, a cysteine protease isolated from *C. papaya* [45]. They compared the procoagulant property of papain with trypsin, a digestive enzyme. They found that trypsin was able to coagulate blood indirectly by activating prothrombin to thrombin, whereas, papain did not activate prothorombin, instead act directly on fibrinogen to form fibrin. Although the anticoagulant activity of latices from ficus species was reported, there is no further evidence to substantiate this finding [46,47]. The field did not get much attention till Ritcher, et al. (2002) reported the activation and inactivation of coagulation factor-X by ficin [48]. Where, ficin reduced the activated partial thromboplastin time and the prothrombin time of normal plasma but not of plasma deficient in factor-X. Furthermore, ficin converted Factor-X (FX) to activated Factor-X (FXa) by consecutive proteolytic cleavage. It specifically cleaves the heavy chain of factor-X between Leu178 and Asp179, Arg187 and Gly188, and Arg194 and Ile195 to release carboxy-terminal peptide. After this report, Rajesh, et al. (2005) showed the procoagulant activity of *C. gigantea* latex extract associated with fibrinogenolytic activity mediated by cysteine proteases [2]. They found that *C. gigantea* latex cysteine proteases induce clotting of platelet poor plasma as well as dissolve fibrin of the formed clot. Furthermore, they analyzed the cleavage pattern of purified human fibrinogen, and fibrin by *C. gigantea* latex proteases. There was selectivity in the order of hydrolysis of fibrinogen and fibrin subunits. This selective hydrolysis of fibrinogen and fibrin subunits could be the reason for clot-inducing and clot-dissolving activities of *C. gigantea* latex cysteine proteases. Recent literature survey indicated that there are several latex extracts and over 10 purified proteases (including both cysteine- and serine-proteases) from different plant latices have been reported for their involvement in blood coagulation/fibrinolysis (Table 1).

Both the cysteine and serine proteases from plant latices exhibit procoagulant action. There are only two reports on anticoagulant activities of plant latex [46,47]. However, it is not clear whether the anticoagulant activity is due to the proteases or other constituents present in the plant latex. Cysteine proteases of plant latices are shown to exhibit selectivity towards coagulation factors and induce specific actions [48,49]. Whereas, plant latex serine proteases appear to be non-specific and their mechanisms of procoagulant action is yet to be understood. For instance, latex glycoprotein (LGP), a serine protease isolated from *S. grantii* latex neither forms fibrin from fibrinogen nor induces clot formation in congenital factor X-deficient plasma [50]. Likewise, hirtin, a serine protease isolated from *E. hirita*, is shown to hydrolyze synthetic substrates specific for thrombin but is not observed for direct fibrinogen-clotting activity [51]. These studies clearly suggest that LGP and hirtin lack thrombin-like activity. Similarly, there are few other serine proteases isolated from plant latices that act as procoagulants, but their mechanism of action is not understood. Overall, these serine proteases are shown to hydrolyze fibrinogen, they lack direct fibrinogen-clotting activity (thrombin-like activity), which is observed for plant latex cysteine proteases [49,50]. These observations indicate that plant latex cysteine proteases and serine proteases are different with respect to their procoagulant mechanisms and interference with blood coagulation cascade. The site of action of some of the plant latex proteases on blood coagulation cascade is summarized in Figure 1.

Thrombin-like activity of plant latex proteases

Thrombin-like enzymes are a class of proteases that have the capacity to induce fibrin clots and resembles at least in part to that of thrombin hydrolysis of fibrinogen [52-54]. They specifically hydrolyze the Aα and/or Bβ chains of fibrinogen and release fibrinopeptide A and/or fibrinopeptide B, respectively leading to the formation of fibrin clot [52,55]. Although fibrinogen-clotting activity of papain was reported by Eagle, et al. for the first time in 1937, they did not show whether papain cleaves fibrinogen similar to the thrombin, releases fibrinopeptides A and B, and induces fibrin formation. However, the study suggests that papain might have thrombin-like activity. We isolated for the first time a thrombin-like enzyme pergularain e I, a cysteine protease from *Pergularia extensa* plant latex and studied the mechanism of its action on fibrinogen molecule. Pergularain e I preferentially cleaves Aα and Bβ chains of fibrinogen and releases fibrinopeptides. The release of these fibrinopeptides is predicted to be due to arginine specific hydrolysis of fibrinogen by pergularain e I. Interestingly, the molecular masses of the two peptide fragments released from fibrinogen by pergularain e I were in close agreement with the molecular masses of 16 amino acid sequence of fibrinopeptide A, and 14 amino acid sequence of fibrinopeptide B released by the action of thrombin [49]. More recently, Russell Doolittle, examined detailed mechanism of thrombin-like activity of papain [56]. Wherein, papain cleaves the Aα and Bβ chains of fibrinogen molecule at specific sites releasing fibrinopeptides similar to thrombin and induce fibrin formation by the polymerization of activated fibrinogen monomers. Additionally, it is shown that papain has factor XIIIa-like activity and catalyzes cross-links between adjacent fibrin monomers similar to factor XIIIa. Intermolecular cross-linking of fibrinogen monomers by papain leads to γ-chain dimers, trimers, and tetramers, similar to thrombin-factor XIIIa-stablilized fibrin. However, papain induce covalent cross-linking between chains in neighboring protofibrils in a 'head-to-tail' fashion by transpeptidation occurs between of α-amino group of γ-Tyr1 of one γ-chain and γ-gly403 of the other, in contrast to 'tail-to-tail' transpeptidation that occurs between γ-Lys406 and γ-Gln398 with factor XIIIa [56]. Other plant latex cysteine proteases that exhibited thrombin-like activity appear to have similar mechanisms of action on fibrinogen. Thrombin-like enzymes are identified and characterized from various sources including snake venoms [55]. However, majority of the thrombin-like enzymes isolated from other sources lack coagulation factor XIIIa-like activity which is observed for papain.

Plasmin-like activity of plant latex proteases

Plasmin is a protease that degrades fibrin into soluble fragments. Plasmin is involved in various physiological processes, including thrombolysis and wound healing [57]. Plasmin-like enzymes are proteases that can hydrolyze insoluble fibrin-clot and mimic plasmin in action/function. Apart from blood clot-inducing activity, both cysteine and serine proteases of plant latices have blood-clot dissolving activities [50,51,58]. Interestingly, most of the plant latex proteases that have procoagulant activity also exhibited fibrinolytic activity (plasmin-like activity). Plasmin-like activity of plant latex proteases is studied with a purified serine protease, LPG isolated from *S. grantii* latex. LGP efficiently hydrolyzed fibrin of plasma-clot as well as thrombin-induced fibrin from fibrinogen. However, the cleavage pattern of fibrinogen and fibrin by LGP is different from plasmin hydrolysis. Although LGP hydrolyzes fibrinogen/fibrin, lack thrombin-like activity but apparently have procoagulant activity. The mechanism of having the dual action of plant latex proteases, clot-inducing and dissolving properties needs to be explored. A possible explanation for this unusual action of plant latex proteases could be the selectivity and order of hydrolysis of coagulation factors. However, further studies are required to understand these dual actions. This unique property is observed only for plant latex proteases and not in mammalian or snake venom proteolytic system. Therapeutically, this property of plant latex proteases might play a role in stop bleeding by inducing clot formation and enhancing the wound healing process by dissolving fibrin deposition around the wound. Purified plant latex proteases and crude plant latex, which affect blood coagulation and fibrinolysis are summarized in Table 1.

Other Pharmacological Activities of PLPs

Other pharmacological activities of PLPs that are scientifically evaluated include wound healing, gastric ulcers healing, anthelmintic and anti-microbial activities. Fibrinolysis is associated with wound healing process which helps in removal of dead tissue around the wound, enhancing proliferation of fibroblasts/epithelial cells, and supplying nutrients to the healing tissue by inducing angiogenesis [59,60]. Wound healing activity of plant latices of *C. gigantea*, *C. procera*, *C. papaya* and *W. tintoria* have been examined using animal models [61-63]. It is shown that wound healing activity of plant latex is mediated at least in part by fibrinolytic activity of PLPs [63]. Studies have also shown the healing properties of PLPs in situations of dermatological trauma [64]. In the case of gastric ulcer healing activity, PLPs mediated an increase in mucus content which fastens intestinal ulcer healing [65]. Furthermore, PLPs are capable of detoxifying gliadin and have shown to be suitable for enzyme therapy in gluten intolerance such as in coeliac disease [66]. Anthelmintic activity of PLPs against gastrointestinal nematodes is demonstrated by studies with papaya, pineapple and fig latex [67-69]. However, there is no clear understanding of the mechanisms of actions on anthelmintic activity of PLPs. PLPs with antimicrobial activities were also identified and reported recently [70].

Furthermore, PLPs (bromelain, ficin and papain) are shown to regulate cell signaling by phosphorylation of extracellular signal regulated kinase (ERK), activation of phosopholipase C (PLC) [60] and protease-activated receptors (PAR) [71]. These studies indicate that PLP shave the potential role to regulate downstream cellular and molecular responses. However, further research has to be carried out to understand these actions in detail.

Toxicities of PLPs

Most of the plant latices are toxic in nature and known to induce contact dermatitis, eye irritation, keratouveitis, edema and hemorrhage [2,72,73]. However, only few of the proteases isolated from plant latex are reported to have deleterious effects. Well characterized plant latex cysteine proteases including bromelain, papain and ficin are lethal to lepidopteron insects and larvae by degrading matrix structural proteins on peritrophic membrane and midgut epithelium [13,74]. Papain was also able to induce allergic response by activating T helper type 2 cells [75,76]. Recently, a cysteine protease (Eumiliin) isolated *Euphorbia milii* shown to induce edema, myonecrisis with leukocyte infiltrate and damaged muscle fibers in the footpad of mice following intraplantar injection [35]. On the other hand, no toxic effects have been observed for serine proteases isolated from plant latices. In general, the toxic effects of plant latex are mainly due to substances other than proteases present in them.

Conclusion

Latices of several medicinal plants have been used in the traditional medicine to stop bleeding from minor injuries and to enhance wound healing. These properties of plant latices are attributed to the action of proteases present in them. PLPs have been found to selectively act on factors of blood coagulation and fibrinolytic system. These actions of PLPs results in inducing/dissolving fibrin clot. It will be interesting to study the involvement of PLPs on other components of hemostasis such as platelet functions. Detailed understanding of the interference of PLPs on hemostasis could be exploited for their usefulness in treatment of hemostatic disorders and other clinical applications, and also as tools in blood coagulations research/laboratories.

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