

## Fibrinogen–Fibrin System Regulators from Bloodsuckers

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Received July 3, 2001

Revision received August 2, 2001

**Abstract**—Thrombin inhibitors from bloodsucking leeches and insects that block conversion of fibrinogen to fibrin are considered. Regulatory mechanisms influencing the fibrinogen–fibrin system in leeches include fibrinogen degradation, inhibition of factor XIIIa, and lysis of fibrin clots. The review also summarizes recent data on plasminogen activator from the vampire bat *Desmodus rotundus* and a role of fibrin as its cofactor.

**Key words:** bloodsuckers, insects, leeches, vampire bat, thrombin inhibitors, fibrinogen, fibrin, fibrinogenolysis, fibrinolysis, factor XIIIa inhibitor, plasminogen activator

The fibrinogen–fibrin system controls hemostatic balance. This system plays a major role in maintenance of blood viscosity under normal conditions and in hemostasis under threats of bleeding [1]. It is activated by various enzymes acting in an opposite manner. Thrombin is responsible for the formation of insoluble fibrin, whereas plasmin is a fibrinolytic and thrombolytic enzyme. Thrombin is formed during activation of vascular–platelet and plasma mechanisms of blood coagulation. Thrombin lacks specific sites (Gla-domain) responsible for binding to plasma membranes of cells involved in the activation of prothrombin complex proteins. It appears in the bloodstream and can interact not only with highly specific cell surface receptors but also with fibrinogen (under a certain blood antithrombin potential). Conversion of plasminogen into plasmin requires plasminogen activators. These include tissue activator and urokinase type activator. The activation of plasminogen preferentially occurs on the surface of a fibrin clot and forming plasmin dissolves blood clots. The activity of plasmin and its activators in blood is controlled by inhibitors,  $\alpha_2$ -antiplasmin and PAI-1 and PAI-2 (Plasminogen Activator Inhibitor) produced by monocytes and endothelial cells. The thrombin-activated plasma carboxypeptidase TAFIa (Thrombin Activated Fibrinolysis Inhibitor) inhibits fibrinolysis (and plasmin activity) by cleaving fibrin C-terminal residues Lys and/or Arg involved in plasmin and plasminogen activator binding [2]; otherwise, plasmin will cause proteolytic degradation

of fibrinogen and other proteins involved in blood clotting. Platelets play an important role in the regulation of this system [3]. Transglutaminase, fibrin-stabilizing factor (factor XIIIa), has an independent function. Its activation is mediated by thrombin, which converts inactive precursor factor XIII into the active form. In the presence of calcium ions, factor XIIIa forms  $\epsilon$ -( $\gamma$ -Glu)-Lys isopeptide bonds between adjacent fibrin monomers and therefore regulates hemostatic properties of forming fibrin. Factor XIIIa also stimulates formation of such bonds between fibrinogen and fibrin, fibrin and  $\alpha_2$ -antiplasmin, fibrin and fibronectin [4].

An evolutionarily conserved mode of feeding of bloodsuckers (insects, worms, mammals) by blood of poikilothermic and homoiothermic animals requires their active intervention into hemostasis of the hosts. The first variant of such intervention represents the most optimal pathway of inhibition of host hemostasis by highly specific biologically active compounds produced by salivary glands of the bloodsuckers [5, 6]. The second pathway includes proteolytic degradation of blood plasma fibrinogen by salivary gland secreted proteases. This blocks thrombin mediated conversion of fibrinogen into fibrin and therefore blocks plasma clot formation. The third pathway includes activation of host blood plasminogen into plasmin by salivary gland secretion followed by degradation of fibrinogen and fibrin.

The mechanism of blood protein degradation by endo- and exopeptidase of bloodsuckers requires prolonged sucked blood digestion in their gut channel. This process is regulated by proteolytic enzyme inhibitors

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secreted by salivary glands and/or gut channel wall [5]. The maintenance of liquid state of sucked blood is an important precondition for successful blood digestion in the gut channels of bloodsuckers. This is regulated by specific fibrinolytic enzymes present in salivary gland secret of some bloodsuckers or by compounds influencing dissolution of fibrin clot. In the present review, we consider components of the fibrinogen–fibrin system of bloodsuckers.

## THROMBIN INHIBITORS

Thrombin completes a cascade of enzymatic reactions of the plasma component of hemostasis. However, thrombin not only converts fibrinogen into fibrin clot, but it also regulates the activity of blood coagulation factors and stimulates platelet reactions. Thrombin directly influences hemostatic functions of vascular endothelium and regulates non-hemostatic functions of blood cells [7, 8]. Thus, thrombin inhibition is an effective tool for regulation of blood coagulation and modulation of various complex thrombin-mediated functions.

Since salivary gland secretion of bloodsuckers contains inhibitors of early stages of the hemostatic process (platelet adhesion and aggregation, activator of factor Xa and factor Xa itself), thrombin-inhibitory activity of bloodsucker secretions may inhibit not only blood coagulating activity of this enzyme but also its related functions.

**Leeches.** *Hirudin* produced by medicinal leeches *Hirudo medicinalis* is the most known natural antithrombin. This polypeptide of molecular mass ~7 kD consists of 65–66 amino acid residues. Leeches produce a family of peptide iso-inhibitors which share 85–90% homology with the amino acid sequence of hirudin.

Hirudin is a unique thrombin inhibitor forming a tight stoichiometric complex with the enzyme. It is the only inhibitor that blocks thrombin activity at very low (picomolar) concentrations. Such unique efficacy is explained by the specific mechanism of multiple enzyme–inhibitor interaction which was recognized during analysis of corresponding crystals. In contrast to other known inhibitors of serine proteinases, which contact with the primary binding pocket of the active site, the region of thrombin contact with hirudin is located near this pocket. Hirudin inhibits both free and fibrin clot-bound thrombin [9]. Studies with recombinant hirudin (the gene encoding hirudin was cloned in 1986 [10]) resulted in the appearance of various structural variants of the natural inhibitor; they all share one common property, non-sulfated Tyr63. The latter determined a common name of these inhibitors as “de-sulfohirudins” or “desirudins” [11]. In contrast to the natural inhibitor, recombinant hirudins are characterized by lower affinity to thrombin although anticoagulant properties are the same as in the natural inhibitor. Besides recombinant

hirudin, shorter peptides resembling the structure of functional domains of hirudin have been synthesized; they also exhibit antithrombin activity [12].

**Bufrugin.** This thrombin inhibitor was isolated from the cephalic region of *Hirudinaria mannilensis*, a leech belonging to the same family as the medicinal leech. This protein of molecular mass ~7 kD shares ~66% homology with medicinal leech hirudin. In the test of partial thromboplastin time, bufrugin has the same anticoagulant activity as hirudin. The recombinant variant of this inhibitor was obtained using DNA cloning and expression of cDNA in *E. coli* [12, 13].

**Haemadin.** This tight-binding thrombin inhibitor of molecular mass ~5 kD was isolated from the leech *Haemadipsa silvestris*. Although the amino acid sequence of this inhibitor lacks homology with hirudin, the mechanisms of thrombin inhibition by these inhibitors are the same. Expression of the haemadin gene in *E. coli* cells provided recombinant protein possessing the same biological activity as the native inhibitor [14].

**Theromin.** This potent thrombin inhibitor was isolated from the leech *Theromyzon tessulatum*. It is a homodimer that consists of 67 amino acid residues (including 16 cysteine residues). The amino acid sequence of theromin lacks homology with any known thrombin inhibitor. Besides thrombin inhibition, theromin was shown to decrease in a dose-dependent manner lipopolysaccharide-induced activation of human monocytes and granulocytes [15].

**Granulin.** A cysteine rich (~20%) polypeptide of 6 kD was isolated from the leech *Hirudo nipponia*. The amino acid composition of this acidic peptide (pI 3.75) is close to granulin (or epithelin). It also inhibits thrombin activity [16].

**Insects.** Antithrombin agents have also been isolated from various insect bloodsuckers. They have been found in some species of assassin bugs (Reduviidae). Prolixin was isolated from *Rhodnius prolixus*; triatomin and maculatin were isolated from *Triatoma infestans* and *Eutriatoma maculata*, respectively. Like hirudin, they exhibit anticoagulant activity by blocking thrombin; the mechanism of their action is similar to hirudin and therefore they may be a modification of the same type of inhibitors.

**Prolixin,** the thrombin inhibitor isolated from *Rhodnius prolixus*, has been studied in detail. This highly specific inhibitor is a protein of molecular mass 11 kD consisting of 103 amino acid residues. The recombinant form of this inhibitor was obtained by expression in *E. coli*. Homology of amino acid residues 6–48 and 57–101 inside this sequence suggests two-domain structure of this protein. The amino acid sequence and two-domain structure refer indicate that this inhibitor is a Kazal type inhibitor. Prolixin forms 1 : 1 stoichiometric complex with thrombin. The inhibition constant for both native and recombinant proteins is  $2 \cdot 10^{-13}$  M [12].

**Triabin.** This thrombin inhibitor was isolated from saliva of the bloodsucking bug *Triatoma pallidipensis*. It forms a noncovalent complex with thrombin in molar ratio 1 : 1. Triabin effectively inhibits thrombin-induced platelet aggregation and prolongs thrombin and partial thromboplastin coagulation time, but it is less effective in inhibiting amidolytic thrombin activity assayed with low molecular mass chromogenic substrate. Triabin totally inhibits trypsinolysis of thrombin; this effect is attributed to blockade of an anion-binding exo-site (ABE-1). In a dose-dependent manner, triabin inhibits the effect of thrombomodulin on thrombin. These data suggest that triabin directly acts at the anion-binding exo-site. Determination of the N-terminal amino acid sequence was used to obtain cDNA, and four isoforms of this protein containing 142 amino acid residues were recognized. Recombinant triabin expressed by *E. coli* cells was found in their periplasm; it was active in the test of thrombin-induced platelet aggregation and inhibited fibrinogen hydrolysis by thrombin with  $K_i = 3$  mM [17].

The potent and specific thrombin inhibitor *TTI* (*Tsetse Thrombin Inhibitor*) was found in extracts of salivary glands of the tsetse fly (*Glossina morsitans morsitans*). This is a low molecular mass peptide (3.53 kD). It does not have any homology with known anticoagulants. TTI is a stoichiometric thrombin inhibitor ( $K_i = 584 \cdot 10^{-15}$  M). It can inhibit thrombin-induced platelet aggregation [18].

**Tabanin.** This highly effective inhibitor was isolated from salivary glands of the bloodsucking female gadfly *Tabanus bovinus* [12]. The mechanism of its action is similar to that of hirudin.

**Anophelin.** This polypeptide of molecular mass 6.34 kD was isolated from salivary glands of the mosquito *Anopheles albimanus*. It inhibits thrombin-induced platelet aggregation, esterase activity of thrombin (assayed with synthetic substrate), and fibrinogen cleavage by thrombin [19]. Anophelin is a reversible competitive thrombin inhibitor ( $K_i = 5.87 \pm 1.46$  pM) forming a tight stoichiometric complex with  $\alpha$ -thrombin. Ionic interactions influence the inhibitory activity. In the presence of 0.15 and 0.4 M NaCl, the  $K_i$  values increased by 17.6- and 207-fold, respectively. Studies of the interactions of anophelin with  $\alpha$ - and  $\gamma$ -thrombin revealed that anophelin inhibits both the catalytic site and anion-binding exo-site 1 of thrombin [20].

Secretion of salivary glands of some bloodsucking flies (Diptera: Simuliidae) possesses anticoagulant activity. Extracts of salivary glands of *Simulium argus* Williston and *Simulium vittatum* Zetterstedt exhibit antithrombin activity [21].

**Ticks.** Thrombin inhibitors were isolated from salivary glands of ticks of the genus *Ornithodoros*: *Ornithodoros moubata* and *Ornithodoros savingnyi*. They were named ornithodorin and savignin, respectively.

**Ornithodorin.** This is a potent ( $K_i = 10^{-12}$  M) and highly selective thrombin inhibitor. This protein is char-

acterized by two-domain structure. Each domain has some similarity with Kunitz pancreatic trypsin inhibitor and with TAP (Tick Anticoagulant Peptide) isolated from the same organism [22]. Like hirudin, the N-terminal part of the ornithodorin polypeptide chain binds near the catalytic site of thrombin, whereas the C-terminal domain interacts with the exo-site of fibrinogen recognition [22, 23].

**Savignin.** This is a polypeptide of molecular mass 12.43 kD. It inhibits thrombin-induced platelet aggregation but does not influence ADP- or collagen-induced platelet aggregation. Kinetic experiments revealed that savignin is competitive thrombin inhibitor that is more selective for  $\alpha$ -thrombin ( $K_i = 4.89 \pm 1.39$  pM) than for  $\gamma$ -thrombin ( $K_i = 22.3 \pm 5.9$  nM). It is inactive with respect to plasmin, factor Xa, and trypsin [24].

Thrombin inhibitor was also isolated from the tick *Ixodes ricinus*; it was named *ixin*. The specific antithrombin activity of this mini-protein is 250 U per mg protein [25].

## REGULATORS OF FIBRINOGENOLYSIS AND FIBRINOLYSIS

Under conditions of physiological hemostasis there is a dynamic equilibrium between fibrin formation by thrombin, its stabilization by factor XIIIa, and the fibrin degrading system. It should be noted that stabilized fibrin is much more resistant to the action of blood proteolytic enzymes than fibrinogen and non-stabilized fibrin.

Various antithrombins found in saliva and/or extracts of the bloodsuckers together with inhibitors of platelet–vascular and plasma hemostases not only maintain anticoagulant potential of the salivary gland secretions but also play a role of blood preservatives in the gut channel of the bloodsuckers.

Little is known on the degradation of fibrinogen and fibrin by secretions of bloodsuckers. Here we summarize data obtained using proboscic leeches *Haementeria*, jaw leech *Hirudo medicinalis*, and the bloodsucking vampire bat. We did not find any information on the presence of such biological activity in the salivary gland secretions of the bloodsucking insects.

**Leeches.** The giant proboscic leech *Haementeria ghilianii* can be 50 cm long and its proboscis is about 10 cm. Using this proboscis the leech sucks blood and blood coagulation is blocked in the proboscis by secretion produced by four salivary glands [26]. Blood (15 ml) passes along the proboscis at the rate  $\sim 0.14$  ml/min. The secretion of these leeches does enter the wound, and this explains lack of bleeding of the bitten animals [27]. Besides factor Xa inhibitor [28], the salivary gland secretion contains an enzyme known as hementin. The latter is responsible for proteolysis of blood fibrinogen with formation of products which block conversion of fibrinogen

into fibrin catalyzed by thrombin [29]. Since fibrinogen is involved in formation of platelet aggregates (acting as a bridge between separate cells), this prevents platelet aggregation induced by ADP and collagen. Hementin can also induce deaggregation of platelet aggregation induced by ADP, but not collagen [30]. Hementin has a low affinity to fibrin. However, taking into consideration that the sucked blood contacts the salivary gland secretion and passes the proboscis quicker (about 1 min) than it is required for spontaneous blood coagulation accompanied by fibrin formation, it was suggested that hementin can cause fibrinogen degradation and therefore leave forming thrombin without its natural substrate; besides, fibrinogen degradation prevents platelet aggregation [27]. So hementin is the single exogenic regulator of hemostasis produced by *Haementeria ghilianii*. However, salivary gland secretion of these leeches contains factor XIIIa inhibitor, tridegin, which readily forms a complex with the A- and B-subunits of factor XIIIa. The presence of tridegin together with hementin in the secretion questions the lack of fibrin formation in the leech proboscis and/or in the gut channel during blood storage. It is possible that this inhibitor prevents formation of stabilized fibrin, which is hydrolyzed by hementin at very low rate. Inhibitors of proteolytic enzymes, inhibitors of trypsin, plasmin,  $\alpha$ -chymotrypsin, and granulocyte elastase, were found in extracts of salivary glands of *Haementeria ghilianii* [31]. Perhaps, they are involved in preservation of the sucked blood and regulate its digestion like proteinase inhibitors of the medicinal leech (*Hirudo medicinalis*) [5].

**Hementin.** This is a neutral Ca-dependent metalloproteinase with pH optimum at 7.5 [32]. It consists of a single polypeptide chain with molecular mass 80-120 kD [33]. The amino terminal sequence was determined to be TTLTE-PEPDL [34]. Hementin can induce proteolytic degradation of fibrinogen [29] with  $K_m$   $1.0 \pm 0.1 \mu\text{M}$  [32]. As in the case of plasmin, fibrinogen degradation by hementin results in Y-, D-, and E-fragments. However, a unique action of hementin consists in initial proteolytic attack of  $\alpha$ -helical regions connecting the D- and E-domains of fibrinogen, and three peptide bonds are cleaved (Asn102-Asn103 in the  $A\alpha$ -chain, Lys130-Gln131 in the  $B\beta$ -chain, and Pro76-Asn77 in the  $\gamma$ -chain). In contrast to plasmin, splitting out 111 amino acid residues from the C-terminal region of  $A\alpha$ -chain occurs very slowly [35]. Hementin-induced fibrinogen degradation results in formation of three pairs of products with molecular masses 320 and 300, 225 and 200, and 157 and 132 kD. In each pair the fragment of higher molecular mass contains intact C-terminal regions of  $A\alpha$ -,  $B\beta$ -, and  $\gamma$ -chains. The fragment of 225 kD contains intact  $A\alpha$ -,  $B\beta$ -, and  $\gamma$ -chains of the initial fibrinogen. Products of hementin-induced fibrinogen degradation inhibit fibrinogen coagulation by thrombin [29]. However, hementin cannot induce degradation of isolated chains of fibrinogen. This suggests the requirement of native fib-

rinogen conformation for recognition by the enzyme [33]. Hementin-treated fibrinogen cannot undergo thrombin-induced coagulation even in the presence of blood plasma proteinase inhibitors [29]. Hementin does not influence the activity of plasma proteins other than fibrinogen. The level of blood coagulation factors II, V, VII-XII, prekallikrein, and high molecular weight kininogen remains unchanged in the presence of hementin [32]. Hementin can lyse fibrin clots; however, its fibrinolytic activity is less pronounced than the fibrinogenolytic one [29].

**Tridegin.** This polypeptide consists of 66 amino acid residues (molecular mass 7.6 kD). It shares high homology with two isoforms of ornatin, antagonist of glycoprotein IIb/IIIa (platelet receptor). Ornatin was isolated from leeches *Placobdella ornata* (related to *Haementeria ghilianii*). Some homology (33%) exists between tridegin and ghilanten, inhibitor of factor Xa also isolated from the leech *Haementeria ghilianii*. However, tridegin does not inhibit platelet aggregation (as ornatin) and does not block activity of factor Xa (as ghilanten) [36]. Tridegin is the only natural inhibitor, which blocks both the activity of blood plasma fibrin-stabilizing factor and platelet fibrin-stabilizing factor of factor XIIIa (IC<sub>50</sub> = 0.44  $\mu\text{g/ml}$ ). (The latter represents the catalytic A subunit of factor XIIIa.) Tridegin also inhibits activity of tissue transglutaminase (IC<sub>50</sub> = 1.7  $\mu\text{g/ml}$ ). It does not influence (even at high concentration) activity of other thiol-dependent enzymes such as papain, bromelain, and cathepsin G [37]. The inhibitory effect of tridegin on tissue transglutaminase depends on calcium ions, whereas inhibition of plasma factor XIIIa is Ca<sup>2+</sup>-insensitive. Tridegin inhibits cross-linking (formation of isopeptide bonds) between fibrin and PAI-2 (see above) stimulated by factor XIIIa or tissue transglutaminase. This identified Gln83 and Gln86 in PAI-2 as amino acid residues involved into formation of isopeptide bonds with fibrin and revealed the mechanism responsible for the location of PAI-2 on fibrin, where it inhibits fibrinolysis provoked by urokinase type plasminogen activator [38]. Tridegin does not exhibit total anticoagulant activity. It blocks only the phase of fibrin stabilization [36]: the formation of  $\epsilon$ -( $\gamma$ -Glu)-Lys isopeptide bonds between  $\gamma$ - $\gamma$ - and  $\alpha$ - $\alpha$ -chains fibrin does not occur and therefore it is not accompanied by the formation of D-D-dimer. Lack of isopeptide bonds leads to rapid lysis of plasma clot, which is stimulated by streptokinase, tissue plasminogen activator or hementin [39]. Plasma clots formed in the presence of tridegin are more sensitive to lysis by hementin (time required for 50% lysis in the presence and absence of hementin was 16 and 22.3 h, respectively) [40]. Study of lysis of clots formed from platelet rich plasma revealed that in the presence of tridegin the effect of fibrinolytic enzymes was the same as in platelet poor plasma, whereas lysis of platelet-containing clots occurred slower. This suggests an important role of platelets in the resistance of

plasma clots to fibrinolytic enzymes and importance of cross-linking in this process [39].

All these data on the effects of hementin and tridegin suggest synergic action of these components of salivary glands of *Haementeria ghilianii*. They may be considered as promising thrombolytic agents.

**Hementerin.** This protein exhibiting fibrino(genolytic) activity was isolated from salivary gland extracts of the leech *Haementeria depressa*. Highly purified hementerin consists of a single polypeptide chain with molecular mass of 80 kD. This  $\text{Ca}^{2+}$ -dependent metalloproteinase specifically hydrolyses fibrin(ogen) irrespectively to activation by plasminogen. N-terminal octapeptide sequence shares 80% homology with hementin. However, these proteins differ in kinetic constants and mode of fibrin(ogen) cleavage. Hementerin cleavage of  $\text{A}\alpha$ -,  $\text{B}\beta$ -, and  $\gamma$ -chains of fibrinogen yields degradation products with molecular masses ranging from 270 to 67 kD; they differ from degradation products obtained after fibrinogen cleavage by plasmin and hementin. Hementerin can hydrolyze stabilized fibrin and lyse blood clots at a lower rate than plasmin [41].

**Destabilase.** Secretion of salivary glands of the medicinal leech *Hirudo medicinalis* does not contain proteolytic activity; it is also unable to activate plasminogen [42]. However, leech therapy is known to be very effective in the treatment of various thrombophlebitis [43]. In fact, leech secretion does not affect non-stabilized fibrin; however, stabilized fibrin is lysed during prolonged incubation (for 40 h and even longer) at 37°C and efficacy of lysis depends on the degree of fibrin stabilization. In contrast to monomeric fibrin, the resulting products do not undergo polymerization after dilution with saline [42]. These results suggested the existence of some enzyme specifically hydrolyzing endo- $\epsilon$ -( $\gamma$ -Glu)-Lys isopeptide bonds accompanied by formation of modified fibrin monomers. These modified monomers may appear after the spontaneous depolymerization of destabilized fibrin. Such depolymerization stems from charge redistribution, which occurs after isopeptidolysis: instead of Gln and Lys residues initially involved in isopeptide bond formation (during fibrin stabilization) Glu and Lys appear. The enzyme responsible for such activity in the salivary gland secretion of the medicinal leech is called destabilase [42]. Its molecular mass (determined by SDS-PAGE) is 12.3 kD [44].

Under intravenous injection, partially purified destabilase preparations exhibited thrombolytic properties and thrombi preformed in the rat were lysed by 75 and 100% in 67 and 137 h after intravenous injection of destabilase, respectively [45]. The low rate of thrombolysis correlated with low rate of vascular wall repair. Inconsistency between these parameters during urgent thrombolytic therapy with streptokinase and tissue plasminogen activator is often (in 30% of cases) complicated by repeated thromboses, which are not seen during the leech therapy

of thrombophlebitis. The latter may be attributed to the thrombolytic properties of the leech secretion.

D-D-dimer, a protein of molecular mass 190 kD, is the substrate of destabilase. This protein contains fragments of all three chains of monomer fibrin ( $\alpha$ ,  $\beta$ ,  $\gamma$ ). The D-D-dimer represents a fragment of stabilized fibrin; it contains two isopeptide bonds formed by Lys405 and Gln397 in C-terminal regions of  $\gamma$ -chains [46]. Destabilase causes monomerization of D-D-dimer [47], and there is a nonlinear dependence of the reaction rate on substrate concentration [48]. Sequence analysis revealed the identity of three N-terminal amino acids from the  $\gamma$ - $\gamma$ -chains of D-D-dimer and  $\gamma$ -chains of formed D-monomer indicating preservation of the integrity of these polypeptide chains. These results agree with the hypothesis that the isopeptide bonds are cleaved during the monomerization of D-D-dimer by destabilase [49, 50]. The stabilized fibrin is also a substrate of destabilase, which catalyzes hydrolysis of isopeptide bonds connecting  $\gamma$ - $\gamma$ - and  $\alpha$ - $\alpha$ -chains of this protein [47].

Decoding of the primary structure of this enzyme allowed the synthesis of primers and the revelation of a family of three genes encoding destabilase; they encode proteins differing by some substitutions in the primary structure [51, 52]. Attempts to express the gene encoding destabilase in *E. coli* or yeast cells were not successful. Recombinant protein containing seven disulfide bonds was elaborated as inclusion bodies and it was impossible to solubilize them. Only expression in a baculoviral system provided cell extract containing catalytically active recombinant destabilase exhibiting monomerization of D-D-dimer [51]. The comparison of the amino acid sequence of destabilase revealed high homology of this protein with invertebrate lysozyme. Besides D-D-dimer monomerizing activity, it also exhibited lysozyme activity with respect cell walls of *Micrococcus lysodeikticus*. Repeated expression of the destabilase gene in the baculoviral system revealed lysozyme activity in the cell extracts; it was demonstrated that the sensitivity of the lysozyme test was significantly higher than the D-D-dimer monomerizing one. For example, lysozyme activity can be detected when  $4.5 \cdot 10^6$  active molecules per cell are present, whereas D-D-dimer monomerizing activity is detected only at the level not less than  $10^8$  active molecules per cell [53]. So until recently, it remained unclear whether these activities represent a characteristic property of one or two proteins, and what is the ratio between D-D-dimer monomerizing and lysozyme activities in the cell extracts. Recently these two activities were separated using reversed-phase chromatography of highly purified protein using a C18 column; these activities were also separated by HPLC of salivary gland secretion of the medicinal leech using Superose S-12 gel filtration [54]. Further experiments are required to demonstrate whether these activities can be attributed to two different proteins or to isoforms of one protein. It is known that destabilase

is very sensitive to external effects; for example, low concentrations of any detergents convert destabilase into a highly active proteinase that can hydrolyze not only D-D-dimer, but also casein and bovine serum albumin [55].

*$\gamma$ -Glutamyl transpeptidase.* This protein catalyzing cleavage of the synthetic substrate  $\gamma$ -Glu-pNA (*p*-nitroaniline) was isolated from salivary gland secretion and gut channel content of the medicinal leech *Hirudo medicinalis*. It consists of 600 amino acid residues and has molecular mass of 65.52 kD. The complete nucleotide sequence of the gene coding this protein and primary structure of the protein are revealed. The N-terminal region of  $\gamma$ -glutamyl transpeptidase shares 41.5% identity with a large subunit of human blood  $\gamma$ -glutamyl transpeptidase; the C-terminal region of the leech enzyme shares 48.9% identity with the small subunit of the human blood enzyme [56]. Binding of this protein to concanavalin A suggests its glycosylation. SDS-PAGE revealed that this enzyme can cleave  $\alpha$ - $\alpha$ - and  $\gamma$ - $\gamma$ -chains of stabilized fibrin which are linked by  $\epsilon$ -( $\gamma$ -Glu)-Lys isopeptide bonds. The appearance of additional bands suggests that proteolysis also occurs. The properties of this enzyme are similar to those of bovine  $\gamma$ -glutamyl transpeptidase [56].

*Proteinase AhP (A. hydrophyla proteinase).* This enzyme is secreted by symbiotic bacteria of the medicinal leech, *Aeromonas hydrophyla*, which inhabit the leech gut channel [57]. AhP is Zn-dependent proteinase of molecular mass 19 kD containing one zinc atom per molecule; it is sensitive to inhibition by 1,10-phenanthroline. Iodoacetate, leupeptin, DFP, PMSF, pepstatin, and  $\alpha_2$ -macroglobulin do not influence AhP activity. The N-terminal amino acid sequence shares 50% identity with some known Zn-dependent proteinases. AhP selectively cleaves the peptide bond after the second Gly residues in the sequence Gly-Gly- $X_{X_1}$ - $X_{X_2}$ . The dimer of  $\gamma$ -chain of fibrin containing the sequence Gly-Gly-Ala-Lys- $N_{\epsilon}$ - $\gamma$ -Glu in the region of isopeptide bond formation ( $\epsilon$ -( $\gamma$ -Glu)-Lys) is the substrate of this enzyme. In stabilized fibrin, AhP hydrolyzes the peptide bond Gly-Ala and this is accompanied by the formation of two "pseudo  $\gamma$ -monomers" [58]. In contrast to stabilized fibrin, fibrinogen is not a substrate for AhP because it contains unsubstituted Lys residue in the sequence Gly-Gly-Ala-Lys, which is a potential region for cross-linking; this provides a conformation of the fibrinogen molecule that differs from that of fibrin. With substrate Suc-Gly-Gly- $N_{ph}$ -CONH<sub>2</sub> (where  $N_{ph}$  is *p*-nitrophenylalanine)  $k_{cat}/K_m$  is 140-1000 M<sup>-1</sup>·sec<sup>-1</sup>. The presence of AhP accelerates lysis of stabilized fibrin by tissue activator of plasminogen [58]. A similar mechanism was recognized for the matrix metalloproteinases stomelysin and martilysin, and membrane matrix metalloproteinase type I [59].

Attempts to apply the same mechanism to D-D-dimer monomerizing activity of destabilase are not correct because the latter is not a metalloproteinase enzyme (unpublished observation).

*DSPA (Desmodus Salivary Plasminogen Activator).* This protein activating conversion of plasminogen into plasmin was isolated from saliva of the South American vampire bat *Desmodus rotundus* [60]. It exists as four genetically and biochemically distinct isoforms each of which has a certain combination of structural fragments (domains) also typical for urokinase and tissue activators of plasminogen. They are the so-called finger (F) domain, epidermal growth factor-like (E) domain, kringle (K), and protease (P) domain. The latter is typical for serine proteinases. The isoforms are designated as FEKP (DSPA $\alpha$ -1 and  $\alpha$ -2), EKP (DSPA $\beta$ ), and KP (DSPA $\gamma$ ) [61]. They all represent single chain glycosylated proteins with molecular masses of 49, 42, and 40 kD. The primary structure of DSPA (FEKP) shares 85% homology with tissue plasminogen activator (t-PA) [62]. In the absence of fibrin, neither form can activate conversion of Glu-plasminogen into plasmin, and the  $k_{cat}/K_m$  value is only 4 M<sup>-1</sup>·sec<sup>-1</sup> (with t-PA this parameter is significantly higher, 42 M<sup>-1</sup>·sec<sup>-1</sup>). However, in the presence of fibrin monomers the activating effect of DSPA $\alpha$ -1 and  $\alpha$ -2 sharply increases (the  $k_{cat}/K_m$  value is 970 M<sup>-1</sup>·sec<sup>-1</sup>). In the presence of non-stabilized and stabilized fibrin this effect is much higher (54,000 and 174,000 M<sup>-1</sup>·sec<sup>-1</sup>, respectively). Similar catalytic behavior was also typical for t-PA in the presence of fibrin-2 [63]. This implies the important role of binding of DSPA $\alpha$ -1 and  $\alpha$ -2 with fibrin. In fact, non-protease domains and the protease domain of this activator irreversibly bind to fibrin, but the mechanisms of this binding are different [64]. The cofactor effect of fibrin is known to be determined by its interaction with the activator and plasminogen [65]. In t-PA, the F-domain and kringle-2 are involved in the interaction with fibrin. In the case of DSPA $\alpha$ -1, its F-domain also interacts with fibrin; since it lacks kringle-2, low affinity sites of E- and K-domains are also involved in this interaction. Involvement of these domains is supported by the fact that DSPA $\beta$  and DSPA $\gamma$  are homologous to DSPA $\alpha$ -1 by 89 and 90%, respectively, but lacking the F-domain are also stimulated by fibrin (although to a lesser extent) during activation of plasminogen [61]. Comparison of the characteristic features of DSPA and t-PA is important because DSPA $\alpha$ -1 has some advantages over this widely used thrombolytic agent. Fibrin stimulates the catalytic efficacy ( $k_{cat}/K_m$ ) of DSPA $\alpha$ -1 up to 100,000-fold, whereas in the case of t-PA this stimulation is "only" 550-fold. The selectivity of the fibrin effect (evaluated as fibrin/fibrinogen ratio of the bimolecular rate constants of plasminogen activation) is 13,000, 6,500, 250, 90, and 72 for DSPA $\alpha$ -1, DSPA $\alpha$ -2, DSPA $\beta$ , DSPA $\gamma$ , and t-PA, respectively [61]. High molecular weight products of fibrin degradation can also act as cofactors during plasminogen activation. The effect of TAFIa reduces their efficacy with respect to DSPA $\alpha$ -1 by 215 times, whereas t-PA activation was 90 times less after treatment with TAFIa. This suggests the important role of

F-domain binding sites for fibrin and products of its degradation during DSPA $\alpha$ -1 activation [66]. In contrast to t-PA, intravenous administration of DSPA to animals did not cause activation of blood plasma plasminogen and degradation of fibrinogen and factor VIII. So DSPA administration is much safer in terms of possibility of increased bleeding which often accompanies t-PA injections [67, 68]. Preclinical and clinical trials revealed certain advantages of DSPA over t-PA, such as extremely high affinity for fibrin and prolonged half-life time in blood plasma (2.8 h for DSPA versus 4-8 min for t-PA); thus, a single dose of DSPA may be sufficient for thrombolysis [69]. So, DSPA is considered now as an ideal thrombolytic agent [70, 71] like tenecteplase, lanoteplase, staphylokinase, and saruplase [69].

This review provides convincing evidence that adaptation to feeding with vertebrate blood and its subsequent digestion by bloodsuckers occurs via directed synthesis of highly specific biologically active compounds in salivary glands of these animals. Studies of the mechanisms of their action on certain components of the hemostatic process can be used to regulate particular stages of hemostasis. Recombinant analogs of these compounds are already used in clinical practice (e.g., hirudin) [72] or intensively studied in preclinical and clinical trials: DSPA [69], antistasin, and TAP as factor Xa inhibitors from the leech (*Haementeria officinalis*) and the tick (*Ornithodoros moubata*), respectively [73], and also inhibitors of adhesion and platelet aggregation from leeches [74]. Thus, it is important to extend the use of exogenous regulators of hemostasis as effective and safe agents for the treatment of various cardiovascular diseases.

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