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REVIEW

Proteinase Inhibitors from the Medicinal Leech *Hirudo medicinalis*

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Abstract—The medicinal leech *Hirudo medicinalis* produces various types of proteinase inhibitors: bdellins (inhibitors of trypsin, plasmin, and acrosin), hirustasin (inhibitor of tissue kallikrein, trypsin, α-chymotrypsin, and granulocyte cathepsin G), tryptase inhibitor, eglins (inhibitors of α-chymotrypsin, subtilisin, and chymasin and the granulocyte proteinases elastase and cathepsin G), inhibitor of factor Xa, hirudin (thrombin inhibitor), inhibitor of carboxypeptidase, and inhibitor of complement component C1s. This review summarizes data on their primary and tertiary structures, action mechanisms, and biological activities.

Key words: medicinal leech, bdellins, eglins, hirustasin, tryptase inhibitor, factor Xa inhibitor, hirudin, carboxypeptidase inhibitor, component complement inhibitor, trypsin, chymotrypsin, thrombin, kallikrein, subtilisin, granulocyte elastase, granulocyte cathepsin G

Bloodsucking leeches represent a special category of annelids related to various orders and families of the class *Hirudinea*. They are exclusively fed by blood of poikilothermic and homeothermic animals. Adaptation to this "mode of nutrition" resulted in the evolutionary selection and fixation of specific mechanisms typical of bloodsuckers. They include prevention of clotting of sucked blood and its prolonged conservation in the gut channel of the leech. The salivary glands play an important regulatory role in the functioning of these mechanisms. In medicinal leeches (*Hirudo medicinalis*), salivary gland ducts open to a special channel that exists in each of 90 sharp denticles located on each of three jaws [1]. Using these denticles, the leech bites the skin of its victim and simultaneously injects salivary gland secretion. Part of this secretion is adsorbed on the surface of the damaged blood vessel of the host, whereas another part is mixed with blood flowing out of the wound. Sucked blood containing the major proportion of the secretion is accumulated in the gut channel, which is located along the whole body of the leech. This channel contains many appendices that serve as stores of sucked blood. Once having sucked in blood, the leech may function for a long time (sometimes up to one year or even longer). Blood digestion occurs very slowly in the distal part of the gut channel; this process involves exo- and endopeptidases secreted by bacterial symbionts living in the gut channel. The low rate of degradation of blood proteins in the leech gut channel is regulated by proteolytic enzyme inhibitors secreted by the gut wall [2]; they are also found in the salivary gland secretion [3]. This review summarizes data on the structure and mechanisms of action of these inhibitors in the medicinal leech *Hirudo medicinalis*.

Proteinase inhibitors from the medicinal leech represent a group of proteins that can block catalytic activity of proteolytic enzymes of various classes. They possess different structures and mechanisms of proteinase inhibition.

Serine proteinases possess a wide spectrum of biological functions; they are involved in numerous physiological processes from food digestion and regulation of blood clotting to modification of extracellular matrix. They play an important role in the regulation of the nervous and immune systems. This explains why inhibitors of serine proteinases are one of the best-studied groups of proteinase inhibitors. The main function of these inhibitors is the elimination of the proteolytic enzymes from biological systems; this includes an interaction of the enzyme–inhibitor complex with receptors via a special recognition site of the receptor molecule [4]. Although proteolytic enzyme inhibitors produced by the medicinal leech differ in structure from the classic serine

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proteinase inhibitors, they have similar functions. In this review, we describe known groups of proteolytic enzyme inhibitors found in the medicinal leech.

BDELLINS

Bdellins are inhibitors of trypsin, plasmin, and sperm acrosin. They are distributed over the whole body of the medicinal leech. The highest concentration is found in the region of the external genital organs. This suggests that bdellins are involved in reproductive functions of the medicinal leech [5]. Bdellins were initially found by Fritz et al. [6] in crude hirudin preparations isolated from whole leeches. They identified bdellins as two related groups of proteins possessing the same activity but differing in anion-exchange chromatography behavior, amino acid composition, and molecular masses. Proteins eluted from DEAE-cellulose by the initial buffer (0.1 M sodium acetate, pH 6.0) were denominated bdellins A and proteins eluted from DEAE-cellulose by the same buffer containing 0.4 M NaCl were denominated as bdellins B. The molecular masses of bdellins A and B determined by gel filtration are 7 and 5 kD, respectively [7]. Equilibrium chromatography on DEAE-cellulose resulted in the separation of multiple forms of bdellins A and B. According to their elution, they were denominated from A1 to A6 and B1 to B6. Bdellins A and B are potent inhibitors of trypsin, plasmin, and sperm acrosin with K_i values within the concentration range from 10^{-7} to 10^{-10} M. They do not block catalytic activity of chymotrypsin, tissue and plasma kallikrein, and subtilisin. Bdellins A and B form equimolar complexes with corresponding enzymes, and the P_1 position of the reactive site of these inhibitors contain a lysine residue (Lys8) [5].

Type B bdellins. Using polyacrylamide gel electrophoresis, Seemuller et al. [8] found that bdellins B have apparent molecular masses of 20 and 38 kD. In contrast to the low molecular weight forms, these bdellins were denominated as high molecular weight bdellins group B (HMB). Later it was demonstrated that HMB fraction III (molecular mass of 20 kD) exhibits the highest inhibitory activity [9]. The N-terminal amino acid sequence of this bdellin corresponded to the sequence of low molecular weight bdellin B-3. The eighty amino acid residue C-terminal sequence of HMB displays an unusual composition of amino acids including 15 Asx, 25 Glx, 6 Gly, 1 Val, 26-27 His, and 4 Lys [9]. It remains unclear whether the low molecular weight bdellin B-3 is formed from HMB (or, perhaps, from a higher molecular weight precursor) under physiological conditions in the medicinal leech, or HMB is cleaved during the isolation procedure.

Inhibitory activity of low molecular weight bdellin B-3 and HMB with respect to trypsin, plasmin, and acrosin is characterized by the same K_i values, $<10^{-10}$ M. This suggests that the enzyme affinity for the inhibitor does not depend on the length of its polypeptide chain. The long C-terminal fragment of HMB night be involved in physiological binding of the inhibitor to cell membranes [9]. Although bdellin B-3 is a Kazal-type inhibitor, its properties differ from those of classic representatives of this group such as secreted bovine pancreatic trypsin inhibitor [10] or the third domain of silver pheasant ovomucoid [11].

In contrast to classic inhibitors of this group, bdellin B-3 contains only 37 amino acid residues between fixed half-cystines, whereas other members of this group usually contain 46-56 amino acid residues between the first and the last half-cystines. It is one of the shortest Kazal-type inhibitors. This protein possessing compact structure is considered as the first member of a subfamily of non-classic Kazal-type inhibitors [9]. The location of disulfide bonds and spatial structure of bdellin B-3 [5] are consistent with models of classic Kazal-type inhibitors [12, 13]. A proteinase-binding loop including cysteine residues (16-35) and Asn33 is connected with the protein core; it forms a conservative structure responsible for tight binding to trypsin [9].

Type A bdellins, bdellastasin. For a long time, the structural features of type A bdellins remained uncharacterized. In a recent study, Moser et al. provided extensive information on these proteins [14]. They used the most active fraction of bdellin A (bdellin A 2,3) described by Fritz et al. [7] as the starting material. This highly purified fraction was employed for determination of primary structure of bdellin A. This protein (molecular mass 6333 daltons) contains 59 amino acid residues; it is stabilized by five disulfide bonds. It is a homolog of antistasin, the first member of a new family of serine proteinase inhibitors produced by the Mexican leech *Haementeria officinalis* [15]. Therefore, bdellin A was renamed bdellastasin to avoid confusion with bdellin B [15]. Antistasin is a cysteine-rich protein of molecular of 17 kD. It inhibits the activity of the serine enzyme factor Xa (K_i = 0.5 nM). Its primary structure contains two homologous domains. Antistasin does not have close similarity with any known proteinase inhibitor. It is the prototype of a new family [16] characterized by strict conservative localization of 10 cysteine residues [17]. The amino acid sequence of bdellastasin shares some homology with the first and second domains of antistasin (29.8 and 28.7%, respectively).

Moser et al. [14] expressed bdellastasin in *Saccharomyces cerevisiae* [14]. The recombinant protein exhibited the same physicochemical parameters, amino acid sequence, and the inhibitory activity with respect to plasmin and trypsin as the wild protein; however, the K_i for plasmin inhibition by the recombinant protein was twofold less than that for the wild-type bdellastasin [14]. The conformation of bdellastasin was analyzed using circular dichroism spectra in the far UV region. These data indicate that both the wild and recombinant bdellastasins

contain random coil elements. Both types of this protein lack α-helices and β-sheet structures [14]. Bdellastasin complexes with bovine or pig β-trypsin crystallize as wedge-shaped or tetrahedral crystals containing six or one molecules per asymmetric unit, respectively.

Like antistasin, the single domain bdellastasin consists of two subdomains. The interaction between bdellastasin and trypsin is limited by the C-terminal subdomain of the inhibitor (it involves the primary binding loop that includes amino acid residues Asp30-Glu38) [18]. In contrast to other antistasin-type trypsin inhibitors containing an Arg residue at the P_1 position, the reactive center of bdellastasin contains Lys34 at the P_1 position. Bdellastasin inhibits trypsin (K_i = 1 nM), plasmin (K_i = 24 nM), and the trypsin-like enzyme acrosin [14]. It does not affect the activity of factor Xa, thrombin, plasma kallikrein, or chymotrypsin. Several models of bdellastasin complexes with some proteinases (or their fragments) were built using data of X-ray analysis. These include bdellastasin complexes with trypsin, plasmin fragment, μ-plasmin, or mycoplasmin containing the A chain of the enzyme (which consists of 20 amino acid residues) and the B chain (229 residues) linked with the former by two disulfide bonds [18]. These models demonstrate that the recognition of bdellastasin by trypsin and mycoplasmin occurs via a canonic principle of binding with proteinase inhibitors. Comparison of these three-dimensional structures with antistasin structure revealed that in contrast to antistasin binding to factor Xa, the inhibitor to trypsin and plasmin is accompanied by some shift of the P_5-P_3 segment of the loop responsible for their primary binding; this determines the different specificity of bdellastasin and antistasin [18].

HIRUSTASIN

Hirustasin isolated from extracts of the medicinal leech [19] also belongs to the same family of antistasin-like inhibitors of serine proteinases. Using cation-exchange chromatography and isoelectrofocusing ($pI_{hirustasin}$ = 9.6), hirustasin was separated from trypsin inhibitors present in the extracts. Subsequent use of affinity chromatography on anhydrotrypsin-Sepharose and high performance liquid cation-exchange chromatography produced two isoforms of this protein that differed by only one amino acid residue at the C-terminus (AKASQ and AKAS). The authors suggested that these two isoforms are formed by limited proteolysis of a higher molecular weight precursor; it is also possible that carboxypeptidase cleaves the glutamine residue before or during purification of this protein. The hirustasin molecule (molecular mass 5869) contains 55 amino acid residues including 10 cysteines. The locations of these cysteines in the polypeptide chain correspond to the positions of cysteine in the first and second domains of antistasin from the Mexican leech *Haementeria officinalis*, ghilianten from the Amazon leech *Haementeria ghilianii*, and guamerin from the Korean leech *Hirudo nipponia*. The primary structure of hirustasin shares some homology with the first and second domains of antistasin (27 and 32%, respectively). Ten cysteine residues of hirustasin molecules form five disulfide bonds that determine the compactness of spatial structure of this inhibitor. Two subdomains are recognized in hirustasin molecules. The C-terminal subdomain (24-53 amino acid residues) contains two disulfide bonds, whereas the N-terminal subdomain (3-23 amino acid residues) contains three disulfide bonds. Wild and recombinant hirustasins have the same N-terminal amino acid sequence, molecular mass, and biological activity [20]. The positions of S–S bonds in hirustasin molecules were determined using X-ray analysis of crystals of free recombinant hirustasin and its complex with tissue kallikrein. The N-terminal part contains disulfide bonds between cysteine residues 6-17 and 11-22, whereas the C-terminal part contains disulfide bridges between cysteine residues 24-44, 29-48, and 33-55 [20].

Hirustasin contains Arg30 in the P_1 position, and the Arg30-Ile31 peptide bond has been identified as the reactive site of this inhibitor [19]. This bond is hydrolyzed after prolonged incubation of hirustasin with trypsin. As in antistasin, the reactive site of hirustasin contains an Arg residue; the eight amino acid residues in P_5-P_3' positions are also identical to those found in the inhibitory domain of antistasin. There are only two conservative substitutions in the P_3 and P_2 positions: His28-Arg and Arg32-His in hirustasin and antistasin, respectively [19]. However, in contrast to antistasin, hirustasin does not inhibit factor Xa activity, but it does inhibit tissue kallikrein, trypsin, chymotrypsin, and neutrophil cathepsin G [19]. The ability of hirustasin to inhibit tissue (but not plasma) kallikrein is a very important property of this protein. Tissue kallikrein is one of a group of serine proteinases having rather narrow substrate specificity [21]. It is also included into the family of S1 peptidases (known as the trypsin family) and the subfamily of glandular kallikrein [22]. Tissue kallikreins catalyze the release of highly active kinins, kallidin or lysyl-bradykinin, from kininogens, by cleaving bonds Met-Lys and Arg-Ser [23]. Kinins have a wide spectrum of biological effects (realized via specific receptors on target cells) including vasodilatation, hypotension, and relaxation or contraction of smooth musculature. The tissue kallikrein–kinin system is involved in the maintenance of normal blood pressure [24]. The pathophysiological importance of kallikrein is determined not only by uncontrolled high level of kinins. For example, human glandular kallikrein-1 is similar to an antigen typical for prostate. It does not release kinins from kininogens and is directly linked to malignant tumors and their metastasizing [25]. An increased level of tissue kallikrein has been found in human cell carcinomas and breast cancer [26, 27].

The primary binding loop for tissue kallikrein is located in the region of amino acid residues 26-32, whereas the secondary binding loop is located in the region of amino acid residues 46-48 of the C-terminal subdomain [28]. Among elements of secondary structure, only β-sheets (two in the N-terminal and one in the C-terminal domains) was found [29].

The C-terminal subdomains of two molecules form a tight dimer in a region of antiparallel β-sheet structures [28]. In spite of compact packing, hirustasin (as well as bdellastasin and antistasin) is a rather flexible molecule. This flexibility is important for the conformational adaptation recognized by corresponding proteinase. For example, binding with kallikrein changes the relative orientation of the subdomains. This change is accompanied by a turn of the primary binding loop by 180° and *cis-trans* isomerization of Pro47 in the secondary binding loop.

Using recombinant hirustasin, it was shown that the interaction of hirustasin with tissue kallikrein is accompanied by its preferential cleavage at the peptide bond Arg30-Ile31. Crystals of hirustasin–kallikrein complexes are stable only for 4-5 days; then they change their morphology and begin to dissolve. Incubation of hirustasin with kallikrein for 6 days was accompanied by reduction of the content of the inhibitor as determined by SDS electrophoresis. Taken together, these data suggest temporal inhibition of tissue kallikrein by hirustasin [20]. Under this type of inhibition, prolonged incubation results in the appearance of catalytic activity in the enzyme–inhibitor complex. This temporal inhibition may be explained by prolonged proteolytic degradation of a modified form of the inhibitor that is more sensitive to proteolysis than the native inhibitor [30]. Such behavior was found for trypsin complexes with Kazal-type inhibitors (hen egg ovomucoid, bovine secreted trypsin inhibitor, pig pancreatic secreted trypsin inhibitor type I, and human pancreatic secreted trypsin inhibitor) [31-33]. Antistasin also exhibits temporal inhibition of blood plasma Xa factor [34].

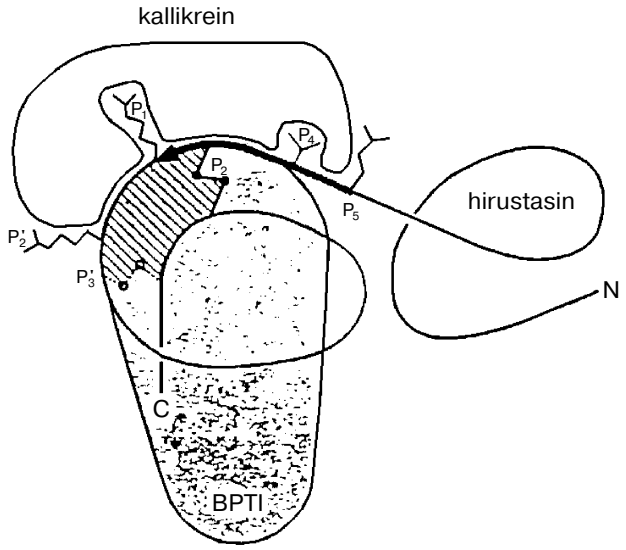

Fig. 1. Mechanism of the interaction of tissue kallikrein with hirustasin and bovine pancreatic trypsin inhibitor (BPTI). Two disulfide bridges in the C-terminal part of hirustasin are shown by solid and broken lines. Hatched area shows hydrophobic regions of hirustasin. Hirustasin residues P_3', P_2', P_1, P_2, P_4, and P_5 and tissue kallikrein pockets for binding with P_1 and P_4 are indicated [28].

Analyzing crystal structures of kallikrein–hirustasin and kallikrein–aprotinin (bovine pancreatic trypsin inhibitor, BPTI) complexes [35], Uson et al. [28] found significant differences (Fig. 1). Only the C-terminal domain of hirustasin interacts with this proteinase. Kallikrein recognizes hirustasin by forming antiparallel β-sheet with this inhibitor. Compared to aprotinin, hirustasin has longer binding loop; this allows fixing the inhibitor P_4 site (Val127) in the kallikrein-binding pocket. The latter (including residues 169-174) is adapted to hirustasin binding. The interaction with kallikrein also involves the P_5 site of hirustasin. The kallikrein-binding pocket for the P_4 site of hirustasin is not involved in the complex formation between kallikrein and aprotinin. (Because of Pro13, the polypeptide chain of aprotinin has a sharp turn which excludes the possibility of its interaction with this pocket.) In contrast to the weak hydrogen bond between Lys15 and Asp189 of aprotinin and kallikrein molecules, respectively, the position of the hirustasin P_1 site (Arg30) in the kallikrein binding pocket is stabilized by hydrogen bonds with His217 and Asp189 of kallikrein (Fig. 1).

TRYPTASE INHIBITORS

Tryptase, a trypsin-like serine proteinase, is a tetrameric protein. Being a basic component of secretory cytoplasmic granules of mast cells, it is involved in catabolism of extracellular matrix proteins [36]. Tryptase plays an important role in the pathogenesis of allergic and inflammatory states related to mast cell dysfunction such as asthma, some other lung diseases, rheumatoid arthritis, psoriasis, etc. [37-39]. Among serine proteinases, human tryptase is a unique enzyme in that in human plasma and extracellular space it exists in catalytically active form [40, 41]. Tryptase is insensitive to natural proteinase inhibitors influencing the activity of other trypsin-like enzymes, such as α_1-proteinase inhibitor, antithrombin III, C-1 esterase inhibitor, antileukoproteinase, and α_2-macroglobulin [40, 42]. Only diisopropylfluorophosphate, tosyllysylchloromethyl ketone, and benzamidine derivatives are known tryptase inhibitors [43]. In 1994, Sommerhoff et al. isolated a tryptase inhibitor from extracts of the medicinal leech

Hirudo medicinalis [44]. This inhibitor, denominated LDTI (leech-derived tryptase inhibitor), exists in three isoforms. Isoforms A and B, separated only by reversed-phase chromatography, contain 42 amino acid residues, whereas the third isoform C contains 46 amino acid residues. The molecular masses of these isoforms are 4340 (A and B forms) and 4738 daltons (C-form). It has been suggested that LDTI is a fragment of a larger protein molecule or the mature proteins is shortened at C-terminus due to possible action of accompanying carboxypeptidases. Comparison of amino acid sequences of LDTI-A and bdellin B-3 revealed that these proteins share 55% homology, and 19 of 42 amino acids residues of LDTI-A are identical to the corresponding positions in bdellin B-3. Analysis of similarity with other proteins also revealed that 18 and 19 amino acids are identical to domains of rhodniin, a thrombin inhibitor from the blood-sucking insect *Rhodnius prolixus* [45]. LDTI as well as bdellin B-3 [9] are non-classic Kazal-type inhibitors. They are characterized by a strictly determined location of six cysteine residues and a fixed number of amino acid residues between them. A variable site between the first and second cysteines containing six amino acid residues less than the third domain of silver pheasant ovomucoid (the classic Kazal-type inhibitor) is the only exception. The primary structure of LDTI and bdellin B-3 contains one additional amino acid residue between the fourth and fifth cysteines; both inhibitors also lack six amino acid residues between the fifth and sixth cysteines. Thus, the distance between the first and sixth cysteines is unusually short, and LDTI and bdellin B-3 are denominated as the shortest Kazal-type inhibitors [44]. The four monomers of the tryptase molecule form a closed ring structure. Their four active sites are exposed to the internal oval-shaped space. This structure reduces the susceptibility of the active sites to high molecular weight inhibitors and substrates [46]. As a non-classic Kazal-type inhibitor of low molecular weight, LDTI binds to two enzyme monomers (with one of them it binds in the canonical manner via its reactive center loop, involving residues P_4-P_4') [47]. In the complex with tryptase monomer, four N-terminal residues preceding the LDTI-binding segment may change their conformation, and this provides binding "side to side".

The N-terminal amino acid residues of this inhibitor, Lys1 and Lys2, interact with tryptase carboxyl groups Asp143 and Asp144. Realization of these electrostatic interactions may be explained by deletions in the N-terminal region of LDTI compared with classic Kazal-type inhibitors of high molecular weight. These basic residues provide penetration of LDTI inside the central pore of the tryptase tetramer, and this explains the inhibitory potential of LDTI [48]. Another non-classic Kazal-type inhibitor of low molecular weight, bdellin B-3, does not inhibit tryptase because, in contrast to LDTI containing N-terminal Lys1 and Lys2, it has Asp1 and Thr2 [44]. LDTI binds to two active sites of tryptase. Maximal inhibition of tryptase by LDTI depends on the size of substrate used for determination of its catalytic activity. Using low molecular weight substrates such as tosyl-Gly-Pro-Arg-pNA, inhibition was 50%, whereas in the case of another substrate containing 27 amino acid residues and only one hydrolyzable bond, tryptase inhibition by LDTI was 75% [49].

These differences can be explained by the fact that LDTI binds to two of four active sites of the tryptase tetramer, leaving two active sites vacant for binding and proteolytic cleavage of short substrate [44]. Data on LDTI inhibition of cleavage of high molecular weight kininogen (114 kD) by tryptase and LDTI inhibition of a tryptase-dependent mitogenic effect (by more than 90%) [44] seem to support this suggestion.

The interaction of LDTI with two active sites of the tryptase tetramer determines the tight binding behavior of this inhibitor (K_i = 1.4 nM) [44]. This interaction is realized via a binding loop that includes a region of the N-terminal amino acid residues (from 6th to 12th). This loop is linked to an α-helical site of the inhibitor molecule by two disulfide bridges. Inhibition of trypsin and chymotrypsin also involves the LDTI binding loop (K_i values are within the nanomolar range). In LDTI, this region contains the following amino acid residues: Cys6 (P_3), Pro7(P_2), Lys8 (P_1), Ile9 (P_1'), Leu10 (P_2'), Lys11 (P_3'). Lys8 is the active site of this inhibitor [50]. The effect of LDTI is rather specific: besides trypsin and chymotrypsin, it does not inhibit other serine proteinases (or the inhibition is very weak).

LDTI is the first tight-binding proteinase inhibitor of human mast cell tryptase. It effectively blocks tryptase-induced cleavage of vasoactive intestinal peptide, His-Met, and kininogen. It also inhibits the mitogenic activity of tryptase and its direct cellular effects. LDTI may be potentially used as a pharmacological probe for the evaluation of the pathophysiological role of mast cell tryptase in asthma, arthritis, periodontal diseases, skin diseases, and impairments of blood clotting.

In this connection, the problem of elaboration of an effective leech tryptase inhibitor for medical use becomes especially important. Auerswald et al. expressed the LDTI gene in *E. coli* and yeast [51]. The resulting recombinant protein (r-LDTI) had the same molecular mass, amino acid composition, and electrophoretic mobility as the wild-type LDTI. It effectively inhibited tryptase (K_i = 1.5 nM) and trypsin (K_i = 1.6 nM) and proliferation of human keratinocytes and fibroblasts (IC_{50} of 0.1 and 1 nM, respectively) [52]. Higher concentration of r-LDTI (20 μM) blocks replication of HIV-1 virus in HUT-78 cells [51], this being directly linked to tryptase catalytic activity [53].

It is suggested that the leech secretes LDTI to block protective proteolytic enzymes of mast cells, which are usually released during parasite infection of the skin.

The structure of LDTI-C was used as a prototype for the development of artificial recombinant Kazal-type inhibitor that could effectively block not only tryptase, trypsin, and chymotrypsin, but also thrombin [54]. Using functional phage display, several LDTI mutants (with mutations at P_1-P_4' positions of the reactive site) have been constructed. The library consisted of $5.2 \cdot 10^4$ mutants of the recombinant phage M13 including various variants of LDTI constructions. Three clones were selected by their ability to interact with thrombin. New variants of LDTI (2T, 5T, and 10T) were obtained using expression in yeast. Two of them inhibited thrombin, with K_i values of 302 nM (2T) and 28 nM (5T). LDTI 10T interacted with thrombin but did not inhibit it. These LDTI variants inhibited trypsin activity with K_i values of 6.4 nM (2T), 2.1 nM (5T), and 12 nM (10T), but they were inactive with respect to factor Xa, plasma kallikrein, and neutrophil elastase. At 0.5 μM, LDTI 2T and 5T caused 1.8- and 2-fold increase of the time required for blood clot formation. In contrast to hirudin, these inhibitors influence thrombin activity only by interacting with its active site. So, the most promising LDTI variant (LDTI 5T) might be used in the future for construction of such monovalent thrombin inhibitors [54].

EGLINS

These are low molecular weight proteins isolated from extracts of the medicinal leech. They inhibit the activity of α-chymotrypsin, chymase, subtilisin, and the neutrophil proteinases elastase and cathepsin G. They were initially isolated and described in 1977 as two preparations (of molecular weight 8073 and 8099 daltons) denominated eglin *b* and eglin *c* [55]. It was later shown that eglin also inhibits proteinases A and B from *Streptomyces griseus* [56]. Lack of cysteine residues is the characteristic feature of eglins (containing 70 amino acid residues), which are highly resistant to denaturing effects of acids and heating [57]. Iso-inhibitors eglin *b* and eglin *c* differ by only one amino acid residue (at 35 position eglin *b* and *c* contain His and Tyr, respectively); this is due to the point mutation *e* (a single base substitution in the eglin gene) [58]. Recombinant eglin *c* was expressed in *E. coli* [59, 60]. Data on the inhibitory activity of eglins *b* and *c* with respect to various proteolytic enzymes are summarized in the table.

K_i values for inhibition of some proteinases by eglins *b* and *c*

Proteinase	K_i, M	
	eglin *b*	eglin *c*
α-Chymotrypsin	$3 \cdot 10^{-10}$ [5]	$7 \cdot 10^{-10}$ [5]
Subtilisin	$2 \cdot 10^{-10}$ [5]	$1.2 \cdot 10^{-10}$ [5]
Neutrophil elastase	$2.3 \cdot 10^{-10}$ [5]	$2 \cdot 10^{-10}$ [5]
Neutrophil cathepsin G	$2.5 \cdot 10^{-10}$ [5]	$2.8 \cdot 10^{-10}$ [5]
Mast cell chymase		$4.45 \cdot 10^{-8}$ [64]

Inhibition of human leukocyte elastase [61], chymotrypsin, and subtilisin [62] is characterized by the second order rate constants of 10^6 and 10^7 $M^{-1} \cdot sec^{-1}$. High affinity of serine proteinases for eglin *c* results from very low dissociation constant (10^{-4} sec^{-1}) of the proteinase–inhibitor complex [63]. High K_i value for the inhibition of mast cell chymase by eglin *c* (table) suggests that the latter protects the leech against penetration of this enzyme from the skin mast cells of the host used by the leech for blood sucking [64]. The spectrum of inhibitory activity of eglin *c* explains its high anti-inflammatory activity [65]. Nanomolar concentrations of recombinant and mutant forms of eglin *c* inhibit NS3-proteinase of hepatitis C virus; this results in production of non-infective viral particles [63].

Eglin *c* belongs to the potato inhibitors-I family, which also includes barley inhibitors CI-1 and CI-2.

The reactive site of eglins contains Leu45. Most serine proteinase inhibitors are characterized by the so-called "standard mechanism" of inhibition [47, 66]. According to this mechanism, the reactive center binding loop of the inhibitor binds at the active site of an enzyme. (This region of the inhibitor has structural similarity with specific substrates of the enzyme). The conformation of the reactive center–binding loop system is maintained by the rest of the inhibitor molecule or by disulfide bridges (as in the case of pancreatic trypsin inhibitor [67]) or by non-covalent interactions (as demonstrated for the potato inhibitors-I family [68, 69]).

The structure of free eglin *c* was studied using two-dimensional NMR [70] and X-ray analysis of inhibitor crystals [71]. Crystals of eglin complexes with subtilisin [65, 72-74], α-chymotrypsin [75], and termitase [76] have also been investigated. According to these data, the tertiary structure of eglin *c* consists of a hydrophobic core and a surface loop (residues 40-48) responsible for proteinase binding.

INHIBITOR OF FACTOR Xa

In the cascade of protein activation of plasma homeostasis, blood clotting factor Xa catalyzes the conversion of prothrombin to thrombin. This requires the presence of calcium ions, factor V on the surface of activated platelets, or fragments of damaged endothe-

lial or smooth muscle cells. The activating prothrombin complex is called prothrombinase. The resulting thrombin catalyzes the conversion of a soluble plasma protein, fibrinogen, into insoluble fibrin, which spontaneously polymerizes giving rise to both hemostatic and pathological thrombi [77]. Pathological thrombi appear during thrombosis of superficial and deep veins, disseminated intravascular clotting (DVC syndrome), and in some cerebrovascular and cardiovascular diseases [78]. For prevention of unwanted blood clotting, various direct and indirect anticoagulants are employed. Indirect anticoagulants, vitamin K antagonists, block the synthesis of prothrombin and prothrombin complex proteins. The activity of a direct anticoagulant, heparin, is regulated by its cofactors, antithrombin III (ATIII) or by heparin cofactor II. Heparin–ATIII inhibits thrombin and factor Xa, and the degree of inhibition depends on the molecular mass of the heparin. High molecular weight heparin–ATIII preferentially inhibits thrombin, whereas the low molecular weight form inhibits factor Xa [79]. However, administration of heparin–ATIII to patients provokes bleeding and thrombocytopenia, and long-term treatment causes osteoporosis [80]. This stimulates the search of effective new inhibitors of thrombin and factor Xa free from these side effects.

In 1995, Rigbi et al. isolated factor Xa inhibitor (FXaI) from diluted saliva of the medicinal leech [81]. It is a protein of 13-14 kD; it consists of 85 amino acid residues, and 14 cysteine residues form seven disulfide bonds. This inhibitor forms a tight equimolar complex with factor Xa; the IC_{50} for inhibition of amidolytic activity of factor Xa (k_{cat} = 130 sec^{-1}, K_m = 15 μM) was 1 pM. However, inhibition constants of prothrombinase activity of factor Xa (conversion of prothrombin to thrombin) were higher (72-120 pM) [82]. The complete amino acid sequence of this inhibitor was deduced using its cDNA [83]. Recombinant FXaI (r-FXaI) was obtained during gene expression of one of the FXaI isoforms. r-FXaI (molecular mass 14.4 kD) contains 133 amino acid residues, and 22 cysteines are obviously involved into formation of 11 disulfide bridges. r-FXaI shares 42% homology with wild-type FXaI and 50% homology with antistasin [82].

r-FXaI selectively inhibits the activity of factor Xa and trypsin; it does not influence plasmin and thrombin. The amidolytic activity of factor Xa is less sensitive to r-FXaI inhibition (K_i ~ 10 μM) than its prothrombinase activity (K_i ~ 0.04 mM). However, the amidolytic activity of trypsin exhibits much higher sensitivity to r-FXaI (K_i ~ 7 nM).

Like heparin, r-FXaI protects experimental animals against vein thrombosis. Although heparin and r-FXaI have the same effect on the time of bleeding in the experimental animals, the latter is an even more effective antithrombotic agent than heparin [82].

HIRUDIN

Hirudin is a highly specific thrombin inhibitor, forming a tight noncovalent stoichiometric complex with this proteinase [84]. It is effectively employed in clinical practice for medical treatment of diseases characterized by increase in blood thrombin concentration [85]. It was discovered by Hycraft more than a century ago in extracts of the cerebral region of the medicinal leech *Hirudo medicinalis* [86]. In 1903, Jacobi named it hirudin [87]. Hirudin is a glycoprotein that consists of 65 or 66 amino acid residues. It contains three disulfide bridges and has sulfated Tyr63. Hirudin forms a family that comprises more than 20 iso-inhibitors differing in the length of the polypeptide chain and substitutions of some amino acid residues [88]. They share about 20% homology, and some hirudin isoforms lack antithrombin activity [89, 90]. In 1976, Bagdy et al. estimated the complete covalent structure of hirudin [91], which was subsequently confirmed by Dodt et al. in 1984 [92]. One year later Dodt et al. reported the location of disulfide bonds in the hirudin molecule [93]. In 1986, the first reports on the preparation of recombinant desulfated hirudin appeared [94-96].

In the tertiary structure, three functional regions are recognized: a compact domain near the N-terminus of the molecule stabilized by three disulfide bonds, which form the so-called "nucleus" (amino acid residues 6-39), a short peptide (amino acid residues 1-5), and the C-terminal domain (amino acid residues 40-65). The short N-terminal fragment (amino acid residues 1-5) and the C-terminal domain exhibit some flexibility with respect to the "nucleus" [97-99].

Understanding the mechanism of thrombin inhibition by hirudin requires consideration of the structural features of this enzyme. Thrombin, a trypsin-like serine proteinase (EC 3.4.21.5), is a glycoprotein of molecular mass 36.6 kD (human thrombin) that consists of two polypeptide chains. The light A-chain including 49 amino acid residues is connected to the B-chain by two disulfide bonds. The B-chain consists of two 259 amino acid residues; it is stabilized by three S–S-bonds. The active site of thrombin is located in the B-chain, and the A-chain is involved in maintenance of its native conformation [100-102]. Thrombin plays a major role in the activation of the blood clotting system [103, 104]. It converts blood plasma fibrinogen into the insoluble fibrin clot and exhibits high specificity for the first anion-binding exosite located far from the catalytic site of the enzyme. Thrombin is involved in the regulation of the hemostasis system proteins: blood clotting factors V, VIII, and XIII, proteins C and S, and complement system components. Thrombin also interacts with receptors of blood cells (platelets, monocytes, neutrophils) and the vascular system (endothelium and smooth muscle cells). Compared to trypsin, thrombin contains three amino acid substitutions in the primary binding pocket. It also has the insert

Lys-Arg-Gly between amino acid residues 188 and 189. This explains the peptidase efficacy of thrombin with respect to peptide bonds formed by Arg rather than Lys residues typical for tryptic cleavage of substrates [101]. The active site of thrombin is located in a deep slot, and this is the other characteristic feature of this proteinase [102, 105]. Hydrophobic amino acid residues form a binding pocket ("nonpolar binding site") in the active site. This pocket can interact with aromatic amino acid residues in subsite P_3 of thrombin substrates and inhibitors [102, 105]. In contrast to chymotrypsin, thrombin contains longer amino acid stretches exposed to the surface; they are involved in the interaction of thrombin with such physiological ligands as fibrinogen, fibrin, thrombomodulin, heparin cofactor II, and platelet receptors [102, 105]. The second anion-binding site required for the interaction of thrombin with heparin is located on the opposite side of the protein globule [102, 106]. Both anion-binding sites are involved in the activation of factors V and VIII by thrombin [107]. Although thrombogenic and bioregulatory effects of thrombin are very important for homeostasis, an excess of thrombin in the blood circulation can cause dangerous impairments of the cardiovascular and other systems. Hirudin is the only exogenous specific thrombin antagonist that can inhibit all the functions of this proteinase [103].

Hirudin interacts with the active site of thrombin and binds near the primary binding pocket. The N-terminal peptide forms short parallel β-structure with a thrombin segment (the region includes amino acid residues Ser214-Gly219) that is close to its catalytic site. Hydrophobic residues of the first three N-terminal amino acids (Val-Val-Tyr) in the active site slot block substrate access. The negatively charged C-terminal fragment of hirudin (amino acid residues 54-65) binds to positively charged and hydrophobic fragments of the fibrinogen-binding exosite of thrombin. Thus, the interaction of hirudin with thrombin results in blockade of two regions of the active site that are important for fibrinogen binding (substrate- and anion-binding regions). This two-site interaction, called bridge binding, is specific for thrombin [108]. The compact globular N-terminal domain of hirudin forms two ionic couples with the thrombin surface, Asp5(hirudin)–Arg221(thrombin) and Glu17(hirudin)–Arg173(thrombin), and two hydrogen bonds. These contacts block access of high molecular weight inhibitors and

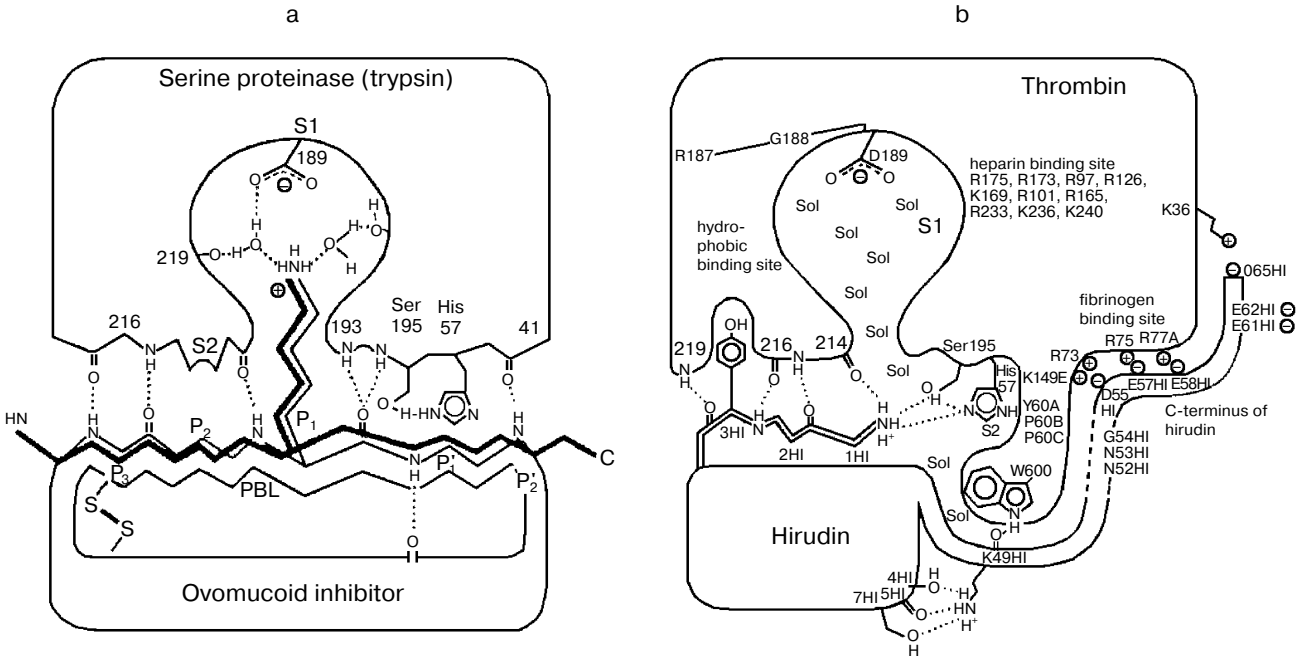

Fig. 2. Comparison of mechanisms of trypsin and thrombin inhibition by low molecular weight inhibitors, ovomucoid and hirudin, respectively. a) Trypsin–ovomucoid complex. The canonic proteinase binding loop (PBL) is located like low molecular weight substrate (indicated by solid line). The inhibitor interacts with the proteinase in a substrate-like manner by forming hydrogen bonds with the substrate binding region. This is accompanied by formation of anti-parallel structure between the P_1-P_3 site of the inhibitor and the trypsin segment including amino acid residues 216-218. Ser195 of the catalytic site of trypsin is in "sub-van der Waals contact" with carbonyl oxygen of the cleaved peptide bond. b) Thrombin–hirudin complex. The N-terminal sequence including three amino acid residues (1HI-3HI) interacts with thrombin segment Ser214-Gly219 via formation of parallel β-sheet. Active site Ser195 is not blocked, and water molecules fill the specific binding pocket. The C-terminal tail of hirudin forms a loop that is located along the characteristic site of thrombin (Tyr60A-Trp60D) and binds to the exosite of thrombin via salt bridges; this exosite is responsible for fibrinogen recognition [110].

substrates to the active site of thrombin [102]. Figure 2 shows the mechanisms of thrombin inhibition by hirudin in comparison with trypsin inhibition by ovomucoid, a classic Kazal-type inhibitor [109].

Thus, hirudin is a unique thrombin inhibitor. Only hirudin is able to block thrombin proteolytic activity at picomolar concentrations. Such unique specificity of hirudin is explained by a previously unknown mechanism of its multiple interactions with thrombin. In contrast to known serine proteinase inhibitors, which preferentially contact the catalytic center of the target proteinases, the region of thrombin interaction with hirudin is located far from its catalytic site. Hirudin forms a tight complex with thrombin (K_d = 10^{-14} M), and numerous contacts between thrombin and hirudin found during analysis of the corresponding crystals are the molecular basis of such a complex. The formation of the thrombin–hirudin complex follows a two-stage mechanism described by the following scheme [110]:

$$E + I \underset{k_2}{\overset{k_1}{=\!=\!=\!=\!=\!=\!=\!=}} E\,I \underset{k_4}{\overset{k_3}{=\!=\!=\!=\!=\!=\!=\!=}} E\,I^*,$$

where E and I are thrombin and hirudin, respectively. The first stage represents the interaction of the C-terminal fragment of hirudin with thrombin, which depends on the ionic strength of solution. The first stage results in the formation of the complex EI; this does not involve the substrate binding region of the active site of thrombin. The latter is involved into the second stage of thrombin–hirudin interaction accompanied by formation of the complex EI*.

Experiments with recombinant hirudin have produced various structural variants of the natural inhibitor; they all have non-sulfated Tyr63, and this property give rise to their common name as "desulfo-hirudins" or "desirudins" [111].

In contrast to natural hirudin, the recombinant hirudins are characterized by somewhat lower affinity for thrombin (K_d = 10^{-13} M), but they have the same anticoagulant properties as the natural inhibitor. Hirudin inhibits not only free thrombin but also the enzyme bound to fibrin clot [103]. Hirudin is widely used in clinical practice [112]; increased bleeding observed in patients only after high doses of this inhibitor limits its medical use.

CARBOXYPEPTIDASE INHIBITOR

In 1998, a new carboxypeptidase A inhibitor was isolated from the medicinal leech. It is called LCI (leech carboxypeptidase inhibitor). LCI exists in two isoforms of molecular masses 7.3 and 7.2 kD, respectively. The former consists of 66 amino acid residues, whereas the latter consists of 65 amino acid residues. These isoforms differ only by the presence or absence of C-terminal Glu. The LCIs contain eight cysteine residues (involved into formation of four disulfide bonds) and nine proline residues [113]. A heptapeptide in C-terminal region of this protein shares homology with carboxypeptidase inhibitors from potato and tomato (the Solanaceae family); it exhibits lower homology with the C-terminal region of a carboxypeptidase inhibitor from the parasitic nematode *Ascaris suum*. The spatial structure of this inhibitor contains five long β-sheet structures and one short α-helix. The same motif was found in the LCI complex with human carboxypeptidase A2 [114].

LCI cDNA has been expressed in *E. coli*. The recombinant protein is equivalent to the wild protein isolated from the medicinal leech [113]. Data of CD and NMR spectroscopies suggest that the recombinant LCI is a highly structured globule; 52 amino acid residues (the region between the first and the last cysteine residues) form its nucleus, whereas nine and five amino acid residues from the N- and C-termini, respectively, form two tails. The compact structure of the protein globule provides stability of this protein over a wide range of pH and temperature and resistance to denaturation by urea.

Recombinant LCI is a competitive inhibitor of various pancreatic carboxypeptidases including bovine carboxypeptidase A1 (K_i = 0.25-0.48 nM), human carboxypeptidase A2 (K_i = 0.17-0.78 nM), pig carboxypeptidase B (K_i = 0.27-0.51 nM), and human plasma carboxypeptidase B (K_i = 0.1-0.2 nM) [113]. Since inhibition does not depend on preincubation time, it is likely that the inhibitor immediately binds to these enzymes. The mobile C-terminal tail of the LCI molecule is suggested to be the primary site interacting with metalloproteinases. Binding to carboxypeptidases results in immediate cleavage of the C-terminal Glu of LCI (which does not affect inhibitory activity). The amino acid residue next to Glu is orientated to a Zn atom, and this is critical for carboxypeptidase inhibition. NMR analysis revealed that after binding to human carboxypeptidase A2, the C-terminal region of LCI becomes rigid and the interaction between this carboxypeptidase and LCI is substrate-like [114]. The LCI nucleus core is a secondary region for binding to the enzyme as was demonstrated for the carboxypeptidase inhibitor from potato [114]. However, due to the smaller nucleus, the latter is unable to form additional effective secondary binding sites with the enzyme. This explains the higher inhibitory activity of LCI than potato inhibitor with respect to various carboxypeptidases (K_i values of 0.2-0.4 versus 1.5-5.0 nM, respectively) [115].

LCI is the only carboxypeptidase inhibitor found in leeches. If it is a component of salivary gland secretion, the following speculation is plausible. LCI can block the

metalloproteinase-dependent kinin hydrolysis at the place where the leech bites the skin. This will increase the kinin-induced bloodstream important for leech blood sucking. LCI may be also involved into removal of blood clots (if they have been formed during blood sucking) by blocking plasma carboxypeptidase B, thus slowing fibrinolysis [116, 117].

INHIBITORS OF COMPONENTS OF THE COMPLEMENT SYSTEM

The complement system is a complex of proteins that are activated in response to antigen–antibody complex formation; the cascade of reactions of limited proteolysis results in the damage to bacterial cells. The system also includes cell receptors of fragments of the complement system components and membrane proteins that regulate activation [118]. The biological role of factors and subcomponents of the complement system is not limited to damage of cell membranes on which components of the complement system are fixed and activated. Products of complement activation can regulate various functions of numerous cells, and therefore the search for natural regulators of this enzymatic system may have practical application.

In 1988, it was shown that the secretion of salivary glands of the medicinal leech can block activation of the complement system via classic and alternative mechanisms [119]. Now the C1s enzyme inhibitor of C1 complement subcomponent has been isolated from extracts of the medicinal leech. It can bind to complement-fixing sites of antibodies (IgG and IgM) on the cell surface and initiates activation of the complement system (authors' unpublished data). Binding to antigen–antibody complex results in activation of C1s (a trypsin-type zymogen) by active subcomponent C1r. This is accompanied by the cleavage of a double-stranded molecule (molecular mass 85 kD) without release of the cleaved components, which are still bound to the main protein molecule by two disulfide bridges. Activated C1s is a serine proteinase that sequentially cleaves the fourth (C4) and the second (C2) complement components that are attached to an adjacent membrane site and form the active complex, the so-called "C3-convertase" [120].

The inhibitor of C1s proteinase is a monomeric protein of 67 ± 5 kD. It contains hydrophobic fragments and lacks carbohydrate components. Blockade of activation of C4 component by inactive enzyme C1s prevents C2 binding and formation of C3-convertase of the classic pathway of activation of the complement system (authors' unpublished data).

The inhibitor of C1s can be used for medical treatment of pathological states associated with deficit of C1-inhibitor and unwanted activation of the complement system (anaphylactic shock, chronic inflammatory processes, septic states).

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