



Review

Structure and function of invertebrate Kunitz serine protease inhibitors

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ABSTRACT

Kunitz type proteins are an important group of ubiquitous protease inhibitors found spanning the evolutionary tree from microbes to mammals. These proteins can have single or multiple Kunitz inhibitory domains linked together, or associated with other domain types. The Kunitz motif comprises a chain of around 60 amino acid residues stabilized by three disulphide bonds. The inhibitory specificity of the Kunitz domain varies with the particular amino acids at the reactive sites and exhibit canonical inhibition. In vertebrates, Kunitz inhibitors play a major role in inflammatory processes while in invertebrates involve in a range of diverse functional roles. This review discusses the structure, mechanism of action and functions of invertebrate Kunitz inhibitors. Venomous invertebrates such as scorpions, cone snails have either Kunitz inhibitors with both neurotoxic and protease inhibitory activity or typical Kunitz type toxins. In parasitic helminths these inhibitors play a major role in providing protection from host digestive protease enzymes. Several proteins having Kunitz domains in nematodes are involved in collagen biosynthesis while some induce IgE-mediated allergic reactions. Most Kunitz inhibitors in blood sucking arthropods function as anti-coagulant factors and several act as a defense against microbial pathogen invaders.

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Contents

1. Introduction	219
1. Introduction	220
2. Kunitz inhibitor structure	220
3. Mechanism of Kunitz inhibition	220
4. Kunitz inhibition of thrombin	223
5. Functional roles	223
5.1. Serine protease inhibition	223
5.2. Inhibition of other proteases	223
5.3. Involvement in reproductive and allergic activity	224
5.4. A role in defense against microbial infection	224
5.5. Cuticle formation and tissue remodeling	224
5.6. Cytotoxic activity	224
5.7. Involvement in formation of the extracellular matrix	225
6. A phylogeny of Kunitz inhibitors	225
7. Summary	225
Acknowledgments	225
References	225

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1. Introduction

Proteases are found in all living organisms from bacteria to mammals and usually have a narrow spectrum of activity which, if not tightly regulated by protease inhibitors, can inflict serious cellular damage (Armstrong, 2001). Almost all known naturally occurring inhibitors directed toward endogenous cognate proteases are proteins and they target the four major protease classes, namely serine, cysteine, aspartic and metallo-proteinases.

Serine proteases and their cognate inhibitors play fundamental roles in cellular biology. Inhibitors of serine proteases are the most studied and are classified into several families based on sequence homology, location of the reactive site, structural characteristics and mechanism of action. In the main, these inhibitors are grouped into the Kunitz, Kazal, serpin and mucus families (Roberts et al., 1995). Serine protease inhibitors can also be grouped, based on their mechanism of action, into canonical inhibitors, non-canonical inhibitors and serpins (Krowarsch et al., 2003).

Kunitz protease inhibitors are ubiquitous, being found in many organisms, including animals, plants and microbes. According to the MEROPS database, peptidases of the I2 family have been identified in the unicellular choanoflagellate *Monosiga brevicollis* (Rawlings et al., 2012). Plant Kunitz proteins belong to the I3A family of peptidase inhibitors (Rawlings et al., 2004a) which are unrelated to the Kunitz proteins found in animals (Steiner et al., 1965). At least three families of Kunitz inhibitors have been identified in vertebrates which selectively inhibit serine protease activity and they are involved in various anti-inflammatory processes (Shigetomi et al., 2010). In invertebrates, Kunitz proteins, as well as having protease inhibitor activity, are involved in a range of diverse functional roles, reviewed below.

The Kunitz-type motif usually has a peptide chain of around 60 amino acid residues and is stabilized by three disulphide bridges. Bovine pancreatic trypsin inhibitor (BPTI) is the classic member of this family of proteins and was the first Kunitz-type protease inhibitor described (Kunitz and Northrop, 1936). BPTI has relatively broad specificity inhibiting trypsin as well as chymotrypsin and elastase-like serine (pro) enzymes (Ascenzi et al., 2003). Kunitz inhibitors are thus known as BPTI-like proteins and belong to the I2 family of peptidase inhibitors (Rawlings et al., 2004a). Kunitz inhibitors may contain a single domain (e.g. BPTI) or the domain may be repeated twice (e.g. Bikunin), three times (e.g. tissue factor pathway inhibitor-TFPI) or even more (there are 12 domains in the *Ancylostoma caninum* (hookworm) Kunitz protease inhibitor) to form a multi-domain, single-chain inhibitor able to interact independently with several protease molecules at their reactive sites belonging to separate domains.

In addition to their major action in serine protease inhibition, some Kunitz proteases can act as ion channel blockers and are known as Kunitz-type toxins (KTT). They are frequent components of the venoms from poisonous animals including snakes, sea anemones (e.g. *Anthopleura elegantissima* (Peigneur et al., 2011)), cone snails (e.g. *Conus striatus* (Dy et al., 2006)), tarantulas (*Ornithoctonus* spp. (Yuan et al., 2008)), scorpions (*Lychnis mucronatus* (Zhao et al., 2011)) and the cattle tick *Boophilus microplus* (Lima et al., 2010). KTTs can especially block voltage-gated potassium channels which are essential for regulation of various physiological processes such as blood coagulation, fibrinolysis, host defense and action potential transduction (Paesen et al., 2009). Dendrotoxin (DTX), which is found in the venom of the green mamba snake *Dendroaspis angusticeps* (Dreyer, 1990), is a potent potassium channel blocker and neurotoxin that enhances acetylcholine release at neuromuscular junctions and, being a typical KTT, it has no protease inhibitor activity (Lancelin et al., 1994). However, *Ra*-KLP isolated from the salivary gland of the brown ear tick *Rhipicephalus appendiculatus* activates maxiK ion channels suggesting a potential

mechanism for regulating host blood supply during feeding but shows no significant protease inhibitor activity (Paesen et al., 2009).

2. Kunitz inhibitor structure

Kunitz inhibitors are a class of serine protease inhibitors which possess one or more Kunitz domains, characterized by a conserved spacing between their cysteine residues, a typical disulphide bonding pattern (Laskowski and Kato, 1980), and are a paradigm for the canonical inhibition of serine proteases (Bode and Huber, 1992). The Kunitz domain is an ancient and widespread domain with a disulphide rich $\alpha + \beta$ fold that is stabilized by three highly conserved disulphide bridges with the bonding patterns C1–C6, C2–C4, and C3–C5 (Fig. 3B). Two of the disulphide bonds (C1–C6 and C3–C5) are required for the maintenance of native conformation (Creighton, 1975) whereas the third (C2–C4) stabilizes the two binding domains (Laskowski and Kato, 1980).

BPTI is the typical model for Kunitz inhibitors (Figs. 1 and 2). The central feature of its folding is a distorted antiparallel β -sheet (formed from residue segments 17–25 and 29–36) which is twisted through 180°. The sheet is closed at one end by a β -turn (residues 25–29) and at the other end by the disulphide bridge (C14–C38). The chain segments 1–14 and 38–58 are folded back along the length of the β -sheet so that the terminal residues 1 and 58 come to lie close to each other near the 25–29 β -turn. Disulphide bridges covalently link the terminal sections to each other (C5–C55) and to the vicinity of the β -turn (30–51) (Pritchard and Dufton, 1999). The Cys5–Cys55 and Cys30–Cys51 bonds are left-handed, while Cys14–Cys38 adopts the unusual right-handed form (Richardson, 1981). The immediate N-terminal sequence is formed into a short helical segment, while the C-terminal segment is formed into a longer and more regular α -helix section (residues 48–56) (Fig. 1). The nature of the fold and the positioning of the three disulphide bridges give the impression of a compact and stable molecule. The structures of known invertebrate Kunitz inhibitors so far described are similar to BPTI apart from two deviants (Paesen et al., 2009; Zhao et al., 2011). The disulphide bonding pattern and reactive site amino acids of different Kunitz type proteins are shown in Fig. 4.

The first natural Kunitz domain protein (Conkunitzin-S1), which has only two disulphide bonds, was identified in the venom of the cone snail (*C. striatus*) which interacts with voltage gated potassium channels and acts as a neurotoxin. Notably, the missing disulphide bond (C2–C4) has no effect on the functional activity of the protein (Monika et al., 2005) and it is structurally similar to the Kunitz fold of dendrotoxin (Dy et al., 2006). A bi-functional Kunitz protein (HWTX-XI), missing the same disulphide bond, has been identified from tarantulas (*Ornithoctonus* sp.); it is a very potent trypsin inhibitor as well as being a weak potassium channel blocker (Yuan et al., 2008). This supports earlier findings that selective reduction of the second disulphide bond (C2–C4) has no effect on the stability of BPTI (Marks et al., 1987) or even its inhibitory properties (Czapinska et al., 2000).

A new Kunitz type venom peptide (SdPI), which apparently adopts a new disulphide bridge at the C terminus and has potent trypsin inhibitory activity with a similar mode of trypsin interaction to other Kunitz-type venoms, has been functionally characterized from the scorpion (*L. mucronatus*) (Zhao et al., 2011).

3. Mechanism of Kunitz inhibition

The standard mechanism of Kunitz inhibition (canonical inhibition) involves a tight, non covalent interaction resembling the enzyme–substrate Michaelis complex. Kunitz inhibitors directly

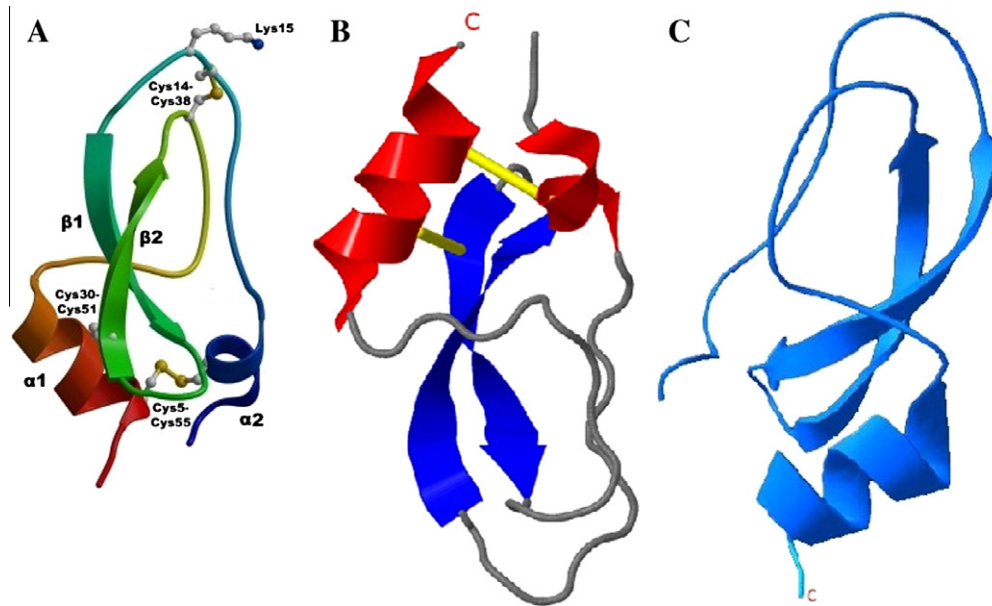


Fig. 1. The crystal structures of: BPTI (A) showing three disulphide bonds (reproduced from Paesen et al. (2009) with permission from Elsevier); Conk-S1 of *Conus striatus* (B) showing two disulphide bonds (reproduced from Spits et al. (2005) courtesy of Proteopedia); and AceKI of *Ancylostoma caninum* (C) (Image courtesy of D. Chu and M. Cappello, Yale University, USA).

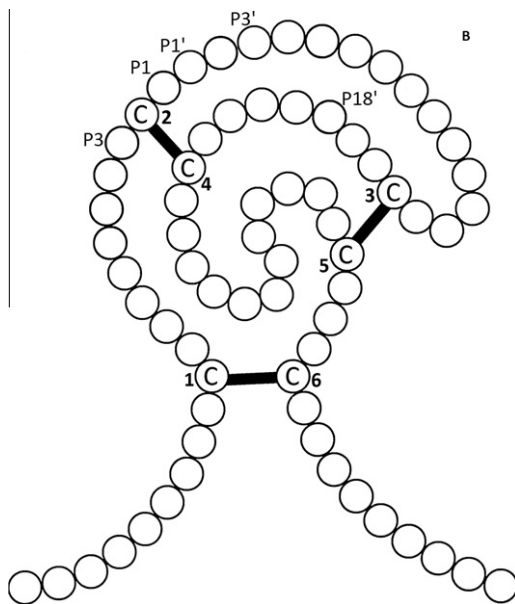


Fig. 2. Predicted folding structure for a single domain Kunitz inhibitor showing the characteristic six cysteine residues, three disulphide bonds and protease binding loop P_3 – P'_3 (modified from Chand et al. (2004)).

block the serine protease active site without any conformational changes and they form an anti-parallel β sheet between enzyme and inhibitor. The binding kinetics and enzyme affinities vary with each invertebrate Kunitz inhibitor. The segment responsible for protease inhibition is called the protease-binding loop. This convex, extended and solvent-exposed loop is highly complementary to the concave active site of the enzyme and spans from position P_3 to P'_3 (Krowarsch et al., 2003). Residues that precede or follow this segment and residues from a sequentially remote region can also contact the enzyme and influence the association energy (Ascenzi et al., 2003). The reactive site P_1 – P'_1 peptide bond is located in the most exposed region of the protease binding loop.

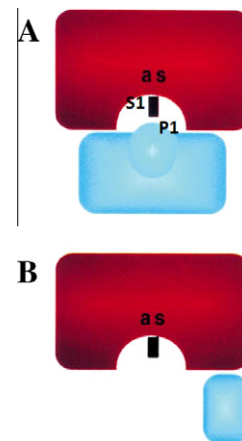


Fig. 3. Binding patterns of proteases with inhibitors. (A) Complex of a Kunitz inhibitor with active site (as) of protease (P_1 binding with corresponding S_1 site of the binding pocket); (B) binding of inhibitor with thrombin (not utilizing the active site of the enzyme). Reproduced from Bode and Huber (2000) with permission from Elsevier.

The interaction between enzyme and inhibitor can be presented in a simplified form as a hydrolysis/resynthesis reaction of the P_1 – P'_1 reactive site peptide bond. In the complex of protease–inhibitor interaction, 10–17 amino acid residues on the inhibitor site and 17–29 residues of the protease make numerous van der Waals and hydrogen bond interactions. It is generally recognized that the N-terminal side of the reactive site (P) is energetically more important than the P' C-terminal side (Perona and Craik, 1997). The recognition provided by the P_1 residue and by those residues in the chain beyond (i.e. P'_1 , P'_2 , P'_3) is more consistent conformationally due to the influence of the disulphide bridge and the start of the β -sheet (Pritchard and Dufton, 1999).

Position P_1 (Schechter and Berger, 1967) is the major determinant of the energetics and specificity of protease recognition by Kunitz inhibitors (Krowarsch et al., 1999). A large part of the enzyme–inhibitor contact is made just by the P_1 residue which pen-

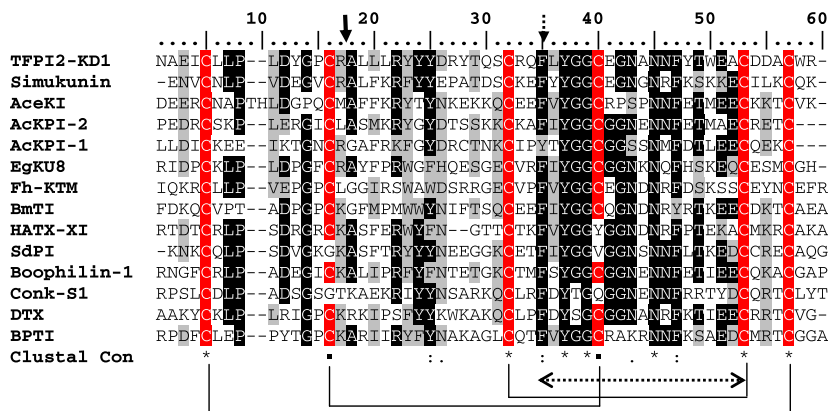


Fig. 4. Amino acid alignment of different Kunitz inhibitors. TFPI2-KD1 (AAA20094.1), first Kunitz domain of Tissue Factor Pathway Inhibitor; Simukunin (ACH56928.1), black fly salivary gland; AceKI (AAD51334.1), *A. Ceylanicum*; AcKPI-1 and AcKPI-2 (AAN10061.1), first and second Kunitz domains of *A. Caninum*; EgKU8 (ACM79010.1), *E. granulosus*; Fh-KTM (AAB46830.1), *F. Hepatica*; BmTI (P81162), *Boophilus microplus*; HATX-XI (D2YZQ9.1), *Ornithoconus* sp., SdPI (GT028613.1), *Lychas mucronatus*; boophilin-1 (CAC82583.1), first Kunitz domain of Boophilin *B. microplus*; Conk-S1 (P0C1X2.1), KTI of *Conus striatus*; DTX (1097974), Dendrotoxin; BPTI (1510193A), Basic Pancreatic Trypsin Inhibitor. The three characteristic disulphide bonds are shown in square brackets, the putative P₁–P₁' site by a black arrow, the P₁₈ by a black dotted arrow and the Kunitz family signature (F*GCG*****F****C) by the dotted double arrow line. The six cysteine residues are highlighted in orange while similar residues are highlighted in black or grey.

etrates deeply into the S1 specificity binding pocket of the protease and this docking of the P₁ side chain inside the S1 binding pocket of the enzyme plays a major role in the energetics of the recognition (Czapinska and Otlewski, 1999) (Fig. 3A). Typical trypsin inhibitors have Arg (R) or Lys (K) at P₁, whereas chymotrypsin inhibitors have Leu (L) or Met (M) (Grzesiak et al., 2000b). The P₁' position also has an effect on the protein–protein association energy that is almost as large as the P₁ position. BPTI-like inhibitors show a great tendency for Ala (A) to occur at the P₁' site and this is seldom replaced

with Gly (G) (Table 1). High-level variability of the P₁' position is observed in non-inhibitory Kunitz proteins such as snake toxins, ion channel blockers and non-canonical inhibitors (e.g. Ornithodorin) and it is likely that the lack of Ala/Gly at this position is one of the key structural features that prevents protease inhibition (Grzesiak et al., 2000a). Furthermore, the P₁₈ position has a highly conserved phenylalanine (F) residue in Kunitz inhibitors which is important in the stabilization of the reactive site structure through internal hydrophobic interactions (Chand et al., 2004).

Table 1
P₁ and P₁' site amino acids of different Kunitz type inhibitory domains with their specific inhibitory enzymes.

Name	Organism	Domain	P ₁ ^a	P ₁ ' ^a	Inhibitory enzymes	References
AceKI	<i>Ancylostoma ceylanicum</i>	1	M	A	Trypsin, chymotrypsin, elastase	Milstone et al. (2000))
Ac-KPI	<i>Ancylostoma caninum</i>	12	R	G	Trypsin	Hawdon et al. (2003)
			L	A	Chymotrypsin	
			K	G	Trypsin	
			M	A	Chymotrypsin	
			K	A	Trypsin	
			K	A	Trypsin	
			K	A	Trypsin	
			K	A	Trypsin	
			R	A	Trypsin	
			K	G	Trypsin	
			D	L	None	
BPTI	<i>Bos taurus</i>	1	K	A	Trypsin, chymotrypsin, elastase	Ascenzi et al. (2003)
Fh-Ktm	<i>Fasciola hepatica</i>	1	L	G	Chymotrypsin	Bozas et al. (1995)
EgKU-8	<i>Echinococcus granulosus</i>	1	R	A	Trypsin, chymotrypsin	Gonzalez et al. (2009)
BmTIsint	<i>Boophilus microplus</i>	1	K	G	Trypsin, plasma kallikrein, Cathepsin L	Sasaki et al. (2006)
TFPI-2	<i>Homo sapiens</i>	3	R	A	Factor VIIa	Bajaj et al. (2001)
			E	G	Factor Xa	
			S	A	None	
Ixolaris	<i>Ixodes scapularis</i>	1	E	S	Factor Xa	Francischetti et al. (2002)
Boophilin	<i>Boophilus microplus</i>	2	K	A	Trypsin, plasmin	Macedo-Ribeiro et al. (2008)
			A	G		
Simukunin	<i>Simulium vittatum</i>	1	R	A	Elastase, Factor Xa, Cathepsin G	Tsujimoto et al. (2012)
Ornithodorin	<i>Boophilus microplus</i>	1	L	G	Thrombin	van de Locht et al. (1996)
TAP	<i>Orithodoros moubata</i>	1	D	S	Factor Xa	Waxman et al. (1990)
HNTX-XI	<i>Ornithoconus</i> sp.	1	K	A	Trypsin	Yuan et al. (2008)
SdPI	<i>Lychas mucronatus</i>	1	K	A	Trypsin	Zhao et al. (2011))
Conkunitzin	<i>Conus striatus</i>	1	T	K	None	Bayrhuber et al. (2005)
21k	<i>Anisakis simplex</i>	1	T	Q	None	Shimakura et al. (2004)
Dendrotoxin	<i>Dendroaspis</i> sp.	1	K	R	None	Dreyer (1990)

^a The amino acids from the multi domain Kunitz inhibitors are placed in order from the first to last domains.

4. Kunitz inhibition of thrombin

Endogenous Kunitz-type inhibitors target a large number of serine proteases, including coagulation factors VIIa and Xa, but not thrombin. By contrast, several two-domain Kunitz inhibitors of the major procoagulant protease have been isolated from both soft and hard blood sucking arthropod ticks but, surprisingly, these anticoagulants do not follow the canonical mechanism of protease inhibition (Corral-Rodriguez et al., 2009) (Fig. 3B).

Thrombin is unique among the serine proteases as it exhibits two large positively charged surface exosites which it utilizes for selection, recognition and binding of specific substrates and inhibitors (Stubbs and Bode, 1995). Ornithodrin is a thrombin inhibitor isolated from the blood-sucking soft tick *Ornithodoros moubata*, having two highly distorted BPTI-like domains (van de Locht et al., 1996). Its C-terminal domain binds to the anion binding exosite I of thrombin. The N-terminal domain also blocks the thrombin active site indirectly; the binding loop does not contact the protease and does not utilize interactions in the S1 pocket. As in a non-canonical hirudin-like manner the N-terminal tail penetrates the thrombin active site and forms a parallel β sheet with the thrombin Ser214–Gly219 segment (Bode and Huber, 2000).

Boophilin from the hard tick *B. microplus* is bi-functional and can interact with serine proteases in a canonical manner and with thrombin in a non-canonical way at the same time (Macedo-Ribeiro et al., 2008). It is composed of two Kunitz domains more closely related to the two N-terminal TFPI (canonical Kunitz module) than to any other invertebrate protein. In contrast to all other natural thrombin inhibitors, boophilin potentially targets other serine proteases such as trypsin and plasmin (Corral-Rodriguez et al., 2009). Crystallization and preliminary crystallographic characterization of the N-terminal Kunitz domain of boophilin have been recently reported (Cereijs et al., 2012).

5. Functional roles

5.1. Serine protease inhibition

To date a number of 12 Kunitz inhibitors having the typical serine protease inhibition function have been characterized from a collection of animals ranging from the sea anemone to man; they play critical roles in several physiological processes (Bode and Huber, 2000). According to NEMBASE, a resource for nematode transcriptome analysis, more than 100 EST clusters predicted to contain Kunitz domains have been identified in parasitic and free living nematodes (Gonzalez et al., 2009). The majority of these domains appear to be present in a diverse group of molecules containing Kunitz and other domains. An example is GP300 which is a thrombospondin-like immunodominant and phosphorylcholine-containing glycoprotein consisting of six closely packed Kunitz domains at the C-terminus. It displays trypsin protease inhibitor activity and has been identified in *Dictyocaulus viviparus*, *Haemonchus contortus* and *Caenorhabditis elegans* (Kooyman et al., 2009). The fact that GP300 localizes to the cuticle and subcuticle of the two parasitic nematodes suggests it may play a role in vivo in inhibiting proteolytic damage by host digestive enzymes, although its function in *C. elegans* is unknown.

The first Kunitz type inhibitor isolated from a nematode was from *Anisakis simplex*; localized to the upper digestive tract of the larva, it was shown to have very high identity with soybean trypsin inhibitor (STI) and inhibited trypsin and elastase (Morris and Sakamori, 1994). Two Kunitz inhibitors were later identified from canine hookworms (*Ancylostoma* spp.). One comprised a single domain KI (AceKI) from *Ancylostoma ceylanicum* which was shown to have inhibitory activity against trypsin, chymotrypsin and pancreatic

elastase (Milstone et al., 2000). Furthermore, AceKI plays a role in pathogenesis causing hookworm-induced malnutrition and growth delay (Chu et al., 2004). The other was a 12 domain KI (Ac-KPI) from *Ancylostoma caninum*, 11 of which are active as chymotrypsin and trypsin inhibitors while domain 12 is inactive as a protease inhibitor, suggesting it may be an extracellular matrix protein (Hawdon et al., 2003). Kunitz inhibitors are more abundant in the transcriptome of *Necator americanus* adult worms (Rabelo et al., 2009) than in *Ancylostoma* spp., but they are not well characterized (Cantacessi et al., 2010). One *N. americanus* peptide has been shown to have some structural features in common with both the BPTI and DTX families of Kunitz inhibitors (Daub et al., 2000). Putative Kunitz inhibitors have also been identified in *Brugia malayi* (Ghedini et al., 2007) and more recently, by small RNA sequencing, in *Ascaris suum* (Wang et al., 2011a).

A single domain Kunitz inhibitor (Fh-KTM) has been identified in the trematode liver fluke *Fasciola hepatica*; it has 3-hydroxyproline residues and hence is different from other Kunitz molecules (Bozas et al., 1995). Indirect immunofluorescence and immunogold labeling revealed that Fh-KTM is an abundant molecule within the parasite, localized to the gut, the parenchymal tissue and the tegument of adult worms. Serine protease inhibition assays revealed that Fh-KTM exhibited little or no inhibition against chymotrypsin, kallikrein, urokinase or key serine proteases of the blood coagulation pathways. However, Fh-KTM was able to inhibit trypsin even though the P₁ reactive amino acid of Fh-KTM is a leucine residue. Several other putative Kunitz inhibitors have been shown present in other trematodes including *Schistosoma mansoni* (Berriman et al., 2009) and *Schistosoma japonicum* (Hu et al., 2003) but none have, as yet, been characterized.

Eight Kunitz inhibitor members were identified from EST (Expressed Sequence Tags)-based transcriptome analysis of the larval protoscolex stage of the cestode *Echinococcus granulosus* (Gonzalez et al., 2009). Of these, one Kunitz molecule (EgKU-8) was shown to be a high affinity trypsin inhibitor. Furthermore, transcriptome studies of other cestode species, including *Echinococcus multilocularis* and *Taenia solium*, have identified several other members of the “cestode Kunitz family” (Gonzalez et al., 2009). Three ESTs have also been identified in a *Taenia saginata* cDNA library as encoding putative Kunitz inhibitors (Lundström et al., 2010).

The process of blood coagulation primarily involves serine proteases with protease inhibitors functioning as negative regulators. The Kunitz protein, tissue factor pathway inhibitor (TFPI), inhibits the initial reactions of blood coagulation by the formation of factor Xa then subsequently factor VIIa-tissue factor complex (Bajaj et al., 2001). Blood feeding arthropods produce antihemostatic factors in their saliva including several Kunitz inhibitors. These include tick anticoagulant peptide (TAP), from the soft tick, *O. moubata*, which inhibits only factor Xa (Waxman et al., 1990), ixolarin from the hard tick and Lyme disease vector, *Ixodes scapularis*, which has two Kunitz domains and inhibits the factor VIIa-tissue factor complex (Francischetti et al., 2002) and many more including penthalarin, hemalin, savignin, amblin, monobin and rhipilin 1 (Corral-Rodriguez et al., 2009; Francischetti et al., 2009; Gao et al., 2011). A recently identified single domain KI (simukunin) from the black fly (*Simulium vittatum*) inhibits factor Xa, elastase and cathepsin G suggesting that it regulates both blood clotting and inflammatory responses of the host (Tsujiimoto et al., 2012).

5.2. Inhibition of other proteases

The Kunitz family also includes inhibitors of cysteine and aspartyl proteinases (Rawlings et al., 2004b). The first description of cysteine protease inhibition by *B. microplus* suggested residues at the P₁, P₃ and P_{3'} positions of the Kunitz inhibitor (BmTIsint) are the important primary structural features involved in the inhibitory

process (Sasaki et al., 2006). Furthermore, SmCI a tri-domain bifunctional Kunitz protein identified in the tentacle crown of the marine annelid *Sabellastrata magnifica* inhibits both metallo-carboxypeptidase carboxypeptidase A and serine proteases, such as trypsin and pancreatic elastase; the third domain of SmCI is a typical trypsin inhibitor containing Lys and Gly residues at positions P₁ and P₁', respectively (Alonso-del-Rivero et al., 2012).

5.3. Involvement in reproductive and allergic activity

A study of adult male and female worms of three *Ancylostoma* spp. (*A. braziliense*, *A. caninum* and *A. ceylanicum*), using previously identified 12 domain and single domain Kunitz inhibitors, showed that transcription was consistently higher in the former for both genes (Costa et al., 2009). Quantitative RT-PCR data confirmed these findings with the highest differential transcription being recorded for the 12 domain Kunitz type gene (Costa et al., 2009), suggesting this Kunitz protein is involved with endogenous serine proteases associated specifically with biological processes in the adult male worm, possibly related to reproduction.

Third stage larvae of the nematode *A. simplex* infect marine fish and invertebrates. When the larvae are ingested orally via seafood, they invade the stomach wall or intestine causing the disease anisakiasis but they can also induce IgE-mediated allergic reactions. The major allergen, identified in the larvae, is a heat stable protein (21k allergen/Ani s 1); it has IgE reactivity and a Kunitz motif but is devoid of trypsin inhibitory activity. It is an isoform of another previously identified (24k) *A. simplex* allergen and it may prove useful as an anti-allergen vaccine (Kobayashi et al., 2008; Shimakura et al., 2004).

5.4. A role in defense against microbial infection

In addition to the anticoagulant function of Kunitz protease inhibitors in blood feeding arthropods, several studies of different model systems suggest that they also have a defense role against microbial challenge. Stimulation of *Drosophila melanogaster* with

bacteria or fungi resulted in an increase in gene expression of two Kunitz proteins (De Gregorio et al., 2001). Expression of a Kunitz protease inhibitor from *B. microplus* was found to be up-regulated in response to *Babesia bovis* infection (Rachinsky et al., 2007) and a five-domain Kunitz-type molecule from the tick *Dermacentor variabilis* has been shown to have bacteriostatic properties, limiting rickettsial colonization of host cells, as well as functioning as an anticoagulant (Ceraul et al., 2008). A Kunitz-type inhibitor (BmSPI-1), present in the cocoon silk of the silkworm *Bombyx mori*, may be involved in cocoon protection against microbial proteases produced by potential predators and microbes. (Nirmala et al., 2001).

5.5. Cuticle formation and tissue remodeling

Blister-5 (BLI-5) is a protein with many features linked to the collagen biosynthetic pathway and it plays a critical role in cuticle biogenesis in nematodes through its interaction with subtilisin-like processing enzymes (such as BLI-4). It is a secreted protein having a Kunitz-type pancreatic protease inhibitor domain and a completely nematode-specific EB (Pfam PF01613) domain with eight conserved cysteine residues, and is found in association with the Kunitz domain (Page et al., 2006). First studied in *C. elegans*, homologues of BLI-5 have now been identified in several parasitic species, including *H. contortus* and *B. malayi*, spanning different nematode clades. Notably, although the BLI-5 proteins have Kunitz domains, serine protease assays have suggested that they in fact act as proteases, actively enhancing serine protease activity, as key residues from the Kunitz motif, necessary for protease inhibition, are absent from the Kunitz domains of BLI-5 (Steppek et al., 2010).

Gm KTPI, isolated from *Galleria mellonella* larvae, was the first Kunitz protein shown to be associated with extracellular regulated kinase signaling and cell proliferation (Lee et al., 2010).

5.6. Cytotoxic activity

Bioactive compounds of great interest are found in the saliva of hematophagous organisms. A transcript for amblyomin-X, a serine

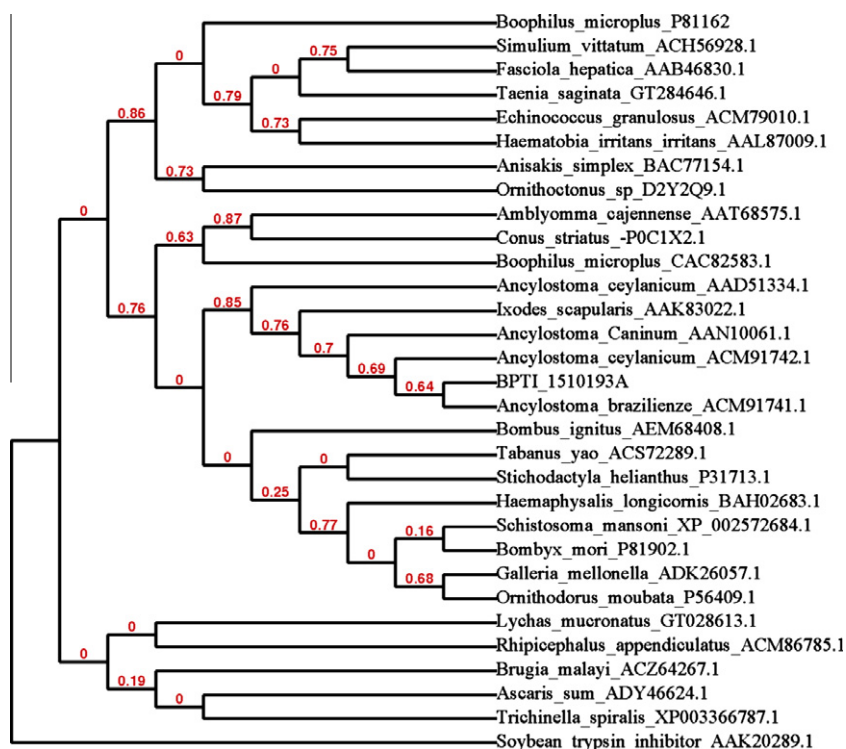


Fig. 5. Phylogenetic tree of invertebrate Kunitz inhibitors.

protease Kunitz-type inhibitor, was first isolated from a cDNA library derived from the salivary glands of the tick *Amblyomma cajennense* (Batista et al., 2010). The expressed protein is able to inhibit Factor Xa in the coagulation pathway and also promotes cytotoxic activity on several tumor cell lines, although the exact mechanism of its action is not yet fully understood. (Chudzinski-Tavassi et al., 2010). These properties are similar to the earlier described Factor Xa inhibitor, with two Kunitz domains, isolated from the *Ixodes scapularis* tick (Francischetti et al., 2002).

5.7. Involvement in formation of the extracellular matrix

Basement membranes are networks of interactive multidomain proteins with high local concentrations of components. Cells interact with the adjacent extracellular matrix (ECM) during cellular movement. Kunitz domains are found in the ECM, usually as multiple domains or in combination with other domains. Papilins are a family of secreted homologous ECM glycoproteins which occur widely from nematodes to man. Their hallmarks are a papilin cassette, consisting of one complete TSR domain, a specific unique cysteine-rich region and a string of *n* partial TSR domains, followed by Cys-rich repeats, Kunitz domains, Ig-C2 loops and a characteristic carboxy-terminal domain, where the number of copies of each of these domains varies between different papilins (Kramerova et al., 2000). However, neither native nor recombinant papilin from *Drosophila* were able to inhibit serine proteases and this is because the amino acid sequence in the reactive site is completely different from the typical Kunitz domain (Fessler et al., 2004). Recent genomic studies have shown the presence of Kunitz type domains in papilins in *A. suum*, *Trichinella spiralis* (Mitrevu et al., 2011) and *Clostridium sinensis* (Wang et al., 2011b).

6. A phylogeny of Kunitz inhibitors

Comprehensive Cladogram phylogenetic analysis of 31 Kunitz inhibitors, comprising a number described in this review together with additional sequences present in GenBank, was undertaken in order to shed light on their evolutionary relationships (Fig. 5). The sequences were aligned using MUSCLE, a bootstrapped maximum likelihood tree was generated using PhyML 3.0 and the analysis was carried out as described (Dereeper et al., 2008), using soybean trypsin inhibitor (STI) as an outgroup.

The phylogeny clearly divides the protein sequences into two distinct branches (clades), with the three proteins of the bottom clade comprising mainly putative Kunitz proteins of nematode origin having domains with distorted disulphide bonds, with one or two bonds missing. SdPI of *L. mucronatus* (GT028613.1) apparently adopts a new disulphide bridge at the C terminal and the ion channel modulator (Ra-KLP) of *R. appendiculatus* (ACM86785.1) has an altered disulphide bonding pattern (Paesen et al., 2009). The phylogenetic tree indicates that other known invertebrate Kunitz proteins, with similar structures to the classical BPTI, fall into one large clade.

7. Summary

Kunitz-type inhibitors are found in a variety of invertebrates and possess one or multiple domains or are found together with other domain types. As well as their major function in serine protease inhibition, some act as Kunitz type toxins (KTT) and they likely have other, as yet unknown, functions. Each Kunitz domain contains six highly conserved cysteines forming three disulphide bridges whereas some functionally active Kunitz inhibitors lack the second disulphide bond (C2–C4). The amino acid residues at the P₁ and P₁' sites play a critical role in inhibition of specific pro-

teases and typical trypsin inhibitors have Arg/Lys and Ala, respectively. Phylogenetic tree analysis can provide insight on common ancestors of putative Kunitz inhibitors but functional assays will be required to provide direct information on their cellular role.

With the increased publication of a large number of draft invertebrate genomes, more Kunitz-type inhibitor genes are likely to be identified by data mining and, with their subsequent biochemical characterization, more light will be shed on their biological function. In turn, this may lead to the identification of new vaccine and drug intervention targets against those invertebrate species that are parasitic.

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