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Isolation and Characterization of Protease Inhibitors from Animal Tissues

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ABSTRACT

The intent of this article is to review protease inhibitors produced from a diversity of animals including cnidarians, annelids, insects, crustaceans, mollusks, fish, amphibians, reptiles and mammals. They can be isolated from various tissues including pancreas, ova, liver, seminal plasma, serum, venom, skin secretion, hemolymph and salivary glands. Animal protease inhibitors display a variety of molecular masses ranging from several to over 50 kilodaltons. Some of them possess different protease specificity and inhibitory constants toward proteases. They play a role in various physiological processes such as reproduction, protection from viral infection, regulation of endogenous and exogenous (pathogen) proteases and anticoagulation activity.

Keywords: trypsin/chymotrypsin inhibitor; chymotrypsin/elastase isoinhibitor; Kazal-type trypsin inhibitor; cysteine protease inhibitor

CONTENTS

INTRODUCTION

Protease inhibitors (PIs) are ubiquitous in nature and are produced by diverse animals as well as plants. There are different types of proteases such as cysteine proteases (such as papain from papayas), metalloproteases (such as thermolysin from bacteria), serine proteases (such as trypsin, chymotrypsin, elastase, subtilisin, thrombin) and aspartic proteases (such as renin), based on their protease specificity. They regulate the activity of various proteases. For instance, antithrombin III is a plasma protein which forms an irreversible complex with thrombin and inactivates the enzyme. Antithrombin III also inhibits other serine proteases in the blood clotting cascade. It inhibits thrombin much more strongly than it inhibits elastase (Berg *et al*. 2007a). Pancreatic trypsin inhibitor is a 6-kDa protein that binds tightly to the active site of trypsin and inactivates it. Alpha1-antitrypsin is a 53-kDa plasma protein that protects tissues from elastase digestion. Genetic deficiency of this enzyme results in emphysema (Berg *et al*. 2007b). In this review the protease inhibitors produced by animals has been discussed.

CNIDARIANS

PIs have been identified in various animal species from lower animals to mammals and humans. This review starts with lower animals and then proceeds to higher animals. Sokotun *et al*. (2006) reported the isolation of a trypsin/chymotrypsin inhibitor from the tropical sea anemone *Radianthus macrodactylus*. It exhibited no reactivity toward other serine proteases including kallikrein, plasmin and thrombin, the aspartic protease pepsin and the cysteine protease papain. The kinetic and thermodynamic parameters for inhibitor-trypsin and inhibitor-chymotrypsin complex formation as reflected by dissociation constant which were 7.38×10^{-8} M for the former and 9.93×10^{-7} M, respectively. Subsequently, Sokotun *et al*. (2007a) employed a chromatographic procedure involving polychrome-hydrophobic chromatography, fast protein liquid chromatography on Superdex peptide and reverse-phase high performance liquid chromatography on Nucleosil C18 to isolate two new serine PIs *Radianthus macrodactylus* inhibitor-1 and inhibitor-2 from the same species of sea anemone. They demonstrate pronounced sequence homology to mammalian, reptilian and coelenterate PIs.

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Invited Mini-Review

The trypsin/chymotrypsin inhibitor from *Radianthus macrodactylus* is a 6.1-kDa Kunitz-type inhibitor with three S-S bonds but lacking Met and Trp residues. The inhibition constant values toward trypsin and α-chymotrypsin were, respectively, 2.49×10^{-9} M and 2.17×10^{-8} M. The inhibitor displays a highly ordered tertiary structure and belongs to mixed α/β or α+β polypeptides. Minagawa *et al*. (2008) isolated from Kunitz-type protease inhibitors from the aerorhagi (aggressive organs) of the sea anemones *Actinia equina*, *Anthopleura aff*, *xanthogrammica* and *Anthopleura fuscoviridis*, one each from the first two species and two from the third species. Their amino acid sequences markedly resemble known Kunitz-type protease inhibitors from the whole bodies of various sea anemone species and bovine pancreas. The six half-Cys residues in the four inhibitors appear at the same positions of the amino acid sequence as known Kunitz-type protease inhibitors. Most of the protease inhibitors were highly active against trypsin and moderately active against another serine protease, plasmin. However, they had no activity against metalloproteases (thermolysin and protease from *Streptomyces griseus*) and cysteine proteases (papain and bromelain).

Kazal2 is a Kazal-type serine protease inhibitor with potent *in vitro* bactericidal activity against *Staphylococcus aureus* produced by epithelial cells and gland cells in *Hydra*. This finding may shed new light on the mechanisms of host defense early in metazoan evolution (Augustin *et al*. 2009).

APEKTx1 is a new member of the type 2 sea anemone peptides targeting voltage-gated potassium channels (K(V)s isolated from the sea anemone *Anthopleura elegantissima*. It contains 63 amino acids cross-linked by 3 disulfide bridges. Similar to the kalicludines from *Anemonia sulcata*, APEKTx1 shares structural homology with both basic pancreatic trypsin inhibitor (BPTI), a very potent Kunitz-type protease inhibitor, and dendrotoxins which are powerful blockers of voltage-gated potassium channels. APEKTx1 selectively and potently blocks K(V)1.1 channels with an IC_{50} value of 0.9 nM. APEKTx1 inhibits trypsin with a dissociation constant of 124 nM. APEKTx1 is a potent and selective blocker of K(V)1.1 channels and a competitive inhibitor of trypsin (Peigneur *et al*. 2011).

Cole *et al*. (2004) initiated a cDNA library screening of the cnidarian, *Cyanea capillata* and identified one cDNA coding for a full-length serine protease inhibitor designated as jellypin. Jellypin resembled serine protease inhibitor from the platyhelminth *Echinococcus multiocularis* and the clade P serine protease inhibitors, suggesting its evolution many years ago. Jellypin, which contained all the functional elements of a serine protease inhibitor, was an inhibitor of the S1 clan SA family of serine proteases. It inhibited human chymotrypsin, cathepsin G, and elastase, forming a SDS stable enzyme/inhibitor complex.

ANNELIDS

From the medicinal leach *Hirudo medicinalis* 13 protease inhibitors with notable sequence resemblance to known serine proteases have been separated (Yanes *et al*. 2005). The majority of these *H. medicinalis* serine proteases are found in different leech trypsin inhibitor families. However, other inhibitors are similar to other families. For instance, the 3,128-Da inhibitor demonstrates a well conserved region (^{7}DENTPCP^{13}) completely identical to *Ascaris lumbricoides* chymotrypsin/elastase isoinhibitor. Two couples of inhibitors with molecular masses of 6,218/6,227 and 5,463/5,325 Da manifest identical sequences within the first 49 and 19 amino acids from the N terminus, respectively, indicating protein degradation or presence of isoforms. The 7,900-Da protein is probably a degraded form of Eglin C or an isoform. Some of the isolated putative serine protease inhibitors display a clear similarity with other inhibitors of the antistasin family previously found in leeches, such as guamerin, piguamerin, bdellin A, and hirustasin.

In the tentacles of the annelid *Sabellastarte indica* Savingny there are at least five different isoinhibitors with inhibitory activity towards trypsin, plasmin, chymotrypsin and kallikrein. They are characterized by heat lability, a high molecular weight, and an isoelectric point in the weakly acid region. They exhibit some similarities to ovomucoid and soybean Kunitz-type inhibitor (Gauwerky *et al*. 1975). The *Sabellastarte indica* inhibitors exhibit a stoichiometric binding ratio of 2:1 for trypsin. Lysine is located at the reactive centre of the inhibitor for interaction with trypsin. The trypsin binding reactive centre is not identical to the chymotrypsin binding reactive centre and does not affect the binding of chymotrypsin. These inhibitors are thus multiheaded, which previously has not been described for invertebrates (Mitteilung 1976).

INSECTS

A serine PI, HiTI, from the thoracic extract of the hornfly *Haematobia irritans irritans*, which is an ectoparasite that sucks blood from cattle had been reported (Azzolini *et al*. 2005). Expression of the recombinant peptide using the ppIC9 expression vector and purification of the recombinant peptide on trypsin-Sepharose yielded a 6.53-kDa peptide with a Ki of 0.4 nM toward bovine trypsin and 1.0 nM toward human neutrophil elastase. HiTI also inhibits the trypsin-like enzyme from *H. irritans irritants* and the Omp T endoprotease of *E. coli*, indicating its role in regulating the endogenous protease and pathogen protease.

The cloning of two locust PIs, each composed of 73 amino acids fusion to the constitutive CaMV 35S promoter, and introduction by *Agrobacterium*-mediated transformation into potato, were described by Kondrak *et al*. (2005). Colorado potato beetle demonstrated retarded growth on transgenic plants than on control plants.

The cattle tick *Boophilus microplus*, which parasitizes farm animals, produces a multifunctional Kunitz-type inhibitor designated as boophilin (Macedo-Ribeiro *et al*. 2008). It contains two canonical Kunitz-type domains, and inhibits thrombin, trypsin and plasmin. The crystal structure of its complex with thrombin has been studied. The mode of binding to the protease is non-canonical. The N-terminal region of the inhibitor binds across the active site of the protease in a parallel fashion. Its negatively charged C-terminal Kunitz domain docks into the basic exosite 1 of thrombin.

Two isoforms of a 13.2-kDa serine protease inhibitor, with inhibitory activity against bovine trypsin and chymotrypsin and the tasar silkworm larval midgut serine protease, were isolated from hemolymph of the tasar silkworm (*Antheraea mylitta*) larva. The chromatographic procedure used entailed affinity chromatography on immobilized trypsin and gel filtration. Its N-terminal amino acid sequence differed from those of previously reported serine protease inhibitors. It possessed only intermediate themostability and pH stability. Its activity was stable from 4 to 65°C and from pH 4.5 to 9 (Rai *et al.* 2010).

Cysteine protease inhibiting activity was detected in the midgut of three phytophagous lepidopteran species. *Trichoplusia ni*, *Heliothis virescens* and *Helicoverpa zea*. The activity was thermostable and stable within the pH range 6-10. The presence of intact disulfide bond(s) is essential to its activity as evidenced by the attenuation of activity in the presence of thiol- reducing chemicals (Li *et al*. 2009).

Watanabe *et al*. (2010) analyzed the sialotranscriptome of the mosquito *Aedes aegypti* and deleted the existence of a 7-kDa Kazal–type trypsin inhibitor in the entire male mosquito and in the salivary glands and carcass of the female mosquito. It exhibited a Ki of 3.8 nM and 0.15 nM toward plasmin and trypsin, respectively. Although, its inhibition of thrombin amidolytic activity was weak, it prolonged prothrombin time, activated partial thromboplastin time and thrombin time. The PI exerts its anticoagulant activity during blood feeding in the female mosquito and plays other roles during the larval and pupal stages.

Meiser *et al*. (2010) studied Kazal-type inhibitors in the stomach of the triatomine *Panstrongylus megistus.* There

were three double-domain inhibitors and one single-domain inhibitor exhibiting sequence identities of up to 91% to the respective domains of Kazal-type inhibitors from other triatomines. The gene of the inhibitors is expressed in the stomach (anterior midgut) but not in the small intestine (posterior midgut), salivary glands or haemocytes.

Leviserpin from the sugar cane weevil *Sphenophorus levis* inhibited bovine trypsin by the formation of the covalent complex serpin-peptidase, as witnessed by SDS-PAGE and mass spectroscopic analysis. The specificity of leviserpin, together with its mRNA coding being transcribed throughout the life cycle of the insect, suggest a possible role in defense mechanism by regulating the action of prophenoloxidase (proPO) activating enzyme (Fonseca *et al*. 2011).

CRUSTACEANS

The Kazal-type serine PI from the black tiger shrimp *Penaeus monodon* was identified from the hemocyte cDNA library and expressed in *E. coli.* The recombinant protein possesses 5 domains and a molecular weight of 29 kDa. It competitively inhibited elastase and subtilisin with a Ki of 3.27 and 0.52 nM, respectively. Trypsin was inhibited by a small extent but chymotrypsin was not affected. Its subtilisin inhibiting activity represents defense activity against pathogenic bacteria (Somprasong *et al*. 2006).

Li *et al*. (2010) identified from a c DNA library of the red swamp crayfish *Procambarus clarkii*, three Kazal-type serine PIs. One of them, hcpc SPI2, was detected mainly in the hemocytes while the other two, hp Pc SPI3 and hp Pc SPI 4, were found in the heart and the hepatopancreas. While all three inhibitors respond to challenge by *Vibrio angeillarum* albeit differently, hcPc SPI2 and hpPc SPI3 probably participate in antiviral immune response, hcPc SPI2 inhibited trypsin with a low potency. It also weakly inhibited subtilisin A and demonstrated antibacterial activity forward *Bacillus subtilis* and *B. thuringiensis.*

The finding suggests that it regulates antibacterial immunity in crayfish.

MOLLUSKS

The plasma of the eastern oyster *Crassostrea virginica* contained a 7.61-kDa serine protease inhibitor with a novel amino acid sequence and six intrachain disulfide bonds. It inhibits trypsin, subtilisin, and perkinsin (major extracellular protease of the protozoan *Perkinsus marinus* parasitic on the oyster) with a Ki of 17.7, 0.29 and 13.7 nM, respectively. The inhibitor binds quickly to the proteases to form weak complexes followed by slow isomerization to produce very tight complexes (Xue *et al*. 2006)

The marine snail *Cenchritis muricatus* produces a 5.48-kDa non-classical Kazal-type inhibitor with three disulfide bonds. Its inhibits trypsin, human neutrophil elastase pancreatic elastase and subtilisin A with values of 11 ± 0.9, 2.6 ± 0.2, 14.5 ± 4.4 and 30.8 ± 1.2 nM, respectively, chymotrypsin, papain, pancreatic and plasma kallikreins and thrombin remained unaffected. The three–dimensional (3-D) model of the inhibitor exhibits characteristics similar to those of Kazal-type inhibitors. A 3-D model of the complex formed between the inhibitor and human neutrophil elastase demonstrates analogous as well as dissimilar contacts at the primany binding sites when compared with the complex formed between trukey ovomucoid third domain and human neutrophil elastase. Additional contacts at the protease – inhibitor interface may contribute to the association energy of the complex. The inhibitor is unique in its potent inhibition of trypsin and elastases (Gonzalez *et al*. 2007).

A quaternary alkaloid, from the cell-free hemolymph of the marine mussel *Perna viridis* that has been challenged with bacteria, potently inhibited serine proteases. The EC_{50} of Ki, Et/Ki, and Et/Km were 102.5 μM, 97.1-104.68 μM, 6.3, and 1.04, respectively. As revealed by Van't Hoff equation, Ki declined as temperature was elevated, and the inhibitor binding was driven entropically (Khan *et al*. 2008).

FISH

Two isoforms of a protease inhibitor were isolated from the seminal plasma of the common carp *Cyprinus carpio* (Wojtczak *et al*. 2007). They possess a molecular weight of 54 and 55.5 kDa, and a carbohydrate content of 12.1 and 12.6%, respectively. The two isoforms have the same first 10 N-terminal amino acids. Immunolocalization of the protease inhibitor in the region around the spermatids indicates a role in regulation of spermatogenesis, production of spermatozoa and testicular tissue from undesired proteolysis and maintenance of the connective tissue in testis.

Mickowska (2009) isolated serine protease inhibitors α1-PI and antithrombin III from blood plasma of the common carp *Cyprinus carpio* and the rainbow trout, *Oncorhynchus mykiss*. The striking sequence similarity of the reactive site loops of the inhibitors suggests homology with other serpins. The association rate constants for interaction with bovine trypsin and chymotrypsin were 2-5.2 × 10^{-6} M^{-1} S^{-1} and the K for human neutrophil elastase exceeded 10^{-7} M^{-1} S^{-1}, for both carp and trout α1-PI. The association rate constants for interaction with bovine thrombin and trypsin exceeded 10^{-7} M^{-1} S^{-1} and was 1.3 × 10^{4} - 7.9 × 10^{5} M^{-1} S^{-1} for both carp and trout antithrombin III.

AMPHIBIANS

A 22-kDa single-chain glycoprotein with trypsin inhibitory activity was isolated from the skin extract of the toad *Bufo andrewski* (Zhao *et al*. 2005a). Ion exchange chromatography, gel filtration, and reverse-phase chromatography were employed for the isolation. The mode of inhibition was competitive. The inhibitor constant Ki was 14 nM and the inhibitor did not exhibit reactivity toward chymotrypsin, elastase and thrombin.

The same group of investigators isolated an irreversible serine protease inhibitor from the skin secretions of the same frog species (Zhao *et al*. 2005b). The 60-kDa single-chain glycoprotein designated as baserpin, was purified by ion exchange chromatography and gel filtration. Its association rate constant with bovine chymotrypsin, porcine elastase, and bovine trypsin were 8.9 × 10^{6}, 6.8 × 10^{6}, and 4.6 × 10^{6} $M^{-1}S^{-1}$, respectively. The inhibitor had no effect on thrombin.

Skin albumin purified from the frog *Bimbina maxima* differs in sequence from the homologous serum albumin by only two amino acid residues. The inhibitor binds trypsin in a 1:1 molar ratio. The equilibrium dissociation constants of the skin and serum albumins were 1.92 × 10^{-9} M and 1.55 × 10^{-9} M, respectively. A noncovalent complex is formed with trypsin through an exposed loop generated by Cys 53-Cys62 disulfide bridge, which comprises the scissile bond Arg58 (P1)-His 59 (P1). No reactivity toward chymotrypsin, elastase, subtilisin, and thrombin was discernible. The distribution of the albumin within the stratum spongiosum of the dermis and around the membrane of epithelial cells indicates a regulatory role in water economy and exchange of metabolites in addition to osmoregulation (Zhang *et al*. 2005).

A single-chain 14.4-kDa serine protease inhibitor with bacteriostatic activity toward *Bacillus subtilis* has been isolated from ova of the odor frog *Rana grahami*. The protein, designated as ranaserpin, was obtained by using a procedure that involved gel filtration on Sephadex G 50, anion exchange chromatography on Resource Q, and reverse phase-HPLC on a C4 column. Its Ki values for elastase, subtilisin and trypsin were, respectively, 2.7 × 10^{-7}, 2.2 × 10^{-8}, and 6.2 × 10^{-8} M. It was suggested that the inhibitor plays a defense role against pathogens and pests (Han *et al*. 2008).

A trypsin inhibitor with 17 amino acid residues and a six-residue loop containing 2 half-Cys residues was isolated from the skin secretion of the frog *Odorrana grahami*. Its overall structure is dissimilar to other serine protease inhib-

itors (Li *et al*. 2008).

A peptide composed of 18 residues with an amidated C-terminus and trypsin inhibitory activity was isolated from frog (*Huia versabilis*) skin secretions. It is a Bowman-Birk type PI. Its precursor has a highly conserved signal peptide, an intervening domain with a high content of acidic amino acids, and a single C-terminal domain encoding the trypsin inhibitor. A synthetic form of the inhibitor with Lys-8 in the P1 position displays highly potent trypsin inhibitory activity (Ki = about 19 nM). The potency declined as Lys-8 was replaced by Arg (Ki = 57 nM) and totally eliminated by substitution with Phe (Song *et al*. 2008).

REPTILES

A Kunitz-type serine PI textilinin-1, which inhibited trypsin and plasmin, was purified from venom of the Australian common brown snake, *Pseudonaja textilis.* Its crystal structure has an overall fold similar to aprotinin. The much weaker plasmin blinding affinity of textilinin-1 in comparison with aprotinin was attributed to the existence of a bulky valine at the p1 site (Millers *et al*. 2009).

King cobra (*Ophiophagus hannah*) venom contained a 6.34-kDa trypsin/chymotrypsin inhibitor. It inhibited chymotrypsin and trypsin with a Ki of 8.48×10^{-8} M and 3.91×10^{-7} M, respectively. It was isolated with a procedure involving gel filtration, affinity chromatography on immobilized trypsin, and reverse phase HPLC. It was expressed in *E. coli* as a maltose-binding fusion protein (He *et al*. 2008).

Richards *et al*. (2011) identified and characterised cystatin-like cysteine-protease inhibitors from elapid snake venoms. One highly conserved isoform highly homologous to family 2 cystatins was detected in the elapid snakes studied. Recombinant *Austrelaps superbus* (Australian lowland copperhead) snake venom inhibited cathepsin L ≅ papain > cathepsin B, but there was no inhibition of calpain or legumain. The evolutionarily conserved Gly-11 of family 2 cystatins, crucial for cysteine protease inhibition, is conserved in snake venom cystatins as Gly-3. The widespread, low-level expression of type 2 cystatins in snake venom, together with the presence of only one highly conserved isoform in each species, imply essential housekeeping or regulatory roles for these proteins (Richards *et al*. 2011).

BIRDS

Serine protease inhibitor with the same N-terminal amino acid sequence was found in the reproductive tract and seminal plasma of the turkey *Meleagris gallopavo.* Affinity chromatography, ion exchange chromatography and reverse phase chromatography were used to isolate the seminal plasma inhibitor while only the first two types of chromatography were employed for purification of the testicular inhibitor. There are two forms or the seminal plasma inhibitor: a virgin form with slower electrophoretic mobility and a modified form with faster mobility (Slowinska *et al*. 2008). The inhibitors belong to the Kazal family (pancreatic secretory inhibitors, mammalian acrosin inhibitors)

Two isoforms, one with a molecular weight of 5938 Da and another with a molecular weight of 6026 Da, were isolated from chicken liver. The procedure included perchloric acid extraction, $(NH_4)_2SO_4$ precipitation, fractionation with ethanol and acetone, get filtration, ion exchange chromatography, and reverse-phase HPLC.

Its association constant values toward bovine trypsin, porcine trypsin, cationic form of chicken trypsin, anionic form of trypsin, and human plasmin were, respectively, 1.1×10^{-9}, 2.5×10^{-9}, 1.2×10^{-10}, 4.5×10^{-8}, and 1.2×10^{-7} M (Kubiak *et al*. 2009)

A 7650-Da secretory trypsin inhibitor with homology to chicken PSTI was isolated from ostrich pancreatic tissue using acid extraction, salt fractionation, chromatography on SP-Sephadex C-50 and QAE-Sephadex A-25 and RP-HPLC. The isoelectric point of ostrich PSTI was 5.7. It inhibited ostrich and commercial bovine trypsin with Ki values of 8.0 $\times 10^{-9}$ and 2.4×10^{-7} M, respectively, while no inhibitory effects were observed with other serine proteases (Zhao *et al.* 1996).

Native and cleaved alpha 1-PIs with a molecular weight of about 66 kDa were isolated from ostrich serum using ammonium sulfate precipitation, and ion exchange chromatography on DEAE-Toyopearl 650 M. Isoelectric focusing of the inhibitor in the pH range 3-10 demonstrated pI values of 4.84 and 4.91, and in the pH range 4-6 the microheterogeneity characteristic of mammalian α1-PIs was observed. Sialic acid, hexoses and hexosamines were present in the inhibitor.

MAMMALS

A 60-kDa glycoprotein serine PI was isolated from ovine serum by $(NH_4)_2SO_4$ precipitation, affinity chromatography on blue Sepharose, gel filtration, and affinity chromatography on Con A-Sepharose. The Phe (350) and Met (356) residues were involved in recognition and inhibition of the serine protease. Glycosylation is related to thermal stability of the inhibitor. The ovine inhibitor was less themostable than its human counterpart due to a lower level of glycosylation. Removal of the carbohydrate moieties of the ovine and human inhibitors by enzymatic means brought about a decline in thermostability (Gupta *et al*. 2008).

Madine Darby canine kidney (MDCK) cells secrete trypsin inhibitors to inhibit replication of influenza viruses since viral infectivity depends on protease activation of the viral glycoprotein hemagglutinin. Trypsin inhibiting activity in the culture supernatant of MDCK cells was separated into a 15-kDa submandibular protease inhibitor and an 11-kDa canine kidney protease inhibitor. The latter exhibits about 64% sequence identity with human secretory leukocyte protease inhibitor (SLPI). The trypsin inhibitors protect host cells from viral infection (Nishiyama *et al*. 2008).

Bovine pancreatic trypsin inhibitor carrying an Arg residue at the P1 position, and porcine pancreatic inhibitor with a Lys residue instead, specifically inhibited Arg-specific cysteine protease gingipain R and Lys-specific cysteine protease gingipain k, respectively. The Ka values were 1.6×10^{6} and 2×10^{4} M^{-1}, respectively. Gingipains R and K are produced by *Porphyromonas gingivalis*, the predominant pathogen causing adult periodontitis (Bania *et al*. 2008).

Recombinant bovine pancreatic trypsin inhibitor protected against CCl_4-induced liver damage in mice as judged by reduction of oxidative stress and inflammation, histopathological changes and serum aminotransferase activities (Yang *et al*. 2010).

By using ion exchange chromatography on DEAE-Sephacel, gel filtration on Sephadex G15, and ion exchange HPLC on a Q anion exchange, a Kazal-type serine protease inhibitor was isolated from murine seminal vesicular secretion (Lin *et al*. 2008). The protein possessed six conserved Cys residues almost identical to those of other Kazal-type serine protease inhibitors. The protein, which was testosterone-regulated, bound to spermatozoa, augmented their motility, inhibited sperm capacitation induced by bovine serum albumin, and prevented sperm-egg interaction *in vitro*. Thus, it appears to be a decapacitating factor. It was immunolocalized in the mucosal epithelium and luminal fluid of seminal vesicles (He *et al*. 2008).

Trypsin inhibitors with a molecular mass of 6.3-7 kDa were purified from equine seminal plasma using $(NH_4)_2SO_4$ precipitation, FPLC-gel filtration on Superose 12, and reverse-phase HPLC on a C18 column. Different isoforms of the trypsin inhibitor were detected in the seminal plasma (Vasconcelos *et al*. 2009).

Trypsin inhibitors with a molecular mass of 6.3-7 kDa were purified from equine seminal plasma using $(NH_4)_2SO_4$ precipitation FPLC-gel filtration on Superose 12 and reverse-phase HPLC on a C18 column. Different isoforms of the trypsin inhibitor were detected in the seminal plasma (Vasconcelos *et al*. 2009)

The N-terminal amino acid sequences and other bioche-

Table 1 Biochemical characteristics of selected animal protease inhibitors.

	Species name	Specificity toward proteases	Molecular mass (kDa)	Ki (Protease)
Sea anemone	*Radianthus macrodactylus*	No inhibitory action on thrombin, papain and kallikrein	6.1	24.9 nM (trypsin) 21.7 nM (α-chymotrypsin)
Hornfly thoracic extract	*Haematobia irritans irritans*	Inhibition of OmpT endoprotease of *E. coli* and trypsin-like enzyme from *H. irritans irritans*	6.53	0.4 nM (bovine trypsin) 1 nM (human neutrophic elastase)
Tasar silkworm larvae	*Antheraea mylitta*	Inhibition of larval midgut trypsin and chymotrypsin as well as bovine trypsin and chymotrypsin	13.2	
Mosquito	*Aedes aegypti*	Weakly inhibits thrombin activity	7	3.8 nM (plasmin) 0.15 nM (trypsin)
Black tiger shrimp	*Penaeus monodon*	Slight inhibition of trypsin No effect on chmotrypsin	29	3.27 nM (elastase) 0.52 nM (subtilisin)
Marine snail	*Cenchritis muricatus*	No inhibition of chymotrypsin, thrombin, papain, kallikreins	5.48	11 nM (trypsin) 30.8 nM (subtilisin) 14.5 nM (pancreatic elastase)
Toad skin	*Bufo andrewsi*	No inhibition of chymotrypsin, thrombin and elastase	22	14 nM (trypsin)
Odor frog ova	*Rana grahami*	No inhibitory action on trypsin and chymotrypsin	14.4	62 nM (trypsin) 22 nM (subtilisin) 270 nM (elastase)
Frog skin	*Huia versabilis*	No inhibitory action on chymotrypsin	2	19 nm (trypsin)
King cobra venom	*Ophiophagus hannah*	No inhibitory action on thrombin and subtilisin	6.34	84.8 nM (chymotrypsin) 391 nM (trypsin)
Chicken liver	*Gallus domesticus*		5.938 6.026	1.1 nM (bovine trypsin) 2.5 nM (porcine trypsin) 120 nM (human plasmin)

Table 2 N-terminal amino acid sequences of selected animal protease inhibitors.

Protease inhibitor	N-terminal sequence	Reference
Rm In I from sea anemone, *Radianthus macrodactylus*	GICSEPIVVGPCKAG	Sokotun *et al*. 2007a
Rm In II from *R. macrodactylus*	GSTCLEPKVVGPCKA	Sokotun *et al*. 2007a
Isoforms a and b of carp seminal plasma protease inhibitor	SLPDTVILNR	Wojtczak *et al*. 2007
Trypsin inhibitor from toad *Bufo andrewsi* skin	EKDSITD	Zhao *et al*. 2005a
Baserpin from toad *B. andrewsi* skin	HTQYPDILIAKPXDK	Zhao *et al*. 2005b
Trypsin inhibitor from frog (*Odorrana grahami*) skin secretion	AVNIPFKVHFRCKAAFC	Li *et al*. 2008
Trypsin inhibitor from skin secretion of frog *Huia versabilis*	SVIGCWTKSIPPRP (FVK-amide)	Song *et al*. 2008

mical characteristics of some animal PIs are presented in **Tables 1** and **2**.

DISCUSSION

Different chromatographic methods were utilized to isolate PIs from animal tissues. In some cases trypsin-Sepharose was used (Azzolini *et al*. 2005; Rai *et al*. 2010). Affinity chromatography on Blue-Sepharose and Con-A Sepharose (Gupta *et al*. 2008), hydrophobic chromatography (Sokotun *et al*. 2007a), gel filtration (Zhao *et al*. 2005a; Sokotun *et al*. 2006; He *et al*. 2008; Rai *et al*. 2010), HPLC (Sokotun *et al*. 2007b), ion exchange chromatography (Zhao *et al*. 2005a), and a combination of these methods have also been used.

PIs have been isolated from different organs and their physiological functions may be related to the tissues from which they originate from or the mode of life of the animal. Mosquito PI manifests an anticoagulant function during blood sucking (Watanabe *et al*. 2010) and have other functions at other stages of the mosquito. PI in seminal plasma of the carp regulates testicular activity (Wojtczak *et al*. 2007). Frog skin PI is involved in osmoregulation (Zhang *et al*. 2005). Other PIs may play a defensive role like hornfly inhibitor (Azzolini *et al*. 2005) and black tiger shrimp inhibitor (Somprasony *et al*. 2006).

It is noteworthy that a quaternary alkaloid from the hemolymph of bacteria-challenged mussel *P. viridis* is a serine protease inhibitor (Khan *et al*. 2008), when the bulk of the aforementioned PIs are proteins and peptides. There is a wide range of molecular masses (**Table 1**) from about several kDa (Gonzalez *et al*. 2007) to over 50 kDa (Zhao *et al*. 2005b; Wojtczak *et al*. 2007). In addition, there are variations in specificity and inhibitor constants toward different proteases (**Table 1**) and also differences in amino acid sequence (**Table 2**), indicating that different proteins are produced by different animals though these proteins serve the same function of inhibiting proteohytic enzymes. Some of them belong to the kazal type (Somprasong *et al*. 2006; Gonzalez *et al*. 2007; Slowinska *et al*. 2008; Lin *et al*. 2008; Watanabe *et al*. 2010), others to the Kunitz type (Macedo-Ribeiro *et al*. 2008; Minagavva *et al*. 2008; Millers *et al*. 2009) while some are of the Bowman-Birk type (Song *et al*. 2008).

CONCLUSION AND FUTURE PROSPECTS

Transgenic plants overexpressing locust protease inhibitors show augmented resistance to Colorado potato beetles than control plants (Kondrak *et al*. 2005). It is possible that animal protease inhibitors can be exploited for the welfare of mankind.

REFERENCES

Augustin R, Siebert S, Bosch TC (2009) Identification of a Kazal-type serine protease inhibitor with potent anti-staphylococcal activity as part of hydra's innate immune system. *Developmental and Comparative Immunology* **33**, 830-837

Azzolini SS, Sasaki SD, Campos IT, S Torquato RJ, Juliano MA, Tanaka AS (2005) The role of HiTI, a serine protease inhibitor from *Haematobia irritans irritans* (Diptera: Muscidae) in the control of fly and bacterial proteases. *Experimental Parasitology* **11**, 30-36

Bania J, Kubiak A, Wojtachnio K, Polanowski A (2008) Pancreatic secretory trypsin inhibitor acts as an effective inhibitor of cysteine proteinases gingipains from *Porphyromonas gingivalis*. *Journal of Periodontal Research* **43**, 232-236

Berg JM, Tymoczko JL, Stryer L (2002a) *Biochemistry* (2nd Edn), W.H. Free-

man and Co, New York, Chapter 10, 289 pp

Berg JM, Tymoczko JL, Stryer L (2002b) *Biochemistry* (2nd Edn), W.H. Freeman and Co, New York, Chapter 10, 284 pp

Cole EB, Miller D, Rometo D, Greenberg RM, Brömme D, Cataltepe S, Pak SC, Mills DR, Silverman GA, Luke CJ (2004) Identification and activity of a lower eukaryotic serine proteinase inhibitor (serpin) from *Cyanea capillata:* Analysis of a jellyfish serpin, jellypin. *Biochemistry* **43**, 11750-11759

Fonseca FP, Ike PT, Assis DM, Icimoto MY, Juliano MA, Juliano L, Puzer L, Henrique-Silva F (2011) Leviserpin: A serine peptidase inhibitor (Serpin) from the sugarcane weevil *Sphenophorus levis*. *The Protein Journal* **30**, 404-412

Gauwerky C, Corman G, Uhlenbruck G (1975) New protease-inhibitors with broad specificity in the polychaet *Sabellastarte indica* (Savingny). *Zeitschrift für Klinische Chemie und Klinische Biochemie* **13**, 429-435

González Y, Pons T, Gil J, Besada V, Alonso-del-Rivero M, Tanaka AS, Araujo MS, Chávez MA (2007) Characterization and comparative 3D modeling of CmPI-II, a novel 'non-classical' Kazal-type inhibitor from the marine snail *Cenchritis muricatus* (Mollusca). *Biological Chemistry* **388**, 1183-1194

Gupta VK, Appu Rao AG, Gowda LR (2008) Purification and biochemical characterization of ovine alpha-1-proteinase inhibitor: Mechanistic adaptations and role of Phe350 and Met356. *Protein Expression and Purification* **57**, 290-302

Han Y, Yu H, Yang X, Rees HH, Liu J, Lai R (2008) A serine proteinase inhibitor from frog eggs with bacteriostatic activity. *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* **149**, 58-62

He YY, Liu SB, Lee WH, Qian JQ, Zhang Y (2008) Isolation, expression and characterization of a novel dual serine protease inhibitor, OH-TCI, from king cobra venom. *Peptides* **29**, 1692-1699

Khan MS, Goswami U, Rojatkar SR, Khan MI (2008) A serine protease inhibitor from hemolymph of green mussel, *Perna viridis*. *Bioorganic and Medicinal Chemistry Letters* **18**, 3963-3967

Kondrák M, Kutas J, Szenthe B, Patthy A, Bánfalvi Z, Nádasy M, Gráf L, Asbóth B (2005) Inhibition of Colorado potato beetle larvae by a locust proteinase inhibitor peptide expressed in potato. *Biotechnology Letters* **27**, 829-834

Kubiak A, Jakimowicz P, Polanowski A (2009) A Kazal-type serine proteinase inhibitor from chicken liver (clTI-1): Purification, primary structure, and inhibitory properties. *International Journal of Biological Macromolecules* **45**, 194-199

Li C, Song X, Li G, Wang P (2009) Midgut cysteine protease-inhibiting activity in *Trichoplusia ni* protects the peritrophic membrane from degradation by plant cysteine proteases. *Insect Biochemistry and Molecular Biology* **39**, 726-734

Li J, Wu J, Wang Y, Xu X, Liu T, Lai R, Zhu H (2008) A small trypsin inhibitor from the frog of *Odorrana grahami*. *Biochimie* **90**, 1356-1361

Li XC, Zhang RR, Sun RR, Lan JF, Zhao XF, Wang JX (2010) Three Kazal-type serine proteinase inhibitors from the red swamp crayfish *Procambarus clarkii* and the characterization, function analysis of hcPcSPI2. *Fish and Shellfish Immunology* **28**, 942-951

Lin MH, Lee RK, Hwu YM, Lu CH, Chu SL, Chen YJ, Chang WC, Li SH (2008) SPINKL, a Kazal-type serine protease inhibitor-like protein purified from mouse seminal vesicle fluid, is able to inhibit sperm capacitation. *Reproduction* **136**, 559-571

Macedo-Ribeiro S, Almeida C, Calisto BM, Friedrich T, Mentele R, Stürzebecher J, Fuentes-Prior P, Pereira PJ (2008) Isolation, cloning and structural characterisation of boophilin, a multifunctional Kunitz-type proteinase inhibitor from the cattle tick. *PLoS One* **3**, e1624

Meiser CK, Piechura H, Werner T, Dittmeyer-Schäfer S, Meyer HE, Warscheid B, Schaub GA, Balczun C (2010) Kazal-type inhibitors in the stomach of *Panstrongylus megistus* (Triatominae, Reduviidae). *Insect Biochemistry and Molecular Biology* **40**, 345-353

Mickowska B (2009) Purification and characterization of alpha(1)-proteinase inhibitor and antithrombin III: Major serpins of rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) blood plasma. *Fish Physiology and Biochemistry* **35**, 231-240

Millers EK, Trabi M, Masci PP, Lavin MF, de Jersey J, Guddat LW (2009) Crystal structure of textilinin-1, a Kunitz-type serine protease inhibitor from the venom of the Australian common brown snake (*Pseudonaja textilis*). *FEBS Journal* **276**, 3163-3175

Minagawa S, Sugiyama M, Ishida M, Nagashima Y, Shiomi K (2008) Kunitz-type protease inhibitors from acrorhagi of three species of sea anemones. *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* **150**, 240-245

Mitteilung Z (1976) Further characterisation of trypsin inhibitors in the polychaet *Sabellastarte indica* (Savigny). *Journal of Clinical Chemistry and Clinical Biochemistry* **14**, 245-251

Nishiyama K, Sugawara K, Nouchi T, Kawano N, Soejima K, Abe S, Mizokami H (2008) Purification and cDNA cloning of a novel protease inhibitor secreted into culture supernatant by MDCK cells. *Biologicals* **36**, 122-133

Peigneur S, Billen B, Derua R, Waelkens E, Debaveye S, Béress L, Tytgat J (2011) A bifunctional sea anemone peptide with Kunitz type protease and potassium channel inhibiting properties. *Biochemical Pharmacology* **82**, 81-90

Rai S, Aggarwal KK, Mitra B, Das TK, Babu CR (2010) Purification, characterization and immunolocalization of a novel protease inhibitor from hemolymph of tasar silkworm, *Antheraea mylitta*. *Peptides* **31**, 474-481

Richards R, St. Pierre L, Trabi M, Johnson LA, de Jersey J, Masci PP, Lavin MF (2011) Cloning and characterisation of novel cystatins from elapid snake venom glands. *Biochimie* **93**, 659-668

Słowińska M, Olczak M, Wojtczak M, Glogowski J, Jankowski J, Watorek W, Amarowicz R, Ciereszko A (2008) Isolation, characterization and cDNA sequencing of a Kazal family proteinase inhibitor from seminal plasma of turkey (*Meleagris gallopavo*). *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* **150**, 207-215

Sokotun IN, Gnedenko OV, Leĭchenko EV, Monastyrnaia MM, Kozlovskaia EP, Mol'nar AA, Ivanov AC (2006) Intertion investigation of trypsin inhibitor from sea anemone *Radianthus macrodactylus* with proteases. *Biomeditsinskaya Khimiya* **52**, 595-600

Sokotun IN, Il'ina AP, Monastyrnaya MM, Leychenko EV, Es'kov AA, Anastuk SD, Kozlovskaya EP (2007a) Proteinase inhibitors from the tropical sea anemone *Radianthus macrodactylus:* Isolation and characteristic. *Biochemistry (Moscow)* **72**, 301-306

Sokotun IN, Leĭchenko EV, Vakorina TI, Es'kov AA, Il'ina AP, Monastyrnaia MM, Kozlovskaia EP (2007b) A serine protease inhibitor from the anemone *Radianthus macrodactylus*: Isolation and physicochemical characteristics. *Bioorganicheskaya Khimiya* **33**, 448-455

Somprasong N, Rimphanitchayakit V, Tassanakajon A (2006) A five-domain Kazal-type serine proteinase inhibitor from black tiger shrimp *Penaeus monodon* and its inhibitory activities. *Developmental and Comparative Immunology* **30**, 998-1008

Song G, Zhou M, Chen W, Chen T, Walker B, Shaw C (2008) HV-BBI-a novel amphibian skin Bowman-Birk-like trypsin inhibitor. *Biochemical and Biophysical Research Communication* **372**, 191-196

Vasconcelos AB, Santos AM, Oliveira JS, Lagares Mde A, Santoro MM (2009) Purification and partial characterization of proteinase inhibitors of equine seminal plasma. *Reproductive Biology* **9**, 151-160

Watanabe RM, Soares TS, Morais-Zani K, Tanaka-Azevedo AM, Maciel C, Capurro ML, Torquato RJ, Tanaka AS (2010) A novel trypsin Kazal-type inhibitor from *Aedes aegypti* with thrombin coagulant inhibitory activity. *Biochimie* **92**, 933-999

Xue QG, Waldrop GL, Schey KL, Itoh N, Ogawa M, Cooper RK, Losso JN, La Peyre JF (2006) A novel slow-tight binding serine protease inhibitor from eastern oyster (*Crassostrea virginica*) plasma inhibits perkinsin, the major extracellular protease of the oyster protozoan parasite *Perkinsus marinus*. *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* **145**, 16-26

Wojtczak M, Całka J, Glogowski J, Ciereszko A (2007) Isolation and characterization of alpha1-proteinase inhibitor from common carp (*Cyprinus carpio*) seminal plasma. *Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology* **148**, 264-276

Yanes O, Villanueva J, Querol E, Aviles FX (2005) Functional screening of serine protease inhibitors in the medical leech *Hirudo medicinalis* monitored by intensity fading MALDI-TOF MS. *Molecular and Cellular Proteomics* **4**, 1602-1613

Yang L, Dong W, Yan F, Ren X, Hao X (2010) Recombinant bovine pancreatic trypsin inhibitor protects the liver from carbon tetrachloride-induced acute injury in mice. *Journal of Pharmacy and Pharmacology* **62**, 332-338

Zhang YX, Lai R, Lee WH, Zhang Y (2005) Frog albumin is expressed in skin and characterized as a novel potent trypsin inhibitor. *Protein Science* **14**, 2469-2477

Zhao Y, Jin Y, Lee WH, Zhang Y (2005a) Isolation and preliminary characterization of a 22-kDa protein with trypsin inhibitory activity from toad *Bufo andrewsi* skin. *Toxicon* **46**, 277-281

Zhao M, Naudé RJ, Muramoto K, Oelofsen W (1996) Purification and characterization of ostrich pancreatic secretory trypsin inhibitor. *International Journal of Peptide and Protein Research* **48**, 174-181

Zhao Y, Jin Y, Wei SS, Lee WH, Zhang Y (2005b) Purification and characterization of an irreversible serine protease inhibitor from skin secretions of *Bufo andrewsi*. *Toxicon* **46**, 635-640