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Dedicated to Professor Bronisława Morawiecka on the occasion of her 70th birthday

Minireview

Serine proteinase inhibitors from insect hemolymph*

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Insect hemolymph, like vertebrate serum, contains several different types of polypeptides that are able to inhibit the catalytic function of proteolytic enzymes, however studies on proteins possessing this capability have been limited to a relatively few species. A comparative examination of the inhibition of trypsin, chymotrypsin, neutrophil elastase and cathepsin G and pancreatic elastase by the hemolymph of 14 insect species belonging to six orders showed great diversity in terms of both total proteinase inhibitory capacity and specificity. Most of the inhibitors examined fall into two groups: low molecular mass proteins (below 10 kDa) related to Kunitz type inhibitors, and proteins of about 45 kDa which belong to the serpin superfamily of serine proteinase inhibitors. This minireview describes the properties, characteristics and possible biological significance of selected inhibitors.

Since it has been proved that proteases play a regulatory role in the majority of fundamental cell processes, ranging from protein synthesis to its degradation, proteolysis is generally accepted as one of the most important biological functions. Evidently, such broad functions of proteolytic enzymes call for very accurate and efficient control.

Amongst the compounds taking part in regulation of proteolysis, endogenous proteinase inhibitors are believed to be very powerful tools of Nature for controlling the activity of proteolytic enzymes. To date, quite a large number of protein proteinase inhibitors has been purified and characterized, however, most of them are inhibitors of serine-type proteinases. These substances appear to be ubiquitous and are often found as major components of cytoplasm, intercellular fluids and secretions [1]. In human plasma, for example, they constitute nearly 10% of the total protein and are considered to be important factors in the control of a variety of critical events associated with coagulation, complement activation, fibrinolysis and the inflammatory reaction [2, 3]. The physiological significance of proteinase inhibitors is however, much broader and is not limited to the cited functions. It has been reported, for instance, that some of this class of proteins are also effective suppressors of the

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Abbreviations: AMCI, *Apis mellifera* chymotrypsin inhibitor; BPTI, bovine basic pancreatic trypsin inhibitor; HLE, human leukocyte elastase; HLTI A and B, tobacco hornworm trypsin inhibitors; LMCI I and II, *Locusta migratoria* chymotrypsin inhibitors; PPE, porcine pancreatic elastase; PSTI, pancreatic secretory trypsin inhibitor; SCI-I, -II and -III, silkworm chymotrypsin inhibitors; TIC, trypsin inhibitory capacity; sw-AT, silkworm antitrypsin; sw-Achy, silkworm antichymotrypsin.

radiation- and chemical carcinogen-induced transformation *in vitro* and *in vivo* [4]. Furthermore, at least some of these polypeptides have growth factor-like activities which are cell or species specific [5, 6].

It has been known for a number of years that insect hemolymph, like vertebrate serum, contains several different types of polypeptides that are able to inhibit catalytic function of proteolytic enzymes, though detailed studies on proteins possessing this ability have only recently been conducted [7].

TOTAL PROTEINASE INHIBITORY CAPACITY AND SPECIFICITY OF SELECTED INSECT HEMOLYMPHS

The concentration of protease inhibitors fluctuates during development [8–10] and varies from species to species [11]. A comparative examination of the inhibition of porcine trypsin and pancreatic elastase (PPE), bovine α-chymotrypsin, and human leukocyte elastase (HLE) and cathepsin G by the hemolymph of 14 insect species belonging to six orders showed great diversity in terms of both total proteinase inhibitory capacity and specificity (Table 1) [11].

Trypsin inhibition

Antitrypsin activity was found in all the studied samples of hemolymph though their inhibitory capacities varied significantly from species to species. The hemolymph samples can be arbitrarily divided into groups of low (up to 100 μg/ml), moderate (110–250 μg/ml) and high (above 300 μg/ml) trypsin inhibitory capacity (TIC). The first two groups include adult hemolymph of all but one species and larval hemolymph of only two of six species sampled, of which *Apis mellifera* exhibited the lowest TIC. The third group shows TIC close to, or comparable to, that of human plasma (650 μg/ml). It comprised hemolymph derived from an adult of one species, *Periplaneta americana* and larvae of four species: *Trichoplusia ni*, *Tenebrio molitor*, *Manduca sexta* and *Zophobas rugipes*.

Electrophoretic separation of hemolymph proteins in edestin-containing polyacrylamide gel, followed by exposure to trypsin, revealed

Table 1
Inhibitory capacity of insect hemolymph against serine proteases

Species	Stage	Protein (mg/ml)	Trypsin (IC)	Chymotrypsin (IC)	Cathepsin G (IC)	HLE (IC)	PPE (IC)
Zophobas rugipes	L	68.0	+++	+	++++	++	++++
Tenebrio molitor	L	66.0	+++	+	+++	++	++++
Derobrachus geminatus	A	27.0	+	+	ND	ND	++
Trichoplusia ni	L	100.0	+++	+	+	+	+++
Manduca sexta	L	21.0	+++	+	+	++	++
Heliothis zea	L	86.0	++	++	+++	+++	+++
Apis mellifera	L	92.0	+	+++	++	ND	+
Romalea guttata	A	24.5	+	++	++	+	+
Taeniopoda eques	A	17.7	++	+	+	+	+
Acheta domesticus	A	12.0	++	+	+++	+	+++
Periplaneta americana	A	46.0	+++	+	++++	+	+
Gromphadorhina portensa	A	45.0	++	+	+	++	+++
Acanthocephala femorata	A	26.0	+	+	ND	ND	+
Euthochta galeator	A	92.0	++	+++	+++	ND	++

L, larva; A, adult; IC, inhibitory capacity (micrograms of enzyme inhibited per 1 ml of hemolymph tested): ++++, 830–1250 μg/ml; +++, 300–720 μg/ml; ++, 110–250 μg/ml; +, 5–100 μg/ml; HLE, human leukocyte elastase; PPE, porcine pancreatic elastase; ND, not detectable.

Table 2
Trypsin and chymotrypsin inhibitors in the hemolymph of insects

Species	Stage	Trypsin inhibitors	Chymotrypsin inhibitors
Heliothis zea	L	6	4
Manduca sexta	L	2	5
Tichoplusia ni	L	2	5
Apis mellifera	L	2	1
Romalea guttata	A	6	3
Acheta domesticus	A	4	3
Euthochta galeator	A	0	3
Taeniopoda eques	A	2	3

L, larva; A, adult. The number of inhibitors was determined after separation of hemolymph proteins by PAGE in the presence of 0.1% edestin.

that the antitrypsin activity of hemolymph of each species can be attributed to several trypsin inhibitors (Table 2). It is worth noting that the hemolymph of *Euthochta galeator* showed no inhibitor bands in spite of the fact that it possessed considerable antitrypsin activity. This could be due either to the Kazal-type temporary trypsin inhibitor which became degraded upon incubation with trypsin, or to the basic protein inhibitor(s) which did not enter into the gel.

Chymotrypsin and cathepsin G inhibition

Chymotrypsin and cathepsin G, despite sharing similar substrate specificities, were found to be inhibited by the hemolymph samples in a different way. In general, susceptibility of chymotrypsin to inhibition by insect hemolymphs was much lower than that of cathepsin G. Only hemolymph of *A. mellifera* and *E. galeator* exhibited high chymotrypsin inhibitory activity (600 µg/ml and 560 µg/ml, respectively). All the remaining species showed rather low or moderate inhibitory capacities. As in the case of trypsin inhibitors, the proteins showing anti-chymotrypsin activity were electrophoretically resolved into several inhibitors (Table 2). The inhibition of cathepsin G was undetectable or barely detectable in the hemolymph of five species, however, the remaining species showed considerable anticathepsin G activity. Especially efficient in inhibiting this enzyme were the hemolymphs of *Z. rugipes* and *P. americana* and their capacities were calculated to be over 1 mg/ml.

Inhibition of porcine pancreatic elastase (PPE) and human leukocyte elastase (HLE)

PPE and HLE, like chymotrypsin and cathepsin G, display similar substrate specificities but their sensitivity to inhibition by insect hemolymphs varied remarkably. For HLE, for example, only the hemolymph of *Heliothis zea* had considerable inhibitory capacity (430 µg/ml) and of the remaining 13 species, four showed moderate, and nine had either a very low or non-detectable anti-HLE activity. Surprising results were obtained concerning the inhibition of pancreatic elastase. This enzyme was inhibited to various degrees by all samples of hemolymph tested. The high inhibitor capacity group comprised as many as six species. In particular, the hemolymphs of *T. molitor* and *Z. rugipes* were very effective in inhibiting this enzyme and their capacities were calculated to be 830 µg/ml and 1250 µg/ml, i.e. comparable to those of the sera of both the mini pig and hamster [12].

PURIFIED INHIBITORS OF SERINE PROTEINASES

Inhibitors of serine proteinases specific primarily towards trypsin and/or chymotrypsin and rarely against other serine type proteolytic enzymes, have been isolated and characterized from relatively few species which are listed in Table 3. Most of these inhibitors fall into two groups: low molecular mass proteins (below 10 kDa) related to vertebrate Kunitz-type inhibitors [13], and proteins of about 40–50 kDa which belong to the serpin superfamily of serine proteinase inhibitors [14].

Kunitz-type inhibitors

Inhibitors that have a certain degree of sequence homology and topological similarity to

Table 3

Serine protease inhibitors from insect hemolymph

Target enzymes	Species	Molecular mass (kDa)	Reactive site P_1-P'_1	Chemical characterization	Name of inhibitor	Inhibitor family	References
Chymotrypsin	*Manduca sexta*	48.0	n.d.	aa[1], N-terminal sequence	–	Serpin	[16]
Chymotrypsin	*Manduca sexta*	47.0	n.d.	aa, N-terminal sequence	–	Serpin	[16]
Trypsin	*Manduca sexta*	46.0	n.d.	aa, N-terminal sequence	–	Serpin	[16]
Pancreatic elastase	*Manduca sexta*	47.0	Ala-Ser	cDNA sequence	Ala-serpin	Serpin	[15, 16]
Trypsin	*Bombyx mori*	41.8	Lys-Val	cDNA sequence	sw-AT	Serpin	[17, 18]
Chymotrypsin	*Bombyx mori*	43.0	Thr-Ser	cDNA sequence	sw-AChyI	Serpin	[17, 19, 21]
Chymotrypsin	*Bombyx mori*	41.0	Phe-Met	sequence	sw-AChyII	Serpin	[20]
Trypsin	*Manduca sexta*	8.3	Arg-Ala (?)	aa, N-terminal sequence	HLTI A	BPTI[2]	[22]
Chymotrypsin, plasmin, PPAE[3]	*Manduca sexta*	9.1	Arg-Ala (?)	aa, N-terminal sequence	HLTI B	BPTI	[22]
Chymotrypsin	*Bombyx mori*	7.0	Phe-Ala	sequence	SCI-I	BPTI	[23, 25]
Chymotrypsin	*Bombyx mori*	7.3	Phe-Ala	sequence	SCI-II	BPTI	[23, 25]
Chymotrypsin	*Bombyx mori*	7.2	Phe-Gly	sequence	SCI-III	BPTI	[23, 24]
Chymotrypsin, plasmin, thrombin, trypsin, PPAE	*Sarcophaga bullata*	6.4	n.d.	sequence	–	BPTI	[28, 29]
Chymotrypsin, PPAE	*Locusta migratoria*	3.7	Arg-Lys	sequence	LMCI I	?	[26, 27]
Chymotrypsin, PPAE	*Locusta migratoria*	3.7	Leu-Lys	sequence	LMCI II	?	[26, 27]
Trypsin, HLE[4] chymotrypsin	*Heliothis zea*	18.0	Arg-X Met-X	aa, N-terminal sequence	Multistatin	PSTI[5]	[30]
Cathepsin G, chymotrypsin	*Heliothis zea*	6.3	n.d.	aa, N-terminal sequence	–	BPTI (?)	[30]
Catepsin G, chymotrypsin, fungal proteinases, PPAE	*Apis mellifera*	6.3	Met-Gln	sequence	AMCI	Ascaris	6
Subtilisin BPN′ fungal proteases	*Bombyx mori*	6.1	Thr-Val	sequence	FPI-F	Bombyx	[31, 32]

[1]Amino acid analysis; [2]Basic pancreatic trypsin inhibitor (Kunitz); [3]Prophenyloxidase activating enzyme; [4]Human leukocyte elastase; [5]Pancreatic secretory trypsin inhibitor (Kazal); n.d.- not determined; [6]Bania *et al.*, unpublished.

bovine basic pancreatic trypsin inhibitor (BPTI) are very common in Nature and have been purified from different sources [1]. Of insect inhibitors, those from the silkworm (*Bombyx mori*), are well documented. The larval hemolymph of this species contains at least 16 kinds of chymotrypsin inhibitors [33] of which only a few have been characterized extensively. Three Kunitz-type chymotrypsin inhibitors named SCI-I, SCI-II and SCI-III were isolated [23] and the complete amino-acid sequence elucidated [24, 25]. Each inhibitor, of molecular mass close to 7.0 kDa, inhibited proteolytic and esterolytic activities of bovine chymotrypsin at 1:1 molar ratio. In addition, SCI-II showed weak antitrypsin activity. SCI-I and SCI-II are basic proteins composed each of 62 amino-acid residues with differences in only two positions with respect to each other, whereas SCI-III is an acidic protein built up of 63 amino-acid resi- dues. All the three inhibitors contain six half cysteines each in the same frame structure as does BPTI except for one amino-acid insertion in the frame of the N-terminal region. The P_1 amino acid of the reactive site in the inhibitors was deduced to be phenylalanine. Two Kunitz-type inhibitors were also found in the hemolymph of the tobacco hornworm, *M. sexta* [22]. The separated proteins named HLTI A (8.3 kDa) and HLTI B (9.1 kDa) are both heat and acid stable, and efficiently inhibit trypsin, chymotrypsin and plasmin at 1:1 molar ratio. HLTI A exists in the hemolymph as a dimer composed of two identical subunits. As binding of one proteinase to HLTI A precludes binding of the other proteinase, it is likely that the inhibitor does not have an independent binding site for each proteinase. Comparison of the N-terminal amino-acid sequence of the *M. sexta* hemolymph inhibitors A and B reveals that these proteins are related, sharing 24 identical residues out of 38 aligned. When compared to BPTI, in HLTI A nearly 36% out of 39 residues aligned were identical, whereas in HLTI B about 40% out of 53 residues occupied identical positions.

Recently, another Kunitz-type inhibitor (6.4 kDa) was isolated from the hemolymph of *Sarcophaga bullata*. The 57 amino-acid sequence of the inhibitor showed that it has six cysteine residues identically spaced to those of BPTI. The inhibitor is active against chymotrypsin, trypsin, plasmin, thrombin and prophenoloxidase activating enzyme [28, 29].

Serpins

The serine proteinase inhibitors that constitute the serpin superfamily, are a group of genetically related proteins exhibiting similarity in amino-acid sequences, overall tertiary structure, and mechanism of inhibition. They differ, however, in their specificity which is determined by the reactive sites that constitute an exposed mobile loop with the P_1-P'_1 residues acting as a bait for a target proteinase [14, 34]. Inhibitors belonging to this superfamily have been found in hemolymphs of two lepidopteran insects, *B. mori* and *M. sexta*. From the silkworm larval hemolymph three kinds of serpins have been isolated and characterized [17–21]; one with antitrypsin activity (41.8 kDa), named sw-AT (silkworm antitrypsin), and two with antichymotrypsin activity: sw-Achy I (43 kDa) and sw-Achy II (41 kDa). The inhibitors react with serine proteinases in the manner observed for the interaction of serpin of vertebrate origin and proteinase. Their complexes with the target enzymes are stable in SDS but dissociate under alkaline conditions. Like in the serpins the reactive sites are localized in the carboxy terminal regions of the molecules. cDNA cloning revealed that sw-AT consists of 376 amino-acid residues. The reactive site for bovine trypsin inhibition was found to be Lys_{343}-Val_{344}. The inhibitor contains no carbohydrate and is homologous with other members of the serpin superfamily. Particularly, a high amino-acid sequence homology (56% identical residues) occurs between sw-AT and alaserpin, the elastase inhibitor from tobacco hornworm hemolymph [15], though the two inhibitors differ in their specificity and show no sequece homology around the reactive site. Two another members of the serpin superfamily from the same species, chymotrypsin inhibitors, have also been extensively studied. The sw-Achy I is a carbohydrate free protein comprising 384 amino-acid residues. Its amino-acid sequence is in about 30% identical to that of sw-AT and alaserpin, and in a somewhat lesser degree to human α_1-antitrypsin and human α_1-antichymotrypsin. The reactive site of the inhibitor was identified as Thr_{343}-Ser_{344} [19]. The second inhibitor is built of 375 amino-acid residues. Its reactive site, determined after enzymatic cleavage followed by sequence analysis, was assigned to the Phe_{340}-Met_{341} pep-

tide bond. It is the first inhibitor among serpins known to have a Phe residue at the P_1 position. The characteristic feature of sw-Achy II is its mosaic structure and very high degree of similarity with sw-AT. In both proteins the amino-acid sequence up to the position 336 is identical but the carboxy terminal regions display only 46% homology.

Of four different serpins isolated from larval hemolymph of *M. sexta* [16], an inhibitor named alaserpin has been studied in details [15, 35]. Alaserpin is a 47 kDa glycoprotein capable of inhibiting porcine pancreatic elastase (K_{ass} 1×10^7 $M^{-1}s^{-1}$) and to a lesser degree bovine chymotrypsin (K_{ass} 7×10^4 M^{-1} s^{-1}). Its amino-acid sequence deduced from the cDNA shows 25–30% identity to most members of the serpin superfamily. Comparison of the alaserpin amino-acid sequence with that of mammalian serpin allowed to predict the P_1 residue to be Ala_{343}. Using site-directed mutagenesis the Ala residue was replaced by lysine or phenylalanine [35]. The resulting recombinant serpins named 1B(A343K) and 1B(A343F), unlike the native protein, inhibited trypsin, plasmin and thrombin, and chymotrypsin and trypsin, respectively. Moreover, they had anticathepsin G activity as well. These results confirmed that Ala_{343} residue is engaged in the reactive site and determines the specificity of the inhibitor. Mutational changes of the P_1 residue or replacement of active site residues greatly influence both the specificity and the potency of the inhibition.

Members of other inhibitor families

Only a few inhibitors belonging to families other than described above have been isolated and characterized. From larval hemolymph of *H. zea* an inhibitor named multistatin was purified [30]. It appeared to be a single chain protein (18 kDa) consisting of 170 amino-acid residues. The inhibitor strongly inhibits human neutrophile elastase (K_i 10^{-11} M), porcine trypsin (K_i 2.1×10^{-10} M), and bovine chymotrypsin (K_i 10^{-9}M) forming 1:1 complexes with each enzyme. The inhibitor reacts independently with HLE and trypsin, with either at 1:1 molar ratio, moreover, the complex (HLE:inhibitor:trypsin) can still inhibit chymotrypsin, although this ability is clearly impaired as compared to the free enzyme. As binding of one proteinase to the inhibitor does not preclude the binding of the other, it seems likely that this protein has independent binding sites for each enzyme. Using chemical modification of the amino-acid residues it was found that the methionine residue defines the antielastase specificity and one arginine resides in the trypsin binding site of the inhibitor. The N-terminal amino-acid sequence of 29 residues suggests a high homology of the inhibitor to the Kazal-type family [13].

An inhibitor specific for chymotrypsin, cathepsin G, fungal proteases and prophenoloxidase activating enzyme was isolated from the larval hemolymph of honeybee, *A. mellifera* (Bania, J., Stachowiak, D., Polanowski, A., unpublished). The inhibitor named AMCI (*Apis mellifera* chymotrypsin inhibitor) consists of 56 amino acids and has a molecular mass of 6.3 kDa. The amino-acid composition includes 10 cysteine residues, but neither tryptophan nor tyrosine. The reactive site established after cathepsin G cleavage at pH 3.5, was found to be Met_{30}-Gln_{31}. The amino-acid sequence shows about 50% homology to that of inhibitors from Ascaris family, and cysteine residues occupy identical positions.

A novel protease inhibitor (FPI-F) highly efficient against fungal protease and subtilisin, but not against trypsin and chymotrypsin, was isolated from the silkworm hemolymph [31]. The inhibitor (6.1 kDa) consists of 55 amino acids including eight cysteine residues. There is no alanine, methionine or tryptophan. Using subtilisin, it was found that this enzyme at pH 3.0 specifically hydrolyzed the peptide bond of the inhibitor at Thr_{29}-Val_{30} and at pH 8.0 resynthetized it. These results show that this peptide bond of the inhibitor is its reactive site. The inhibitor has a unique amino-acid sequence which is not homologous with those of other known proteinase inhibitors of different origin. Based on the amino-acid sequence and both the locations of disulfide bridges and reactive site, FPI-F is considered to be a member of a new family of serine proteinase inhibitors designated as the Bombyx family [32].

The smallest so far known inhibitors in insects are those separated from hemolymph of *Locusta migratoria* by two groups of researchers. One of them has purified two inhibitors consisting of 35 and 36 amino-acid residues named LMCI I and LMCI II, respectively [26]. The second group has isolated three peptides designated

PMP-D2, PMP-C and HI [27]. The first two peptides are very similar to each other and exhibit a 45% sequence homology. The peptide HI showed a 72% strict identity with PMP-D2. It appeared that the sequence of PMP-D2 was identical with LMCI I and that of PMP-C with LMCI II. There is a discrepancy regarding the inhibitory activity of the isolated peptides. According to Boigegrain *et al.* [26], both LMCIs I and II were powerful inhibitors of α-chymotrypsin (K_i 0.25 nM and 0.12 nM, respectively) and weak to medium inhibitors of HLE (K_i > 0.1μM and 0.18 nM, respectively). These results have not been fully confirmed by the other group [27] who showed that PMP-C was indeed a very effective inhibitor of α-chymotrypsin (K_i 0.2 nM) and inhibited human leukocyte elastase with a K_i of 0.12 μM, but the remaining peptides interacted only weakly with α-chymotrypsin and did not inhibit elastase. The reactive site for PMP-C was found to be the Leu_{30}-Lys_{31} peptide bond and that for PMP-D2 and HI, Arg_{29}-Gly_{30}.

PHYSIOLOGICAL FUNCTIONS OF THE INSECT INHIBITORS

Information concerning the physiological roles of serine proteinase inhibitors in insect hemolymph are very meager, although some of their functions have been postulated [36]. First they may protect tissues from the deleterious effects of alimentary tract proteases which may leak into the hemolymph due to bacteria infection. Another potential role is protection of insects from proteases secreted by entomopathogenic microorganisms. Several hemolymph protease inhibitors have been found to be active against some fungal proteinases ([31, 32, 36–37], Bania, J., Stachowiak, D. & Polanowski, A., unpublished). On the other hand, it has been evidenced that fungi produce proteolytic enzymes that enable their penetration through the cuticle [34], and the level of inhibitory activity can increase upon infection of insects with fungi [37]. Presumably, in this case the inhibitors serve for the insects as agents protecting against invaders. One cannot exlude the possibility that insects hemocytes, upon infection with entomopathogens or parasites, may release serine proteinase like mammalian neutrophils do in inflammatory responses. It has been noticed that *M. sexta* hemocytes cultured *in vitro* release into the medium protease(s) inhibited by *M. sexta* serpins [38]. Perhaps the hemolymph serpins regulate such enzymes, in a manner analogous to the regulation of neutrophil elastase and cathepsin G in human blood by α_1-proteinase inhibitor and α_1-antichymotrypsin. Hemolymph protease inhibitors may also function as regulators of endogenous proteinases, as most of the serpins do in vertebrates. A process which the inhibitors may regulate is the activation of the prophenoloxidase activation cascade, which recently has been the subject of active investigations [39, 40]. The prophenoloxidase activating system is considered to be responsible for defensive functions in insects and other invertebrates. In most insects phenoloxidase is present as an inactive proenzyme prophenoloxidase. It is activated as a result of limited proteolysis by a serine proteinase present in hemolymph and cuticule [41, 42]. Under normal physiological conditions the activation is avoided due to the presence in the hemolymph of serine proteinase inhibitors which inhibit the activating enzyme. An endogenous serine proteinase inhibitor possessing the ability to quench the chymotrypsin-mediated prophenoloxidase activation, was isolated from the larval hemolymph of *Sarcophaga bullata* [28]. Thereafter, several inhibitors exhibiting similar properties have been isolated from larval hemolymph of different species [22, 26] (Bania, J., Stachowiak, D. & Polanowski, A., unpublished). The insect prophenoloxidase cascade is known to contain at least two serine proenzymes [42]. Whether hemolymph serine proteinase inhibitors regulate these enzymes similarly to the regulation of blood clotting by vertebrate serpins remains to be elucidated.

REFERENCES

1. Laskowski, M. & Kato, I. (1980) Protein inhibitors of proteinases. *Ann. Rev. Biochem.* **49**, 593–626.
2. Heimburger, N. (1975) Proteinase inhibitors of human plasma. Their properties and control functions; in *Proteases and Biological Control* (Reich, E., Rifkin, D.B. & Shaw, E., eds.) pp. 367–386, Cold Spring Harbor Laboratories, N.Y.

3. Travis, J. & Salvesen, G.S. (1983) Human plasma proteinase inhibitors. *Annu. Rev. Biochem.* **52**, 655–709.

4. Troll, W. & Kennedy, A.R. (1989) Protease inhibitors as cancer chemopreventive agents. *Cancer Res.* **49**, 499–502.

5. McKeehan, W.L., Sakagami, Y., Hoshi, H. & McKeehan, K.A. (1986) Two apparent endothelial cell growth factors from human hepatoma cells are tumor-associated proteinase inhibitors. *J. Biol. Chem.* **261**, 5378–5383.

6. Popik, W., Inglot, A.D., Wilusz, T. & Polanowski, A. (1988) Participation of polypeptide proteinase inhibitors in proliferation of fibroblast. *Arch. Immunol. Therap. Expt.* **36**, 645–653.

7. Eguchi, M. (1993) Protein protease inhibitors in insects and comparison with mammalian inhibitors. *Comp. Biochem. Physiol.* **105B**, 449–456.

8. Kučera, M., Sehnal, F. & Mala, J. (1984) Effect of developmental dearrangements on the proteolytic and protease inhibitory activities in *Galleria mellonella* (Insecta). *Comp. Biochem. Physiol.* **79B**, 255–261.

9. Eguchi, M., Matsui, Y. & Matsumoto, T. (1986) Developmental change and hormonal control of chymotrypsin inhibitors in the haemolymph of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol.* **84B**, 327–332.

10. Aratake, H., Deng, L.R., Fuji, H., Kawaguchi, Y. & Koga, K. (1990) Chymotrypsin inhibitors in haemolymph and eggs of the silkworm, *Bombyx mori*: Developmental changes in inhibitory activity. *Comp. Biochem. Physiol.* **97A**, 205–209.

11. Polanowski, A., Wilusz, T., Blum, M.S., Escoubas, P., Schmidt, J.O. & Travis, J. (1992) Serine proteinase inhibitor profile in the hemolymph of a wide range of insect species. *Comp. Biochem. Physiol.* **102B**, 757–760.

12. Schultz, H., Mohr, M. & Meyer, M. (1989) Elastase and papain inhibition by serum of mammals. *Scand. J. Clin. Lab. Invest.* **49**, 381–388.

13. Laskowski, M., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M.W., Kohr, W.J., Park, S.J., Schatzley, B.L., Schoenberger, O.L., Tashiro, M., Vichot, G., Whatley, H.E., Wieczorek, A. & Wieczorek, M. (1987) Ovomucoid third domains from 100 avian species: isolation, sequences, and hyper-variability of enzyme-inhibitor contact residues. *Biochemistry* **26**, 202–221.

14. Carrell, R.W. & Boswell, D.R. (1986) Serpins: The superfamily of plasma serine proteinase inhibitors; in *Proteinase Inhibitors* (Barrett, A.J. & Salvesen, G.S., eds.) pp. 403–420, Elsevier, N.Y.

15. Kanost, M.R. Prasad, S.V. & Wells, M.A. (1989) Primary structure of a member of the serpin superfamily of proteinase inhibitors from an insect, *Manduca sexta*. *J. Biol. Chem.* **264**, 965–972.

16. Kanost, M.R. (1990) Isolation and characterization of four serine proteinase inhibitors (serpins) from hemolymph of *Manduca sexta*. *Insect Biochem.* **20**, 141–147.

17. Sasaki, T. & Kabayashi, K. (1984) Isolation of two novel proteinase inhibitors from hemolymph of silkworm larva, *Bombyx mori*. Comparison with human serum proteinase inhibitors. *J. Biochem.* **95**, 1009–1017.

18. Tagaki, H., Narumi, H., Nakamura, K. & Sasaki, T. (1990) Amino acid sequence of silkworm (*Bombyx mori*) hemolymph antitrypsin deduced from its homology with serpins. *J. Biochem.* **108**, 372–378.

19. Narumi, H., Hishida, T., Sasaki, T., Feng, D.F. & Doolittle, R.F. (1993) Molecular cloning of silkworm (*Bombyx mori*) antichymotrypsin. A new member of the serpin superfamily of proteins from insect. *Eur. J. Biochem.* **214**, 181–187.

20. Sasaki, T. (1991) Patchwork-structure serpins from silkworm (*Bombyx mori*) larval hemolymph. *Eur. J. Biochem.* **202**, 255–261.

21. Eguchi, M. & Shomoto, K. (1985) Purification and properties of a chymotrypsin inhibitors from hemolymph of the silkworm. *Comp. Biochem. Physiol.* **81B**, 301–307.

22. Ramesh, N., Sugumaran, M. & Mole, J.E. (1988) Purification and characterization of two trypsin inhibitors from the hemolymph of *Manduca sexta* larvae. *J. Biol. Chem.* **263**, 11523–11527.

23. Sasaki, T. (1978) Chymotrypsin inhibitors from hemolymph of the silkworm, *Bombyx mori*. *J. Biol. Chem.* **84**, 267–276.

24. Sasaki, T. (1984) Amino acid sequence of a novel Kunitz-type chymotrypsin inhibitor from hemolymph of silkworm larvae, *Bombyx mori*. *FEBS Lett.* **168**, 227–230.

25. Sasaki, T. (1988) Amino-acid sequences of two basic chymotrypsin inhibitors from silkworm larval hemolymph. *Biol. Chem. Hoppe-Seyler* **369**, 1235–1241.

26. Boigegrain, R.-A., Mattras, H., Brehélin, M., Paroutaud, P. & Coletti-Previero, M.-A. (1992) Insect immunity: Two proteinase inhibitors from hemolymph of *Locusta migratoria*. *Biochim. Biophys. Res. Commun.* **189**, 790–793.

27. Kellenberger, C., Boudier, C., Bermudez, I., Bieth, J.G., Luu, B. & Hietter, H. (1995) Serine proteinase inhibition by insect peptides containing a cysteine knot and a triple-stranded β-sheet. *J. Biol. Chem.* **270**, 25514–25519.

28. Sugumaran, M., Saul, S.J. & Ramesh, N. (1985) Endogenous protease inhibitors prevent undesired activation of prophenolase in insect hemolymph. *Biochim. Biophys. Res. Commun.* **132**, 1124–1129.

29. Papayannopoulos, I.A. & Biemann, K. (1992) Amino acid sequence of a protease inhibitor isolated from *Sarcophaga bullata* detemined by mass spectrometry. *Protein Sci.* **1**, 278–288.

30. Polanowski, A., Wilusz, T., Blum, M.S. & Travis, J. (1994) Two serine proteinase inhibitors from the larval hemolymph of *Heliothis zea*. *Acta Biochim. Polon.* **41**, 178–180.

31. Eguchi, M., Itoh, M., Nishino, K., Shibata, H., Tenaka, T., Kamei-Hayashi, K. & Hara, S. (1994) Amino acid sequence of an inhibitor from the silkworm (*Bombyx mori*) hemolymph against fungal protease. *J. Biochem.* **115**, 881–884.

32. Pham, T.-N., Hayashi, K., Takano, R., Itoh, M., Eguchi, M., Shibata, H., Tanaka, T. & Hara, S. (1996) A new family of serine proteinase inhibitors (*Bombyx mori*) as established from the unique topological relation between the positions of disulfide bridges and reactive site. *J. Biochem.* **119**, 428–434.

33. Fuji, H., Aratake, H., Deng, L.R., Nakamura, M., Kawaguchi, Y. & Sagakuchi, B. (1989) Purification and characterization of a novel chymotrypsin inhibitor controlled by the chymotrypsin inhibitor A (*Ict-A*) gene from the larval hemolymph of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol.* **94B**, 149–155.

34. St Leger, R.J., Durrands, P.K., Charnley, A.K. & Cooper, R.M. (1988) Role of extracellular chymoelastase in the virulence of *Metharisium anisopliae* for *Manduca sexta*. *J. Invertebr. Path.* **52**, 285–293.

35. Jiang, H., Mulnix, A.B. & Kanost, M.R. (1995) Expression and characterization of recombinant *Manduca sexta* serpin-1B and site-directed mutants that change its inhibitory selectivity. *Insect Biochem. Molec. Biol.* **25**, 1093–1100.

36. Eguchi, M., Haneda, I. & Iwamoto, A. (1982) Properties of protease inhibitors from the haemolyph of silkworms, *Bombyx mori*, *Antheraea pernyi* and *Philosamia cynthia ricini*. *Comp. Biochem. Physiol.* **71B**, 569–576.

37. Boucias, D.G. & Pendland, J.C. (1987) Detection of protease inhibitors in the hemolymph of resistant *Anticarsia gemmatalis* which are inhibitory to the entomopathogenic fungus, *Nomuraea rileyi*. *Experientia* **43**, 336–339.

38. Kanost, M.R. (1990) Serine proteinase inhibitors from the serpin gene family in *Manduca sexta* and *Drosophila melanogaster*; in *Molecular Insect Science* (Hagedorn, H.H., Hildebrand, J.G., Kidwell, M.G. & Law, J.H., eds.) pp. 139–146, Plenum, New York.

39. Ashida, M. & Yamazaki, H.I. (1990) Biochemistry of the phenoloxidase system in insects: with special references to its activation; in *Molting and Metamorphosis* (Ohnishi, E. & Ishizaki, H., eds.) pp. 239–265, Japan Sci. Soc. Press, Tokyo/Springer Verlag, Berlin.

40. Ashida, M. & Sasaki, T. (1994) A target protease activity of serpins in insect hemolymph. *Insect Biochem. Molec. Biol.* **24**, 1037–1041.

41. Ashida, M. & Dohke, K. (1980) Activation of pre-phenoloxidase from the hemolymph of the silkworm, *Bombyx mori*. *Insect Biochem.* **10**, 37–47.

42. Yoshida, H. & Ashida, M. (1986) Microbial activation of two serine enzymes and prophenoloxidase in the plasma fraction of hemolymph of the silkworm, *Bombyx mori*. *Insect Biochem.* **16**, 539–545.