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Purification and characterization of a group of five novel peptide serine protease inhibitors from ovaries of the desert locust, *Schistocerca gregaria*

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Abstract The ovary of the desert locust, *Schistocerca gregaria*, contains multiple inhibitors of serine proteases. Five serine protease inhibitors, designated SGPI-1–5 (*Schistocerca gregaria* protease inhibitors) were purified from methanolic extracts of mature ovaries and analyzed by mass spectrometry and amino acid sequencing. The revealed primary structures display amino acid similarities and are related to the serine protease inhibitors identified in the hemolymph of *Locusta migratoria*. All inhibitors show an in vitro inhibiting activity towards α-chymotrypsin. In addition, SGPI-1 displays in vitro inhibiting activity towards trypsin, and SGPI-2 is a potent pancreatic elastase inhibitor. Differences in inhibitory specificities towards the locust endogenous serine proteases can be readily attributed to the amino acid sequence within the active region and also to amino acid residues beyond the P1-P′1 bond. A difference in one or two amino acid residues around the reactive sites results in considerable alteration of the inhibitory specificity. The temporal and spatial distribution of SGPI-1–5 was studied by RP-HPLC analysis. All inhibitors occur in hemolymph, ovaries, testes and fat body of adults but are absent in the gut. They are also present in larval hemolymph and fat body. An antibody raised against SGPI-2 shows positive immunostaining in the ovarian follicle cells.

Key words: Serine protease inhibitor; *Schistocerca gregaria*; Chymotrypsin; Trypsin; Elastase; Insect ovary

1. Introduction

Two different peptides that inhibit trypsin biosynthesis have recently been identified in late vitellogenic ovaries of two carnivorous insect species, the mosquito *Aedes aegypti* and the gray fleshfly *Neobellieria bullata* [1,2]. These peptides, which have no effect on the activity of the enzyme itself, only on its biosynthesis, are termed trypsin modulating oostatic factors (TMOF). Peptides displaying such activity have as yet not been reported in vertebrates. During a search for a peptide displaying trypsin/chymotrypsin modulating activity in a phytophagous insect, *Schistocerca gregaria*, we found not only that such a peptide is present in late vitellogenic ovaries (Janssen et al., unpublished results), but also that the ovary is a rich source of several classical protease inhibitors that have a high affinity for the active site of these serine proteases.

Intrigued by this finding, which, in our opinion, might contribute to a better understanding of the complex process of vitellogenesis and yolk protein degradation during embryogenesis, we have undertaken and completed the purification of these ovarian serine protease inhibitors. This is the first study on protease inhibitors in the ovary of insects. Most of the serine protease inhibitors in insects examined hitherto have been identified or partially characterized from hemolymph extracts and fall into two groups [3]: low molecular mass proteins (below 10 kDa) related to the Kunitz-type inhibitors [4] and proteins of about 45 kDa which belong to the serpin superfamily [5]. The five primary structures we present in this paper differ from both the Kunitz-type and the serpin-type proteinase inhibitors. Together with the three previously identified protease inhibitors in the hemolymph of *Locusta migratoria* [6–8], they make up a new family of 4 kDa serine protease inhibitors, stabilized by three disulfide bridges and displaying different enzyme specificities.

2. Materials and methods

2.1. Materials

2.1.1. Insects. *Schistocerca gregaria* were reared on alfalfa leaves and bran under a photoperiod of 12 h at a temperature which varied from 28°C to 34°C.

2.1.2. Chemicals. Acetonitrile (CH_3CN) of HPLC grade was purchased from Riedel-de Haën (Seelze, Germany). Trifluoroacetic acid (TFA), methanol, ethylacetate and *n*-hexane were from Acros Organics (Geel, Belgium). Dimethylformamide was obtained from Janssen Chimica. Thyroglobulin, (1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Freund's adjuvant were from Sigma. Enzymes (bovine trypsin, bovine chymotrypsin and pancreatic porcine elastase) and substrates (*N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, *N*-α-benzoyl-DL-Arg-*p*-nitroanilide, *N*-α-benzoyl-DL-Arg-β-naphthylamide (BANA), *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and azocasein) were also purchased from Sigma.

2.2. Methods

2.2.1. Purification of the five serine protease inhibitors from Schistocerca ovary extracts. About 1200 pairs of ovaries from 12–14 day females were dissected and immediately placed in an ice-cold methanol/water/acetic acid (90:9:1) solution. They were sonicated and centrifuged for 30 min (10 000 × *g*; 4°C). The methanol was evaporated and the remaining aqueous residue was re-extracted with ethyl acetate and *n*-hexane to remove the bulk of lipids. The organic solvent layer was decanted and the aqueous solution was dried in siliconized round bottom flasks. Subsequently, it was redissolved in aqueous TFA (0.1%) and prepurified on Megabond Elute C18 cartridges (Varian, Harbor City, CA), which had been activated with CH_3CN/H_2O/TFA (80:19.9:0.1) and then rinsed with aqueous 0.1% TFA. The cartridges were eluted with 25 ml of 50% CH_3CN in 0.1% aqueous TFA. Columns and operating conditions for high performance liquid chromatography on a Gilson HPLC system with variable wavelength detector (214 nm) were: (i) Deltapak C18 column (25 × 100 mm) (Waters Associates, Milford, MA), solvent A: 0.1% TFA in water; solvent B: 50% CH_3CN in 0.1% aqueous TFA. Column conditions: 100% A for 10 min, followed by a linear gradient to 50% B in 80 min; flow rate:

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6 ml/min; detector range: 2 absorption units full scale (Aufs); (ii) Deltapak C4 column (7.8×300 mm, 5 μm) (Waters Associates), solvent A: 0.1% TFA in water; solvent B: 50% CH_3CN in 0.1% aqueous TFA. Column conditions for SGPI-1,2: a linear gradient from 0% B to 50% B in 70 min; column conditions for SGPI-3,4,5: 0% B for 10 min, then linear gradient to 25% B in 60 min; flow rate: 1.5 ml/min; detector range: 2.0 Aufs; (iii) SGPI-1 was purified to homogeneity on a Symmetry C18 column (4.6×250 mm) (Waters Associates), solvent A: 0.1% aqueous TFA; solvent B: 50% CH_3CN in 0.1% aqueous TFA. Column conditions: 100% A for 10 min, followed by a linear gradient to 50% B in 60 min; flow rate: 1.5 ml/min; detector range: 0.5 Aufs; (iv) SGPI-2,3,4,5 were further isolated on a Biosep-Sec-S-2000 column (7.8×300 mm) (Phenomenex, CA), solvent A: 95% CH_3CN in 0.01% aqueous TFA; solvent B: 50% CH_3CN in 0.01% aqueous TFA. Column conditions for SGPI-2,3: 40% B for 10 min, followed by a linear gradient to 60% B in 60 min; column conditions for SGPI-4,5: 25% B for 10 min, then a linear gradient to 100% B in 60 min; flow rate: 1.5 ml/min; detector range: 0.2 Aufs.

Fractions were collected every 2 min for (i) and (ii) and peaks were collected manually for (iii) and (iv). After each run, aliquots of individual fractions (containing about 2 equivalents of ovaries) were monitored in an enzyme assay to detect chymotrypsin/trypsin inhibitory activity.

2.2.2. Mass analyses and amino acid sequence determination. An aliquot (1.5 μl) of the final volume of the pure peak containing protease inhibiting activity was subjected to MALDI-TOF analysis [9]. Amino acid sequencing was performed on a ABI476A (Applied Biosystems, Foster City, CA) or on a Beckman LF 3600 TC protein sequencer according to Hewick [10].

2.2.3. Measurement of serine protease inhibitory activity. Except for azocasein, all substrate stock solutions were prepared in dimethylformamide (DMF). HPLC fractions and purified peptides were screened for bovine trypsin, chymotrypsin and porcine pancreatic elastase inhibitory activity as described previously [11]. Inhibitory activity towards endogenous locust gut proteases was measured as follows. Mature male insects were starved for 48 h. Then, gut protease activity was stimulated by a bran diet. After 6 h, the guts were dissected and homogenized in deionized water containing 0.15 M NaCl and 1 mM $CaCl_2$ (1 gut equivalent per ml). Aliquots of 5 μl of gut homogenate were incubated for 45 min in 45 μl Tris-HCl buffer (pH 8.0) with 1.8 nmol each of SGPI-1 and SGPI-2. Control gut homogenates were incubated with Tris-HCl buffer instead of the *Schistocerca* protease inhibitors. 20 μl of each solution was used for native PAGE electrophoresis according to Laemmli [12] to separate the gut enzymes. Gut protease bands were revealed by placing the acrylamide gel bands onto slides with a film of agar-azocasein gel prepared with 0.2 M Tris-HCl buffer (pH 8, 10 mM $CaCl_2$, 1% agar and 0.1% azocasein). To reveal the site of azocasein degradation, the gel was stained in a Coomassie blue R250 (0.1%), 25% methanol, 10% acetic acid solution. Trypsin-like activity was detected by immersing the gel for 20 min in a solution of 0.2 M Tris-HCl buffer (pH 8.0) containing 2 mM BANA, 20 mM NaCl, 2 mM $MgCl_2$ and 0.6% fast black K salt (Sigma). The gel was washed and fixed with 12.5% trichloroacetic acid.

2.2.4. Antibody production. Two peptides, identical to the N-terminal sequence (EVTCEPGTTFKDKC) and to the C-terminal sequence (DGKSAACTLKACPQ) of SGPI-2, were synthesized using Fmoc polyamide chemistry (J.W. Drijfhout, University Hospital Leiden, The Netherlands). Each peptide was then coupled to bovine thyroglobulin (Sigma) using EDC [13]. Characterization by immuno-dot-blot revealed that the SGPI-2 directed antiserum recognized natural SGPI-2, the synthetic N-terminal and C-terminal peptides of SGPI-2 (to which it was raised), but not the synthetic N-terminus of SGPI-3 (CTPGSRKYD).

2.2.5. HPLC analysis of tissue extracts. 200 pmol of the five native purified peptides were injected together on a Gilson HPLC system (detector set at 214 nm) on a Symmetry C18 column (4.6×250 mm) (Waters Associates), solvent A: 0.1% aqueous TFA; solvent B: 25% CH_3CN in 0.1% aqueous TFA. Column conditions were: 100% A, followed by a linear gradient to 100% B in 60 min; flow rate: 1 ml/min; detector range: 0.2 Aufs. Extracts of ovaries, testes, hemolymph, fat body and gut (20 equivalents each) and larval fat body, hemolymph and gut (20 equivalents each) were run under the same conditions. Fractions were collected every minute and assayed for bovine trypsin and α-chymotrypsin inhibitory activity.

3. Results

3.1. Isolation and identification of the serine protease inhibitors from ovary extracts

On the Deltapak C18 preparative column, the inhibitory activity was separated into four major fractions. These fractions were further purified so that five protease inhibitors, designated SGPI-1–5 (*Schistocerca gregaria* protease inhibitors 1–5) could be isolated to homogeneity using a three step HPLC procedure, SGPI-2, 3, 4, and 5 being further isolated on Deltapak C4 and subsequently on Biosep-Sec-S-2000, and SGPI-1 on Deltapak C4 and on Symmetry C18.

Fig. 1 shows the primary structures of the isolated ovarian protease inhibitors. All serine protease inhibitors were fully identified except SGPI-4, which could only be sequenced up to Ser^{35}. Due to lack of material, we were unable to perform an enzymatic digest. For SGPI-1 and SGPI-5, the entire amino acid sequence was obtained in a single sequencing experiment. SGPI-2 could be unequivocally identified up to amino acid 34 (Pro). Cloning of the precursor revealed that the final amino acid is Gln^2 (Vanden Broeck et al., Eur. J. Biochem., submitted). The amino acid sequence of SGPI-3 was only partially obtained by automated Edman degradation. The C-terminal (Leu-Thr-Phe-Ala) residues were obtained by cDNA sequencing of the precursor (Vanden Broeck et al., Eur. J. Biochem., submitted). The mass spectrometric analysis of SGPI-3 revealed a molecular mass of 4130. This value differs by 6 Da from the molecular mass (4136.6 Da) calculated from the amino acid sequence obtained by Edman degradation. Therefore we conclude that the six cysteine residues are involved in three disulfide bridges. Assuming that the six cysteine residues in SGPI-1 likewise form three disulfide bridges, the calculated mass of this peptide corresponds to the experimentally determined mass (3816 Da).

The molecular mass of SGPI-2 was determined to be 3794 Da from the major peak at 3795.1. Assuming that the six cysteines form three intramolecular disulfide bridges, the calculated mass from the amino acid sequence is 3647.8 Da, which differs by 146 Da from the experimentally determined mass. Among the posttranslational modifications reported so far, an *O*-glycosylation by a deoxyhexose (146 Da) is the most probable one. This sugar moiety is most likely present on Thr^9 since during Edman cycle 9 an additional unidentified peak eluting before the Pth-Thr peak arises, which is absent at cycle 8 where only a Pth-Thr peak occurs. Assuming three disulfide bridges and comparing the calculated (3924 Da) and the experimental mass (4070 Da), we can deduce that

Name	Amino acid sequence	Mass (Da)
SGPI-1	EQE**C**TPGQTKKQD**C**NT**C**N**C**TPTGVWA **CTRKGC**PPH	3816
SGPI-2	EVTCEPGTTFKDKCNTCRCGSDGKSAA**CTLKAC**PQ	3794
SGPI-3	CTPGSRKYDGCNWCTCSSGGAWI **CTLKYC**PPSSGGGLTFA	4130
SGPI-4	SEGHCTPNTTFKKDCNTCSCDNDGTAAV**CTLKAC**LS???	4164
SGPI-5	EVNCTPGATFKNKCNTCRCGSNGRSAS**CTLMAC**PPGSY	4070
PMP-C [8]	EISCEPGKTFKDKCNTCRCGADGKSAA**CTLKAC**PNQ	3919
PMP-D2 [8]	EEKCTPGQVKQDCNTCTCTPTGVW G**CTRKGC**QPA	3752
HI [8]	AGECTPGQTKKQDCNTCTCTPTGIW G**CTRKAC**RTT	3716

Fig. 1. Amino acid sequence of the five novel serine protease inhibitors of *Schistocerca gregaria* (SGPI-1–5) and comparison with the sequences of the serine protease inhibitors of *Locusta migratoria* (gaps are introduced to maximize the similarities).

Table 1
Protease inhibitory activity (%) of the five serine protease inhibitors (10 μM)

Serine protease (final concentration)	SGPI-1	SGPI-2	SGPI-3	SGPI-4	SGPI-5
Bovine trypsin (1.6 μM)	100	0	0	0	0
Bovine α-chymotrypsin (0.08 μM)	100	100	100	100	100
Porcine pancreatic elastase (0.8 μM)	0	100	0	0	0

SGPI-5 is posttranslationally modified as well. Here also, the unidentified peak at cycle 9 of the Edman degradation indicates that the deoxyhexose moiety is present on Thr[9].

Since a few C-terminal amino acid residues are missing in the primary structure of SGPI-4, we cannot calculate the exact mass. The presence of the above mentioned peak at the Pth-Thr 10 Edman cycle, which is absent at the Pth-Thr 6 cycle, suggests that SGPI-4 is also glycosylated. From the experimental mass of 4164 Da we can deduce that no more than four amino acids at the C-terminal side remain unidentified.

3.2. Serine protease inhibitory activity

At a concentration of 1 μM, all peptide serine protease inhibitors inhibit chymotrypsin activity in vitro. Trypsin activity is only inhibited by SGPI-1 and elastase activity is inhibited by SGPI-2 (Table 1).

The zymogram of the gut homogenate (Fig. 2) reveals nine bands containing proteolytic activity (lane A1) as demonstrated with casein as general substrate. One of the four major protease bands (pb6) contains trypsin-like activity since it was revealed after incubation with BANA, a substrate for trypsin-like enzymes (lane B1). SGPI-1 has a large spectrum of inhibition since all bands on the zymogram were abolished in its presence (lane A2). In the presence of SGPI-2, six out of nine proteases are inhibited (lane A3). The band containing trypsin-like activity, as revealed with BANA as a substrate (lane B1), disappears in the presence of SGPI-1 (lane B2) but not in the presence of SGPI-2 (lane B3), indicating that SGPI-2 displays no inhibitory activity towards this trypsin-like protease.

3.3. Spatial and temporal distribution of SGPI-1–5

HPLC analysis of different tissue extracts indicates that all serine protease inhibitors occur not only in the ovary but also in testes, hemolymph and fat body (Table 2). They are absent in the gut. Of particular interest is that a gut specific chymotrypsin inhibitor elutes at a retention time of 78 min, which is much later than all five ovarian serine protease inhibitors. In addition, the fat body, hemolymph and testes contain a factor with chymotrypsin inhibitory activity (elution time 72 min), which is also absent in the ovary. These inhibitors, which according to their elution time seem to belong to a different family of protease inhibitors, remain to be purified.

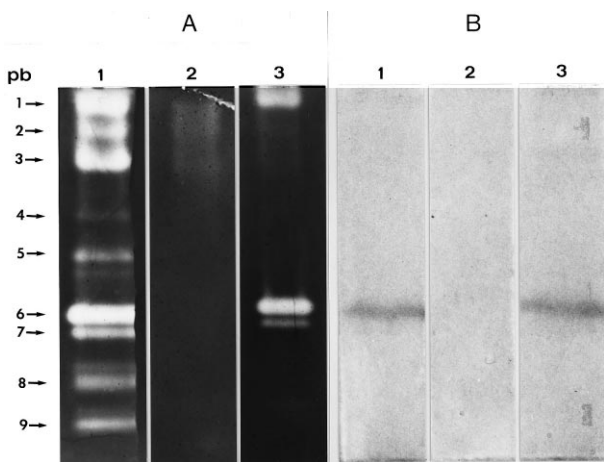

Fig. 2. Inhibitory activity of SGPI-1 and SGPI-2 on insect gut proteases after PAGE electrophoresis and blotting on agar-casein gel (A) and on polyacrylamide-BANA gel (B). Gut homogenate (lanes 1), gut homogenate incubated with SGPI-1 (lanes 2), gut homogenate incubated with SGPI-2 (lanes 3).

3.4. Immunocytochemical localization of SGPI-1-like immunoreactivity

In order to demonstrate that the presence of serine protease inhibitors in the ovary cannot be attributed to the presence of fat body cells in this organ, we prepared a polyclonal antiserum against SGPI-2. By the use of this antiserum, we were able to demonstrate intense labeling in all the follicle cells surrounding the maturing eggs (Fig. 3). The labeling is concentrated in intensively labeled vesicles, which are more pronounced at the apical side close to the oocyte surface than at the basal side of the follicle cells.

4. Discussion

All five ovarian 4 kDa peptides, designated SGPI-1–5, contain six cysteine residues which are involved in three disulfide bridges and therefore belong to the same family. Based on the difference between the calculated mass and the experimental mass (146 Da), we conclude that SGPI-2, 4 and 5 are posttranslationally modified by a deoxyhexose moiety. It is the threonine residue at position 9 or 10 (always on the fifth position after the first cysteine residue) that is glycosylated. The deoxyhexose has been identified as a fucose by Nakakura et al. [7], who previously identified two peptides of the same family in *Locusta migratoria*. It is as yet unclear why some serine protease inhibitors contain this sugar while others lack it. SGPI-3 lacks a threonine at position 9. SGPI-1, however, has a threonine at position 9, but nevertheless it is not glycosylated.

Fig. 1 shows that SGPI-2 (chymotrypsin inhibitor) displays a high degree of sequence similarity (83.33%) with the *Locusta* protease inhibitor designated PMP-C and LMCI-II by two research teams who isolated these peptides independently at the same time [6,7]. Like LMCI-II, SGPI-2 is also a potent inhibitor of porcine pancreatic elastase. SGPI-2 (35 aa) has the same structural region containing the presumed active site, as demonstrated in a detailed kinetic study using synthetic variants [8]. This sequence similarity is also observed in the P4 and P′4 residues of the active site region.

SGPI-1 (35 aa) displays a high degree of sequence similarity (77.14%) with PMP-D2/LMCI-1. However, in contrast to the *Locusta* peptide PMP-D2 which was found to interact only with chymotrypsin and not with trypsin [6,8], SGPI-1 displays strong in vitro inhibitory activity towards bovine trypsin and

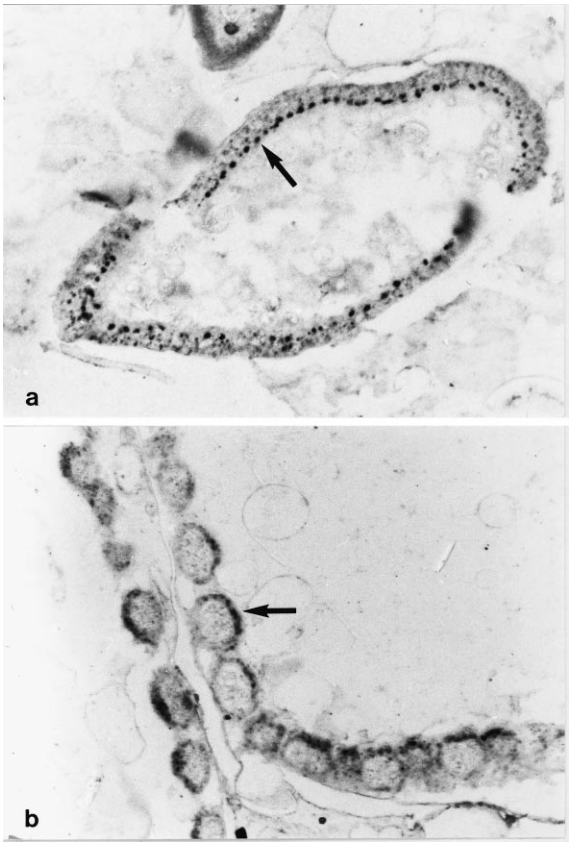

Fig. 3. Cross-sections of ovariole of *Schistocerca gregaria* (a: ×175, b: ×420). SGPI-2-like immunoreactive vesicles (arrows) are present in the follicle cells, surrounding the oocyte.

much less towards chymotrypsin. In addition, the inhibitory activity of SGPI-1 towards a locust endogenous trypsin-like enzyme is in accordance with this effect. The discrepancy is hard to explain especially when we refer to the work of Kellenberger et al. [8] who located the P1-P′1 active site of PMP-D2 in the C^{27}TRKGC^{32} region. SGPI-1 has exactly the same sequence in exactly the same position. This sequence contains a Lys and an Arg residue. Many natural trypsin inhibitors have a Lys as P1 residue and most synthetic substrates for trypsin have an Arg as P1 residue. Recently, it has been demonstrated that changes of the P1 residue can change not only the potency of the inhibition, but also the specificity [8]. Our results show that the specificity of serine protease inhibitors may be determined not only by the nature of the P1 residue as shown by Kellenberger et al. [8], but also by residues beyond the P1-P′1 bond. In this respect it is noteworthy that the P4 residue is Gly in PMP-D2, whereas it is Ala in SGPI-1. The P′4 residue is Gln in PMP-D2, whereas it is Pro in SGPI-1. The P4 residue has been reported to play an important role in the action of some serine protease inhibitors [14].

Comparing the activity of SGPI-2 and SGPI-4, we reach the same conclusion. Although SGPI-4 has exactly the same sequence as SGPI-2 in the reactive site (C^{28}TLKAC^{33}), SGPI-4 displays no inhibitory activity towards pancreatic elastase, whereas SGPI-2 is a strong inhibitor of elastase even when used in lower concentrations. Again, the P4 and P′4 residues are different. Previously it has been shown that an alteration in the P′4 site and even in the P′5 site in a serpin-type protease inhibitor (i.e. PAI-1 plasminogen activator inhibitor 1) drastically changes the interaction with the enzyme (tissue-type plasminogen activator) [15]. A serpin-resistant variant of the t-PA enzyme is fully inhibited by PAI-1-type molecule where the P4′ site (Glu^{304}) is altered with Arg.

RP-HPLC analysis indicates that the isolated ovarian SGPIs are also present in the fat body, the hemolymph and testes but not in the gut. The demonstration of SGPI-2-like immunoreactivity in the follicle cells surrounding the eggs (Fig. 3) indicates that the identification of serine proteases in the ovary cannot exclusively be attributed to a contamination of fat body cells or hemolymph. From this study we cannot conclude whether the ovary is the site of synthesis of some or of all of the inhibitors. It is possible that some of them are taken up from the hemolymph. A molecular biological study on the cloning and expression of some of the inhibitors is being completed at this moment and will address this question (Vanden Broeck et al., Eur. J. Biochem., submitted).

In conclusion, the discovery of SGPI-1–5, in addition to HI, PMP-D2 and PMP-C, indicates the existence of a new family of serine protease inhibitors which differs from the proteinase inhibitors of the Kunitz-type family (mass below 10 kDa) [4,16] and the serpin-type family (mass higher than 45 kDa) [5]. Based on the high sequence similarities we assume that the disulfide pairing is the same in all of the 4 kDa peptide serine protease inhibitors of this family and that they incorporate a cysteine knot and a short three-stranded anti-parallel β-sheet as has been described for PMP-D2 and PMP-C [17–19]. Point amino acid changes in and around the active site are able to change the potency and specificity of the inhibitors. Also towards the insect endogenous serine proteases, they display different specificities as shown by our electrophoresis study. This suggests that they might play a complementary role in the finetuning of the proteases that are implicated in the diverse physiological processes during insect development (molt, immune system, digestion, ovarian development, digestion of yolk proteins, protection against parasites) [20–22].

Table 2
Tissue distribution of serine protease inhibitory activity as revealed by RP-HPLC analysis on a C18 column

Inhibitor	SGPI-1	SGPI-2	SGPI-3	SGPI-4	SGPI-5		
Elution time	24 min	42 min	48 min	52 min	56 min	72 min	78 min
Mass (Da)	3816	3794	4130	4164	4070	unknown	unknown
Vitellogenic ovary	+	+	+	+	+	−	−
Adult fat body	+	+	+	+	+	+	−
Adult hemolymph	+	+	+	+	+	+	−
Adult gut	−	−	−	−	−	−	+
Adult testes	−	+	+	+	+	+	−
Larval hemolymph	+	+	+	+	+	+	−
Larval fat body	+	+	+	+	+	+	−
Larval gut	−	−	−	−	−	−	−

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