



## Functional analysis of a pancreatic secretory trypsin inhibitor-like protein in insects: Silencing effects resemble the human pancreatic autodigestion phenotype

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### ABSTRACT

**Introduction:** In mammalian pancreatic cells, the *pancreatic secretory trypsin inhibitor* (PSTI) prevents the premature activation of digestive enzymes and thus plays an important role in a protective mechanism against tissue destruction by autophagy, a process which may ultimately cause diseases such as pancreatitis and pancreatic cancer. Insects, however, lack a pancreas and so far no PSTI-like peptides are functionally characterized.

**Results:** In several insect species protease inhibitors that structurally resemble the mammalian PSTI were predicted *in silico*. A putative PSTI-like protein (LmPSTI) was cloned and sequenced in the African migratory locust, *Locusta migratoria*. For the first time the expression of an insect derived PSTI-like inhibitor was shown to be restricted to the digestive enzyme-producing organs in insects (midgut and caeca). LmPSTI was produced via a bacterial expression system and was found to be a potent inhibitor of bovine trypsin as well as endogenous locust gut enzymes. In the caeca, RNAi-mediated knockdown of *LmPSTI* resulted in a significantly upregulated expression (2-fold) of locust ATG8 transcripts (an ubiquitin-like protein crucial for autophagosome formation). These findings were confirmed by an ultrastructural study on caeca, revealing the presence of autophagy-related structures in RNAi-treated animals.

**Conclusion:** The results of this study lead us to believe that LmPSTI plays an important role in controlling the proteolytic activity in the digestive system of *L. migratoria*. These findings provide new evidence for the existence of an ancient protective mechanism in metazoan digestive systems and open new perspectives for the study of autophagy-related diseases in the digestive tract.

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## 1. Introduction

Diseases involving the pancreatic system – notably pancreatitis and pancreatic cancer – continue to pose a major public health concern worldwide. In the United States alone, approximately 80 000 people are hospitalized annually due to acute pancreatitis and 40 000 due to chronic pancreatitis, resulting in around 3500 deaths per year (Brown et al., 2008; Everhart and Ruhl, 2009; Gupta and Toskes, 2005). Usually, acute pancreatitis is relatively mild and

resolves on its own, whereas chronic pancreatitis is more severe and is commonly defined as a continuing inflammatory process of the pancreas. Left untreated, chronic pancreatitis greatly increases the risk of developing pancreatic cancer (Raimondi et al., 2010). There are several different causes for pancreatitis, but all lead to a premature intra-pancreatic activation and release of digestive enzymes, resulting in cellular and tissue damage of the pancreas. As a consequence of the incomplete understanding of pathways and mechanisms involved in pancreatic diseases, therapeutic solutions remain inadequate (Wang et al., 2010).

A healthy mammalian pancreas delivers its stored inactive digestive enzyme precursors to the gut. Once secreted in the gut lumen, the digestive enzymes are activated in a cascade-like manner, starting with the cleavage of trypsinogen into the active trypsin by enterokinases. Subsequently, trypsin activates the other pro-proteases needed for the digestion of proteins in the food. To

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prevent premature activation of the digestive enzymes, mammals express a protease inhibitor (PI) in their pancreatic cells called *pancreatic secretory trypsin inhibitor* (PSTI) (Hirota et al., 2006). The amino acid composition and sequence of bovine and human PSTI have been solved by conventional chemistry in the 1960s and 1970s (Greene and Giordano, 1969; Yamamoto et al., 1985). This inhibitor is a member of the Kazal-family and binds to prematurely activated trypsin, instantly interrupting the activation cascade. In patients that suffer from pancreatitis and/or pancreatic cancer, the activation cascade tends to start prematurely, resulting in severe damage to pancreatic tissues. In some cases the intra-pancreatic activation of digestive enzymes has been linked to mutations in the *PSTI* gene (Hirota et al., 2003; Witt et al., 2000). Also, knockout mice deficient in *PSTI* show autophagy-like symptoms in their pancreas and do not survive beyond 15 days (Ohmuraya et al., 2005). Interestingly, a serine protease inhibitor of the Kazal-family with a similar function has recently been identified in an evolutionary distant, cnidarian species, *i.e.* the freshwater polyp *Hydra magnipapillata* (Chera et al., 2006). This suggests an older, shared mechanism in protection against the potentially hazardous effects of digestive proteases. A better insight into this preserved mechanism across the animal kingdom may lead to a deeper understanding of the mechanisms of digestive enzyme regulation in general and of diseases like pancreatitis in particular.

In insects, several previous studies have shown the presence of Kazal-like inhibitors in – among others – *Bombyx mori* (Zheng et al., 2007), *Drosophila melanogaster* (Niimi et al., 1999), *Aedes aegyptii* (Ribeiro et al., 2007), *Triatoma infestans* (Lovato et al., 2006) and *Rhodnius prolixus* (Friedrich et al., 1993). Their biological and physiological functions are diverse, but so far a regulatory role on the activity of insect digestive proteases has not been discovered. In this study, a comparative *in silico* study of the available insect nucleotide sequence data revealed the presence of PSTI-like genes in Insecta (by far the largest class of animal species on planet Earth). Among many newly predicted PSTI-like genes a PSTI-homolog in the African migratory locust, *Locusta migratoria*, (LmPSTI) was identified and further characterized. LmPSTI was cloned, sequenced and its tissue distribution was investigated. Moreover, LmPSTI was produced using a bacterial expression system and its inhibitory effect on bovine and endogenous trypsin was confirmed *in vitro*. Knocking down this PSTI-like inhibitor via RNA-interference resulted in ultrastructural damage to the locust's caeca (the insect's functional equivalent of the mammalian exocrine pancreas) and established – for the first time in an insect – its functional role in a protective mechanism from prematurely activated digestive enzymes, remarkably similar to its role seen in mammals.

## 2. Methods

### 2.1. Rearing of animals

Gregarious migratory locusts, *L. migratoria*, were reared under crowded conditions with controlled temperature ( $32 \pm 1$  °C), light (14 h photoperiod) and relative humidity (40–60%). The animals were kept at high density (>200 locusts/cage) in special wooden cages and were fed daily with fresh grass and rolled oats. For all experiments in this study, freshly molted 5th larval stage animals were grouped on a daily base to exclude possible developmental differences.

### 2.2. cDNA cloning of LmPSTI

#### 2.2.1. RNA extraction and cDNA synthesis

For the initial cloning and sequencing of *L. migratoria* PSTI (LmPSTI) and the subsequent tissue distribution analysis, total RNA

from several tissues was extracted and reverse transcribed into cDNA. To obtain the total RNA, 5th larval stage animals were dissected and their tissues thoroughly washed in Ringer's solution. To maintain the integrity of the RNA, the tissues were immediately snap-frozen in liquid nitrogen and kept at  $-80$  °C. Tissues were subsequently disrupted by means of the MagNA Lyser Instrument in 'Green Beads'-filled reaction tubes (Roche, Indianapolis, IN, USA). Total RNA was extracted with the Lipid Tissue RNA extraction kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. In combination with this extraction procedure, a DNase treatment (RNase-free DNase set, Qiagen) was performed to eliminate potential genomic DNA contamination. Synthesis of cDNA with SuperScriptIII Reverse Transcriptase (Invitrogen) was performed following the manufacturer's recommendations, using random primers and 1 µg of total RNA as starting material.

#### 2.2.2. Cloning and sequence analysis

A putative LmPSTI sequence was derived from EST's retrieved by querying the NCBI database with the human (acc. nr. NP\_003113) and mouse (acc. nr. NP\_033284) PSTI protein sequences. Primers were designed to clone this sequence in a PCR reaction (Fw: 5'-CTCAATATGGACAGAAAGACCCT-3'; Rv: 5'-CAACCGTTTCGCATATCTTAA-3'). Hot-start PCR was run for 30 cycles. Each cycle consisted of a denaturation step for 1 min at 94 °C, an annealing step for 1 min at a specific temperature according to the primer set and an extension step for 1 min at 68 °C, with a final extension step of 7 min at 68 °C. The PCR fragment was then subcloned using the TOPO-TA Cloning Kit for Sequencing (Invitrogen Life Technologies). After plasmid isolation (GenElute HP Plasmid Miniprep Kit; SIGMA), the insert was sequenced on a 3130 Genetic Analyzer (Applied Biosystems). A SignalP analysis ([www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)) was performed to check for the presence of a secretion signal and the subcellular location of this inhibitor was predicted with TargetP ([www.cbs.dtu.dk/services/TargetP](http://www.cbs.dtu.dk/services/TargetP)).

### 2.3. *In silico* analysis

An extensive search in the public NCBI EST-database of insects revealed a multitude of putative Kazal-like inhibitors (*i.e.* the inhibitor family PSTI belongs to). Initial queries used in this search were the human PSTI and LmPSTI protein sequences, but these were progressively complemented with the newly-found insect Kazal-like PI sequences. Only the inhibitors which were expressed in the insects' digestive system were upheld and the amino acid sequences of those inhibitors were aligned with human PSTI using AlignX software (Invitrogen) (see Supplementary data 1).

### 2.4. Real-time RT-PCR transcript profiling

In order to study the tissue distribution of LmPSTI expression, several tissues were dissected from six animals: foregut (FG), midgut (MG), hindgut (HG), caeca (Ca), salivary glands (SG), brain (Br), gonads (Gn), fat body (FB) and malpighian tubules (MT). RNA extraction and cDNA synthesis was performed for each tissue separately as described above. Real-time PCR primers for the derived LmPSTI sequence as well as for the endogenous control (RP49) were designed with Primer Express software (Applied Biosystems). To verify the similar PCR-efficiencies of LmPSTI amplification reactions and the endogenous control, a validation experiment was performed. For this, standard curves were generated for the different transcripts with a serial (5×) dilution of a cDNA mixture of the different tissues. All reactions were run in duplicate on an Abi Prism 7000 Sequence Detection System (Abi Prism 7000 SDS, Applied Biosystems) using the following thermal

cycling profile: 50 °C (2 min), 95 °C (10 min), followed by 40 steps of 95 °C for 15 s and 60 °C for 60 s. After 40 cycles, samples were run for the dissociation protocol (*i.e.* melting curve analysis). In order to compensate for differences in loading and RT-efficiency, RP49 was used as an endogenous control of LmPSTI transcripts. Values were analyzed and normalized relative to RP49 transcript levels by means of the 7000 System SDS (Sequence Detector Software) (version 2.3, Applied Biosystems). Statistical differences were analyzed using one-way ANOVA with a post-hoc Tukey test.

### 2.5. Recombinant production of LmPSTI

For further characterization, the LmPSTI inhibitor was produced as a recombinant protein in the bacterial expression system pMAL (New England Biolabs) (Simonet et al., 2003). This system allows the expression of the protein of interest fused to Maltose Binding Protein (MBP), enabling easy purification by affinity chromatography. Primers were designed to the coding region of LmPSTI (Fw: 5'-TCGCGGCTGGCGGAG-3'; Rv: 5'-**GTAAGCTTCAACCCGTTTCGCATATCTTAA-3'**), yielding an amplicon starting immediately after the signal peptide and including the stop codon. A HindIII restriction site (bold) was added to the 5' end of the reverse primer, allowing directional ligation in the pMAL-p2p vector. After the ligation, the plasmid construct was transformed into BL21 *Escherichia coli* cells (Novagen) and plated onto an LB agar plate containing 10 µl/ml ampicillin. Individual clones were checked for the presence of the correct insertion by means of sequence analysis. Following steps of the production were performed as described by Breugelmans et al. (2009a). The BL21-cells containing the correct insert were grown in a stepwise manner in Overnight Express Instant TB Medium (Novagen) until a suitable density was reached. Subsequently, the cells were collected by centrifugation and the pellet was resuspended in 1 × BugBuster Protein Reagent (Novagen) (5 mg/g pellet), resulting in a mild lysis of the bacterial cells. Simultaneously, per ml BugBuster reagent, 1 µl Benzonase (Novagen) was added. In addition, lysozyme was added (1 kU per ml BugBuster, Novagen) to improve the disruption of the bacterial cell walls. Complete protease inhibitor tablets were added (1 tablet per 50 ml, Roche) to avoid proteolytic degradation. Next, the mixture was incubated (15 min at room temperature, gently shaken). Finally, after centrifugation (16 000 × g, 20 min, 4 °C) the supernatant, containing the fusion protein is decanted. A 5.5 × 50 cm column (D × H, Bio-Rad) was filled with 15 ml of an amylose resin (Bio-Rad). Before loading the crude extract, the column was washed with 8 volumes of Column buffer (1 M Tris–HCl; 0.2 M NaCl; 0.5 M EDTA; pH 7.4) and the crude extract was diluted with Column buffer to a final concentration of ≤2.5 mg/ml. Following steps were performed at 4 °C. The column was loaded with the diluted extract (typically 800–1200 ml) at a flow rate of 2 ml/min and washed with 15 volumes of Column buffer. Finally, the fusion protein was eluted with Column Buffer supplemented with 20 mM maltose at a flow rate of 1 ml/min 30 fractions of 3 ml were manually collected and the protein concentration was measured by means of the Bradford protein assay. Eluted fractions that contained the fusion protein were first pooled and then desalted (NaCl, maltose) and concentrated to a final concentration of about 2–4 mg/ml, using Centriprep Centrifugal Filter Devices (YM-30, Millipore).

LmPSTI was cleaved from MBP by a specific peptidase (Factor Xa) according to established protocol (Breugelmans et al., 2009a) and the molecular mass of the recombinant protein was determined using MALDI-TOF.

### 2.6. Extraction of *L. migratoria* midgut enzymes

Biologically active enzymes were extracted from *L. migratoria* midgut by incubating dissected midgut tissue ( $n = 5$ ) during 1 h in 1 ml Ringer's solution. Next, midguts were removed and the solution

containing the secreted enzymes was used in the *in vitro* assay described below.

### 2.7. Serine protease inhibitor assay

Two types of *in vitro* assays were performed to measure peptidase activity and/or relative inhibitory activity of PI. In the first assay, the inhibitory activity of LmPSTI against peptidases (purified, commercially available bovine enzymes as well as those present in *L. migratoria* midgut extract) was assessed in a modified azocasein assay according to Brock et al. (Brock et al., 1982). Since casein is a chromogenic substrate for almost all peptidases, it allows for the measurement of total proteolytic activity in any given sample. In general, 200 µl of enzyme (or enzyme extract) was added to 200 µl of 1% azocasein (Sigma) and incubated for 45 min at 32 °C. The reaction was terminated by addition of 150 µl of 10% trichloroacetic acid followed by 10 min cooling on ice. After centrifuging at 13 000 rpm for 10 min at 4 °C, 415 µl of the supernatant was centrifuged for a second time (13 000 rpm; 10 min). Then, 14 µl of NaOH (5 M) was added to 90 µl of the supernatant and absorbance was measured at alkaline pH (four technical replicates) at 405 nm. To measure the inhibitory effect of LmPSTI, purified bovine enzyme or locust enzyme extract was pre-incubated for 10 min (32 °C) with either the produced inhibitor or a chemical PI, AEBSF (100 mM), [4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, Sigma]. Then the assay was performed as described above. Prior to the activity assays, bovine trypsin and chymotrypsin were active site titrated with 4-Nitrophenyl 4-Guanidinobenzoate (NPGb).

In the second assay, the trypsin selective substrate N-Benzoyl-Phe-Val-Arg-pNa (Bz-FVRpNA, Sigma) was used to determine the inhibitory activity of LmPSTI against bovine trypsin. To assess the relative inhibitory strength of LmPSTI, AEBSF and the bovine specific Bovine Pancreatic Trypsin Inhibitor (BPTI) were run in parallel experiments. In general, 90 µl of enzyme solution (final concentration 15 nM) and 10 µl buffer (control) or PI (LmPSTI and BPTI: 75 nM; AEBSF: 100 mM) were pre-incubated for 15 min (32 °C) and then added to 100 µl of substrate (1 mM). The absorbance of the mixture was recorded every 10 s at 405 nm over a period of 5 min and the slope of the linear curve ( $Abs_{405\text{ nm}}/\text{min}$ ) is a measure for the peptidase activity in the sample.

In both types of experiments, blanks (replacing enzyme by buffer) were included and the inhibitory activity (in %) was calculated as defined below:

$$\text{Inhibitory activity (\%)} = 100 - [(\Delta Abs_{PI}/\text{min} / \Delta Abs_{\text{control}}/\text{min}) \times 100]$$

### 2.8. RNAi study

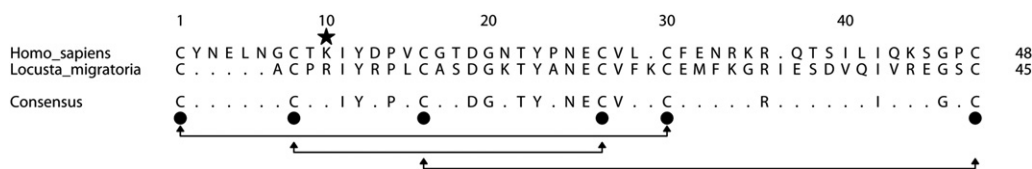
To study the possible function of LmPSTI, the inhibitor was knocked down in an RNAi experiment.

#### 2.8.1. dsRNA synthesis

For the production of LmPSTI dsRNA, a standard PCR reaction was performed using following primers: Fw 5'-**TAATACGACTCACTA-TAGGGCTGGTGTGCTTGGCGGTG-3'**, Rv 5'-**TAATACGACTCACTATAGGCTCGCGCACGATTTCAC-3'**. A T7-promotor sequence (bold) was added to the 5'-end of these primers. The PCR fragment was then used as a template for the production of dsRNA using the MEGAscript RNAi kit (Ambion), following the manufacturer's protocol.

#### 2.8.2. Real-time RT-PCR analysis

Six synchronized fifth instar *L. migratoria* were injected with 10 µl dsRNA solution (400 ng/µl) in the hemocoel. Six control



**Fig. 1.** Alignment of LmPSTI with human PSTI. The amino acid sequences are restricted to the two exterior cysteines. The brackets under the consensus sequence represent the typical 1–5/2–4/3–6 disulfide bridge pattern between the Cys-residues. The P1-residue is indicated with a star.

animals were injected with control solution (*i.e.* elution buffer without dsRNA). An additional control experiment where dsGFP was added to the buffer solution (400 ng/ $\mu$ l) is shown in [Supplementary data 2](#). Four days after injection, the caeca were dissected, total RNA was extracted and transcribed into cDNA as described earlier. Using real-time PCR, the expression of LmPSTI was evaluated as well as that of a homolog of ATG8 (Autophagy-Related Gene 8), an important protein in the autophagy pathway of yeast, where it is essential in the formation of autophagosomes. This protein is also known as Light Chain 3 (LC3) in mammals and is often used as a marker for autophagy (Rose et al., 2006; Umemiya-Shirafuji et al., 2010). Additionally, six animals were deprived of food for the duration of the experiment. Since food deprivation is known to elicit autophagy, these animals served as a positive control. ANOVA with a post-hoc Tukey test was used to recognize statistically significant differential expression levels.

### 2.8.3. Ultrastructural study

Caeca from the animals in the RNAi study were partly preserved for further ultrastructural analysis. Therefore, tissue was fixed in cold 2% glutaraldehyde, buffered at pH 7.3 with 50 mM Na-cacodylate and 150 mM saccharose. Post-fixation occurred in 2% osmiumtetroxide in the same buffer. After dehydration in a graded acetone series, the caeca were embedded in Araldite (Polysciences, Warrington, PA) and sectioned with a Reichert Ultracut E microtome (Reichert-Jung, Austria). Semithin sections of 1  $\mu$ m were stained with methylene blue and thionin and viewed in an Olympus BX-51 microscope (Olympus, Japan). Double-stained thin sections of 70 nm were examined using a Zeiss EM900 electron microscope (Carl Zeiss AG, Germany). Images shown are representative examples from a pool of at least 10 pictures per condition.

## 3. Results

### 3.1. Cloning and sequence analysis

Searching public *L. migratoria* EST-databases predicted a 243 bp (81 aa) long homolog of PSTI. After PCR-amplifying the LmPSTI gene and cloning it into a suitable sequencing vector, the identity of this fragment was verified by sequencing analysis. SignalP predicted a 24 amino acid long signal peptide and according to TargetP this inhibitor follows the secretory pathway (score 0.912).

### 3.2. In silico analysis

Aligning LmPSTI with the human PSTI revealed several conserved amino acids (see Fig. 1). Notably, six cysteine residues are conserved which form a disulfide linkage pattern typical for Kazal-like inhibitors: Cys<sup>I</sup>–Cys<sup>V</sup>, Cys<sup>II</sup>–Cys<sup>IV</sup> and Cys<sup>III</sup>–Cys<sup>VI</sup>. The specificity determining P1-residue is Arg (in *Locusta*) or Lys (in *Homo*), which is indicative of trypsin inhibitors. Aligning LmPSTI with other putative Kazal-like PI sequences found in the digestive system of insects, however, reveals sometimes another residue is found in this place; an often encountered P1-variant is Leu (L), which suggests an inhibitory preference for chymotrypsin, just like Met, Phe and Tyr.

Two species (*R. prolixus* 2 and *Ostrinia nubilalis* 3) have an Asp at this position, suggesting inhibitory activity toward cysteine proteases. Insect Kazal-like PI resembles their mammalian equivalents fairly well, except for the number of amino acids between the first two conserved cysteine residues. In mammals usually six amino acids occupy the space between Cys<sup>I</sup> and Cys<sup>II</sup>, while in invertebrates this is typically limited to just one or two residues.

### 3.3. Tissue distribution analysis

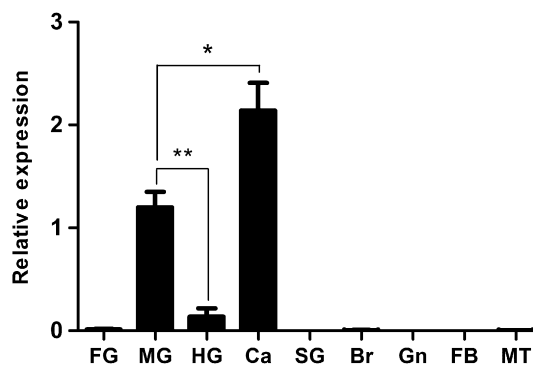
Transcript levels of LmPSTI were quantified by RT-real-time PCR in a number of different tissues of six synchronized 5th larval stage *L. migratoria*. Statistical analysis showed a significantly different expression in different tissues (see Fig. 2). The highest expression was found in the caeca ( $P$ -value < 0.05 compared with the midgut), followed by the expression in the midgut ( $P$ -value < 0.01 compared with the hindgut) and a relatively low expression in the hindgut. There appears to be no or very little expression of LmPSTI in the other tissues.

### 3.4. Recombinant production of LmPSTI

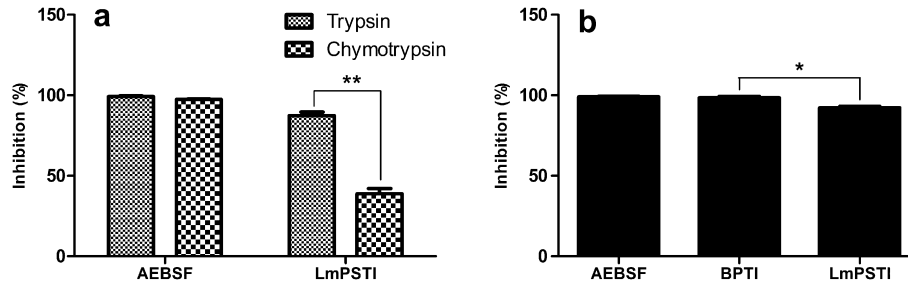
The pMAL Protein Fusion and Purification System was used to obtain sufficient amounts of biologically active LmPSTI protein for further characterization. This expression system allows for the bacterial production of a gene product attached to maltose binding protein (MBP) forming a MBP-LmPSTI fusion protein. By using an optimized protocol, yields up to 15–35 mg/l culture were realized. After cleavage, an accurate estimation of the mass of LmPSTI was obtained by means of MALDI-TOF analysis; revealing a mass of 6491 Da conform to the theoretical mass of LmPSTI.

### 3.5. In vitro activity of recombinant LmPSTI

Initial experiments showed that the inhibitory activity of the MBP-LmPSTI fusion protein does not differ from the cleaved LmPSTI



**Fig. 2.** Relative expression levels of LmPSTI transcripts in different tissues of 5th larval stage *L. migratoria*; foregut (FG), midgut (MG), hindgut (HG), caeca (Ca), salivary glands (SG), brain (Br), fat body (FB) and malpighian tubules (MT). The assay was performed on tissue from six synchronized animals ( $n = 6$ ). The data represent relative quantities  $\pm$ S.E.M., normalized relative to the endogenous control. ANOVA post-hoc Tukey \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Fig. 3.** *In vitro* activity of recombinant LmPSTI. (a) The chemical inhibitor AEBSF was pre-incubated in excess with bovine trypsin and chymotrypsin as a positive control for serine peptidase activity. In parallel, bovine trypsin and bovine chymotrypsin were pre-incubated with LmPSTI in equimolar concentrations (1  $\mu$ M) and tested in an azocasein assay. LmPSTI inhibited trypsin activity by  $\pm$ 85% and chymotrypsin with  $\pm$ 40%. (b) A similar experiment on the trypsin specific substrate N-Benzoyl-Phe-Val-Arg-pNa showed that the proteolytic activity of bovine trypsin was almost completely inhibited by AEBSF (99.06%), while the inhibitory activity of LmPSTI (92.33%) approached that of BPTI (98.57%). The percentage inhibition in both assays was calculated by comparing the activity of enzyme incubated with LmPSTI to the activity of enzyme without inhibitor. Data represent mean values of three repeats  $\pm$ S.E.M. Student's T-test \* $P < 0.05$ ; \*\* $P < 0.01$ .

(Supplementary data 3) therefore all further experiments were performed with purified fusion protein. Initially, the inhibitory activity of the recombinant protein against commercially available bovine trypsin and bovine chymotrypsin was tested in an azocasein assay. Pre-incubating 1  $\mu$ M bovine trypsin with an equimolar amount of LmPSTI fusion protein inhibited the activity of this enzyme by  $\pm$ 85% (see Fig. 3, panel a). Repeating the experiment with bovine chymotrypsin also inhibited enzymatic activity, but to a lesser degree (percentage inhibition of 40%). The difference between the inhibition of trypsin and chymotrypsin was statistically significant ( $P$ -value  $< 0.01$ ). The chemical inhibitor AEBSF was run in parallel with the recombinant inhibitor to reveal possible non-serine peptidase enzymatic activity present in the enzyme solutions.

After establishing LmPSTI as a potent trypsin inhibitor in the azocasein assay, an additional *in vitro* experiment on the trypsin selective substrate N-Benzoyl-Phe-Val-Arg-pNa was performed (see Fig. 3, panel b). The activity of similar concentrations of LmPSTI and BPTI (75 nM) was compared, showing that LmPSTI inhibited trypsin activity with 92% and BPTI was capable of 98% inhibition. These inhibition percentages were statistically significantly different ( $P$ -value  $< 0.05$ ). Like in the azocasein assay, the chemical inhibitor AEBSF was run in parallel and almost completely inhibited trypsin (99%).

Pre-incubating secreted midgut enzymes from *L. migratoria* with a concentration gradient of LmPSTI showed that the proteolytic activity of the midgut enzymes was progressively inhibited as the inhibitor concentration increases (Fig. 4). The highest inhibitor level tested (23.5  $\mu$ M) lowered the activity of the enzymes to the levels seen when the chemical inhibitor AEBSF was added.

### 3.6. RNAi study

To gain more insight in the possible function(s) of PSTI-like proteins in insects, an RNAi-mediated knockdown of LmPSTI was performed. Six 5th instar animals ( $n = 6$ ) were injected in the hemocoel with dsRNA and real-time RT-PCR was used to confirm the effectivity of the knockdown in the caeca. As a negative control six animals ( $n = 6$ ) were injected with buffer solution without dsRNA. An additional six animals ( $n = 6$ ) were starved for the duration of the experiment. Since starvation is known to elicit autophagy, these animals served as a positive control. Real-time RT-PCR was used to observe the expression of the autophagy marker ATG8 in the caeca of the locusts following LmPSTI-knockdown and additionally the ultrastructure of the caeca was examined for any signs of self-digestion.

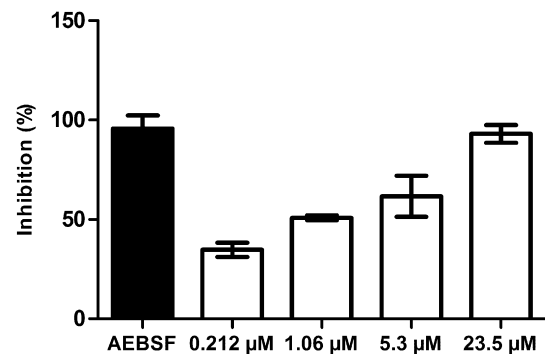
#### 3.6.1. LmPSTI-knockdown and ATG8 expression

As shown in Fig. 5 (panel a) transcript levels for LmPSTI in the caeca were significantly lower four days after injection of the dsRNA (residual expression around 10%,  $P$ -value  $< 0.01$ ). Interestingly, LmPSTI expression was twice as high in starved animals compared to control animals that received food during the experiment (195% of expression in control animals,  $P$ -value  $< 0.01$ ).

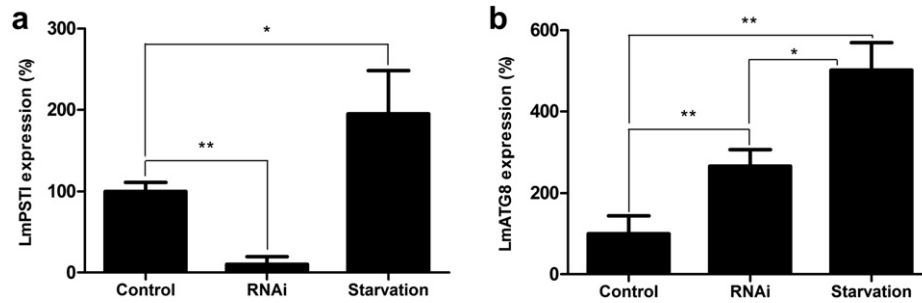
In Fig. 5 (panel b) the effect of the knockdown of LmPSTI as well as the effect of starvation on the expression of an important component in the autophagy pathway (ATG8) was visualized. Highest expression levels of ATG8 were seen in starved animals (502% of expression in control animals,  $P$ -value  $< 0.01$ ). In fed animals, a higher ATG8 expression level was observed in animals injected with LmPSTI dsRNA (266% of expression in control animals,  $P$ -value  $< 0.01$ ), but remained lower than in starved locusts ( $P$ -value  $< 0.05$ ).

#### 3.6.2. Ultrastructural study

Since electron microscopy remains the 'golden standard' for the detection of autophagy, caeca obtained in the RNAi experiment were prepared for an ultrastructural study. Electron microscopy micrographs (see Fig. 6) showed more and larger autophagy-like structures (like the characteristic 'whirl-bodies' and vacuolization) in caeca from starved animals than in caeca obtained from fed animals. Knocking down the expression of LmPSTI coincided with a higher concentration of autophagy-like structures in comparison to fed control animals.



**Fig. 4.** *In vitro* inhibitory activity of LmPSTI against secreted midgut enzymes of 5th larval stage *L. migratoria*. Midgut enzymes were collected and pre-incubated with different concentrations of recombinant LmPSTI ranging from 0.2 to 23.5  $\mu$ M and tested in an azocasein assay. AEBSF was added in excess to determine the percentage of serine protease activity. Data represent mean values of three repeats  $\pm$ S.E.M.



**Fig. 5.** (a) Effect of dsRNA-mediated knockdown and starvation on LmPSTI expression. A significantly reduced expression was observed in RNAi-treated animals compared to controls, while a significantly higher expression was seen in starved control animals. (b) Effect of dsRNA-mediated knockdown of LmPSTI and starvation on ATG8 expression. The highest ATG8 expression was observed in starved animals (502% of control level), the lowest in fed control animals (100%) and an intermediate expression level was seen in RNAi-treated fed animals (266%). The data represents relative quantities  $\pm$ S.E.M. ( $n = 6$ ), normalized relative to the endogenous control (RQ) ANOVA post-hoc Tukey \* $P < 0.05$ ; \*\* $P < 0.01$ .

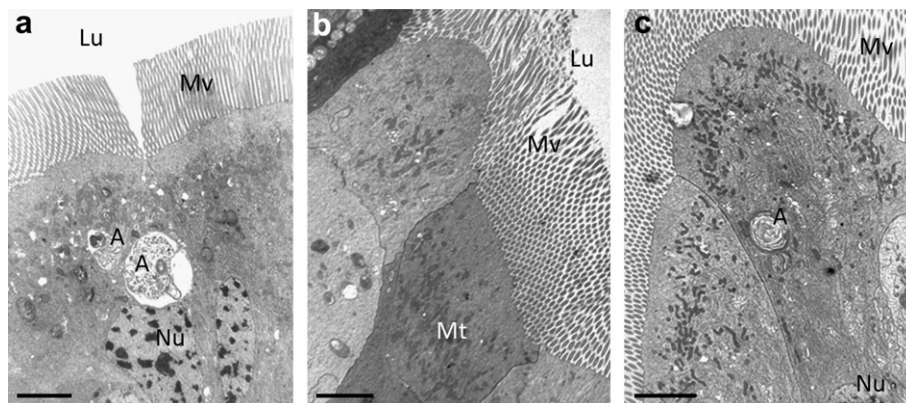
#### 4. Discussion

The mechanisms involved in pancreatitis are still poorly understood, hindering the development of adequate therapeutic solutions. A common factor, however, is the premature activation of the digestive enzymes, resulting in cellular and tissue damage. This activation happens in a cascade-like manner, starting with the conversion of trypsinogen into trypsin, which is then able to activate the other pro-enzymes. To prevent this, mammals express the trypsin inhibitor PSTI in the acinar cells of the pancreas. Recently, a trypsin inhibitor with a comparable function as mammalian PSTI has been identified in the freshwater polyp *H. magnipapillata* (Chera et al., 2006). This is suggestive of an older, shared protective mechanism against the perilous effects of digestive proteases.

A PSTI-homolog in *L. migratoria* was predicted in EST-databases and subsequently cloned and sequenced. The transcript contains six conserved cysteine residues found in nearly all Kazal-type inhibitors where they form a disulphide linkage pattern that is characteristic for this inhibitor family (Cys<sup>I</sup>–Cys<sup>V</sup>, Cys<sup>II</sup>–Cys<sup>IV</sup> and Cys<sup>III</sup>–Cys<sup>VI</sup>) (Rimphanitchayakit and Tassanakajon, 2010). As seen in Fig. 1, the P1 position of LmPSTI is occupied by a positively-charged Arg. Positively-charged amino acids are strongly stabilized by Asp in the catalytic pocket of trypsin-like enzymes, suggesting inhibitory activity toward these proteases. This is in accordance with human PSTI and the PSTI-homolog in *H. magnipapillata*, where a similar preference for trypsin is observed. A preference for trypsin inhibition is a prerequisite for the hypothesized function where, to disrupt the premature activation of zymogens efficiently, trypsin – having a pivotal role as activator of all the other zymogens – has to

be inhibited efficiently. Mammalian PSTI-homologs, for which this protective role has already been proven, indeed exhibit a clear preference for trypsin. A 24 amino acid long signal peptide was predicted by SignalP and according to TargetP-analysis this inhibitor is directed onto the secretory pathway. Considering the suspected role of this inhibitor, this secretion is significant, as it means that LmPSTI is in close vicinity of the inactive digestive enzymes while they are being secreted. For this PI to be a rapid and effective inhibitor of prematurely activated trypsin, a co-localization of PSTI with the other secreted pro-enzymes is essential. LmPSTI being secreted also means that it possibly has a role in protecting the tissue of the digestive system from activated peptidases in the gut, as has been shown for mammals. Further research, however, is needed to confirm this function.

By searching through the available insect EST-data we predicted 47 new Kazal-like inhibitors which are expressed in the digestive system. Analyzing the alignment shown in Supplemental data 1, it is clear other amino acids are also found at the P1 position; Leu is the most common variant and suggests a possible preference for chymotrypsin, just like Met (*Dendroctonus ponderosae* 2), Tyr (*R. prolixus* 3) and Phe (*Sitodiplosis mosellana*). What role – if any – chymotrypsin inhibitors play in preventing the activation cascade of the other pro-enzymes is still unclear. Possibly they protect the cells against damage from earlier activated chymotrypsin or are secreted in the gut to protect the gut tissue from active chymotrypsin. In two insect species (*R. prolixus* 2 and *O. nubilalis* 3) the P1 position is occupied by an Asp, which according to the literature is indicative for an inhibitory preference of caspases (Gupta et al., 2005). Caspases are cysteine proteases involved in apoptosis or programmed cell death.



**Fig. 6.** Ultrastructure of caeca of 5th larval stage *L. migratoria* of (a) starved, (b) control, (c) RNAi-treated animals. Lu (Lumen), Mv (microvilli), Nu (nucleus), A (autophagy-like structures), Mt (mitochondria). Scale bar 5  $\mu$ m.

Interestingly, also in another family of serine protease inhibitors, the pacifastins, inhibitors with an Asp as P1-residue have been discovered (Breugelmanns et al., 2009b). Their exact role has not yet been clarified.

There are a few differences between mammalian and invertebrate Kazal-like inhibitor sequences. For example the number of amino acids between the first two cysteine residues is lower in invertebrates, resulting in a less flexible conformation causing the inhibitors to be more protease specific (Hemmi et al., 2003). These differences (and similarities) are excellently reviewed by Rimphanitchayakit (Rimphanitchayakit and Tassanakajon, 2010).

Real-time PCR analysis of different tissues of 5th larval stage *L. migratoria*, allowed for a study of the distribution of LmPSTI expression. The digestive system is the main organ where this inhibitor is expressed, with the highest expression in the enzyme-producing midgut and caeca. Unlike human PSTI, which can be found in a range of tissues including the gastrointestinal tract, lung, liver, kidney, ovaries and breast milk (Freeman et al., 1990; Marchbank et al., 2009; Shibata et al., 1987), LmPSTI expression seems to be limited to midgut and caeca.

LmPSTI proved to be a potent inhibitor of bovine trypsin (and to a lesser extent) bovine chymotrypsin, confirming the inhibitory specificity predicted from the amino acid sequence. Next, the inhibitory activity of LmPSTI was compared to the activity of a bovine and pancreas specific protease inhibitor, namely BPTI. As expected, BPTI proved to be a strong inhibitor of bovine trypsin. Curiously, LmPSTI approached the inhibitory activity of BPTI. The similar activity of an insect and a mammalian PSTI against mammalian trypsin is another sign that this inhibitor (and thus possibly its role) is more or less conserved in at least these two classes. Since the enzymes used in this standard assay are of bovine origin, the actual target enzymes could possibly be inhibited even more efficiently. Digestive enzymes secreted by the *Locusta* midgut were also tested in a similar azocasein assay. The chemical serine protease inhibitor AEBSE, run in parallel with LmPSTI, showed that about 90% of the digestive enzyme activity in this secrete depends on serine proteases. LmPSTI was able to inhibit all of the proteolytic activity due to these serine proteases indicating a strong affinity for digestive proteases native to *L. migratoria*. Further tests with protease specific substrates will show if this proteolytic activity in secreted midgut enzymes is mainly due to trypsin or to other serine proteases (like chymotrypsin) and whether the inhibitory specificity of LmPSTI for insect enzymes is similar to that seen for bovine enzymes.

In an attempt to unravel the function of PSTI-like inhibitors in insects, the expression of LmPSTI transcript was knocked down using RNAi. As a negative control a group of a 5th instar locusts were injected in the hemocoel with buffer solution. Since we expected an autophagy-like phenotype as a result of this knockdown, a group of animals was starved for the duration of the experiment as a positive control. Starvation is known to elicit autophagy, so any phenotypic responses seen in the RNAi-treated animals could be compared to the phenotype of starved locusts. The percentage knockdown of LmPSTI was evaluated with real-time RT-PCR and after four days the residual transcript level was about 10% compared to the negative control group, indicating a successful knockdown of the transcript. In parallel the expression of a locust homolog of ATG8 in the caeca was studied using real-time RT-PCR. ATG8 is an ubiquitin-like protein required for the formation of autophagosomal membranes and often used to observe autophagy (Rose et al., 2006; Umemiya-Shirafuji et al., 2010). A statistically significant two-and-a-half-fold rise in ATG8 expression was observed in the RNAi-treated animals in comparison with the negative control group. This resembles the effect seen in starved animals where the ATG8 expression was about five times as high as the levels seen in the negative control group.

Starving animals is probably a more effective way of provoking autophagy than knocking down the expression of LmPSTI and a longer knockdown period would most likely increase the phenotypic effect even further. Nevertheless, a significant phenotypic response was observed when knocking down the LmPSTI transcript.

To verify these results the ultrastructure of caeca from the RNAi experiment was studied. Fed, RNAi-treated animals showed a high number of large autophagy-like structures (vacuolization and 'whirl-bodies'), while these were rare in fed control animals. In starved animals, similar structures could be observed. These observations confirmed the results of the ATG8 expression.

An interesting observation was the significantly upregulated expression of LmPSTI in starved animals. The reason for this is not completely clear, but perhaps an increased or longer storage of peptidases in caeca cells – when there is no food present in the gut to digest – heightens the risk of premature enzyme activation and therefore requires a higher level of protection by LmPSTI. This storage-theory is supported by a study of *Manduca sexta* where digestive enzyme secretion was suspended during starvation while the enzyme expression levels remained constant (Broehan et al., 2008).

All in all, we believe these data strongly suggest a protective role of PSTI-like protease inhibitors in insects, much like the function seen in vertebrates. The conservation of this mechanism indicates that insects are interesting experimental animals for obtaining a deeper insight into the regulation of the digestive system in general. Better insight in this mechanism could lead to the development of drugs against diseases of the human pancreatic system, like pancreatitis and pancreatic cancer. Future research in this field is, however, still needed. What is, for example, the role of the multiple isoforms found in many insect species? Do they have additional functions, alongside the protective role? In mammals, other functions for PSTI – as a growth factor or even as a monitor peptide influencing the levels of certain hormones – have been suggested. Whether insects use PSTI-homologs for similar diverse functions remains to be investigated.

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## Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ibmb.2011.04.012.

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