PEPTIDES 29 (2008) 235–241

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The role of hemocytes, serine protease inhibitors and pathogen-associated patterns in prophenoloxidase activation in the desert locust, *Schistocerca gregaria*

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ARTICLE INFO

Article history:
Received 7 June 2007
Accepted 24 July 2007
Published on line 18 January 2008

Keywords:
Innate immunity
Phenoloxidase
Peptide
Serine protease inhibitor
Insect
Hemolymph

ABSTRACT

The prophenoloxidase-activating system is an important component of the innate immune response of insects, involved in wound healing and melanotic encapsulation. In this paper we show that in the desert locust, *Schistocerca gregaria*, hemocytes, challenged with microbial elicitors, are indispensable for the limited proteolytic activation of prophenoloxidase (proPO) in plasma. In addition, we assessed the influence of serine protease inhibitors on the induction of PO-activity in plasma. While soybean Bowman–Birk inhibitor (SBBI) inhibited the PO activation by laminarin-treated hemocytes, the endogenous pacifastin-related inhibitors, SGPI-1 (*S. gregaria* pacifastin-related inhibitor-1) and SGPI-2 did not affect the PO-activity under similar conditions. On the other hand, real-time PCR analysis revealed that the transcripts, encoding SGPI-1–3, were more abundant in the fat body of immune challenged animals, as compared to control animals.

1. Introduction

Although insects lack the elements that compose the adaptive immune response of vertebrates, they manifest effective innate immune responses. This innate immune system includes both humoral and cellular reactions, which operate in a coordinated way to respond to microbial and metazoan challenge. An important component of the humoral immune response in both Hexapoda and Crustacea is the prophenoloxidase-activating system (proPO-AS) [4,13]. This system comprises several components such as pattern recognition proteins (PRPs), a serine protease cascade and the zymogenic proPO. When pathogens succeed in penetrating the cuticular barrier a second line of defense reactions is induced. Pathogen-associated molecular patterns (e.g. peptidoglycan, lipopolysaccharides and β-1-3-glucans) are recognized by PRPs, triggering the rapid activation of a serine protease cascade in the hemolymph. This includes the sequential activation of a yet unknown number of proteases and co-factors, leading to the limited proteolysis of a proPO-activating

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Abbreviations: proPO-AS, prophenoloxidase-activating system; PRPs, pattern recognition proteins; PAP, proPO-activating protease; PI, protease inhibitor; LPS, lipopolysaccharide; SBTI, soybean trypsin inhibitor; SBBI, soybean Bowman–Birk inhibitor; HLS, hemocyte lysate supernatant; SGPI, *S. gregaria* pacifastin-related inhibitor; SGPP, *S. gregaria* pacifastin-related precursor; LMPI, *L.*; *migratoria* pacifastin-related inhibitor.

0196-9781/$ – see front matter
doi:10.1016/j.peptides.2007.07.032

protease (PAP). This enzyme, in turn, catalyzes the proteolytic cleavage of the inactive proPO precursor into the active phenoloxidase (PO). Finally, PO catalyzes the oxidation of phenolic compounds to quinones, which then are converted to melanin through several non-enzymatic steps [4,13]. This so-called melanization reaction is involved in encapsulation, wound healing and cuticle sclerotization. In addition, melanin synthesis includes the formation of toxic intermediary compounds, which help to kill invading microorganisms.

Obviously, a very accurate regulation of the proPO-AS is needed to avoid premature activation. This is partially achieved by synthesizing both PO and its activating enzyme as inactive zymogens that require proteolytic cleavage in order to become active [4,13]. Furthermore, insects and crustaceans contain serine protease inhibitors (PI) in the hemolymph to prevent unwanted activation of this complex system. However, although the presence of serine PI in the hemolymph of insects and crayfish has been associated with the regulation of the proPO-AS for several years [12], only few studies have shown a direct inhibitory effect of PI on PAPs. Both in *Manduca sexta* and *Drosophila melanogaster*, PI belonging to the serpin family have been shown to specifically inhibit proteases, involved in the proPO-activating proteolytic cascade [11,20,21,23]. On the other hand, from the hemolymph of the crayfish *Pacifastacus leniusculus*, an unrelated multimeric PI, called pacifastin, was purified [10] and shown to inhibit the activation of the proPO-activating enzyme [1]. In addition, several monomeric pacifastin-related inhibitors have been purified from locust hemolymph [2,9] and it was shown that these peptides, analogous to the expression of pacifastin in the hepatopancreas, are expressed in the locust fat body [15,22]. Interestingly, pacifastin-related PI from *Locusta migratoria* have been shown to inhibit fungal trypsin [14], suggesting another defensive role for these peptides, i.e. the protection of the insect cuticle against proteolytic degradation by entomopathogenic fungi.

Although the activation of proPO has been studied for many years, the exact site of synthesis and regulation of proPO and its activating enzymes and inhibitors is, except for a few holometabolous insects, not yet fully clarified. Analogously, the role of hemocytes as mediators of the proPO-AS is still controversial. In this study, an *in vitro* assay was used to investigate the influence of microbial elicitors on the proPO-AS in *Schistocerca gregaria* and the different role of haemocyte and plasma proteins in this complex system was further analyzed. In addition the effect of the pacifastin-related hemolymph PI, SGPI-1 (*S. gregaria* pacifastin-related inhibitor-1) and SGPI-2, as putative negative regulators of the proPO-AS in *S. gregaria* was studied. Finally, we investigated the influence of immune challenge on the transcript levels encoding the peptide precursors, SGPP-1 (SGPI-1 and SGPI-2) and SGPP-2 (SGPI-3).

2. Materials and methods

2.1. Rearing of the animals

Desert locusts, *S. gregaria*, were reared under controlled laboratory conditions as described by Vanden Broeck et al. [22]. Unless mentioned otherwise, adult locusts of 10 days old were used in the following experiments.

2.2. Collection and treatment of hemolymph

Thirty minutes before collection of the hemolymph, animals were chilled at 4 °C. Locusts were anesthetized with CO_2 and a leg was amputated. From the bleeding wound, the hemolymph (50 μl/animal) was collected with a pipette and immediately transferred to a falcon tube containing chilled *S. gregaria* Ringer's solution (8.77 g/l NaCl, 0.19 g/l $CaCl_2$, 0.75 g/l KCl, 0.41 g/l $MgCl_2$, 0.34 g/l $NaHCO_3$, 30.81 g/l sucrose, 1.89 g/l trehalose, pH 7.2), obtaining a 1/10 hemolymph dilution. Under these conditions no coagulation was observed. In general, hemolymph from 5 or 10 locusts was pooled to allow for sufficient material to perform a range of tests.

The tubes were centrifuged during 10 min at 4 °C and aliquots of decanted supernatant (plasma) were collected. These plasma samples were used either without any further treatment, or incubated for 1 h at 37 °C with Ringer's solution containing laminarin (Sigma), LPS from *Salmonella typhimurium* (Sigma) or chymotrypsin (Sigma). The remaining hemocyte pellet was washed in *S. gregaria* Ringer's and resuspended in 1.5 ml Ringer's (control) or in Ringer's solution, supplemented with laminarin (Sigma), LPS from *S. typhimurium* (Sigma) or chymotrypsin (Sigma). The addition of chymotrypsin results in the proteolytic activation of proPO to PO and is used to measure the proPO levels. In addition, hemocytes were treated with a mixture of laminarin with one of the following serine PI, SGPI-1, SGPI-2, soybean trypsin inhibitor (SBTI) or soybean Bowman–Birk inhibitor (SBBI) (final concentration: 1.5 μM). The latter two soybean peptides were purchased from Sigma. SGPI-1 and SGPI-2 were produced biosynthetically in our laboratory via a bacterial (*Escherichia coli*) expression system fused to maltose-binding protein (MBP) and the inhibitory activity versus bovine trypsin and chymotrypsin, respectively, was verified as described previously [18].

After an incubation period of 30–60 min at room temperature, the hemocytes were lysed in an ice-water bath by sonication (Soniprep 150, Sanyo) in pulses of 10 s. Cellular debris was removed by centrifugation and aliquots of the hemocyte lysate supernatant (HLS) were used for further experiments. Unless mentioned otherwise, final laminarin, LPS and chymotrypsin concentrations in plasma and HLS samples were 0.5 mg/ml.

2.3. Assay for PO-activity

PO-activity was monitored spectrophotometrically at 492 nm (Multiskan RC V1.5) as the formation of dopachrome. A stock solution of L-DOPA (2 mg/ml, Sigma) was prepared in *S. gregaria* Ringer's. To quantify the effect of the different experimental conditions on the PO-activity in HLS or plasma, 100 μl aliquots were prepared as described above and distributed in a 96-well plate. Next, 100 μl of L-DOPA (2 mg/ml) was added to each well. This solution was mixed 10 s and the absorbance at 492 nm (OD_{492}) was recorded every 10 s during an interval of 90 s. In addition, the effect of HLS, treated with Ringer's solution (control), laminarin or LPS on the activation of proPO in plasma was assessed. Therefore, 50 μl

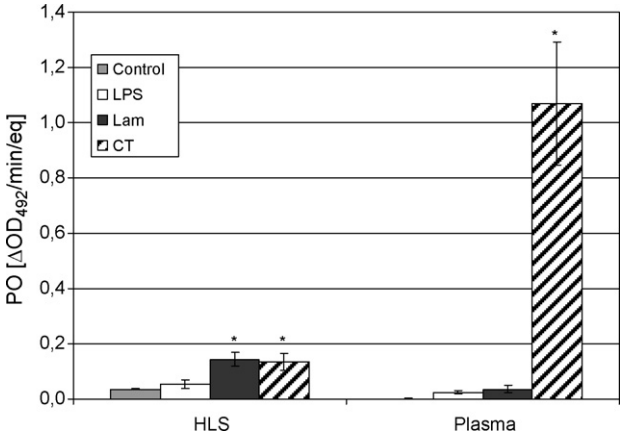

Fig. 1 – PO-activity in plasma and HLS. Per condition, hemolymph from five naïve male locusts was pooled and plasma and HLS were prepared as described in Section 2. The effect of laminarin (LAM), lipopolysaccharides (LPS) and chymotrypsin (CT) on the PO-activity was measured and compared with the control condition (Ringer's solution). Data represent means ± standard deviation ($n = 3$). Bars indicated with '*' are significantly different from the control condition ($P < 0.05$).

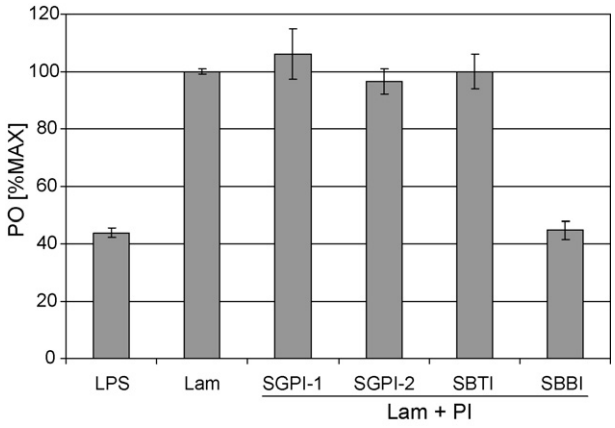

Fig. 2 – Influence of laminarin- and LPS-treated HLS on the plasma PO-activity. The diluted hemolymph of 30 locusts was divided over three tubes (5 ml/tubes) and plasma and HLS were prepared as described in Section 2. The hemocyte pellets were resuspended in 1.5 ml Ringer's solution (not shown), laminarin (0.5 mg/ml) or LPS (0.5 mg/ml). In addition, hemocyte pellets were resuspended in 1.5 ml laminarin (0.5 mg/ml) supplemented with the serine protease inhibitors, SGPI-1, SGPI-2, SBTI and SBBI (1.5 μM). After an incubation period of 30 min, 10 μl hemocyte lysate was added to 50 μl plasma and 40 μl Ringer's solution and this mixture was incubated for 1 h at 37 °C. Then, PO-activity in plasma was measured and is represented as the activity relative to the reference PO-activity of laminarin-treated hemocytes. The figure shows a representative assay with the bars indicating the mean of four identical replicates ($n = 4$) ± standard deviation.

untreated plasma, 10 μl HLS (prepared as described above) and 40 μl Ringer's solution were incubated for 1 h at 37 °C. As a positive control reaction, 10 μl chymotrypsin (2 mg/ml, Sigma) instead of HLS was added. For all experiments the ΔOD_{492}/min ratio was computed via the Genesis V2.16 software (Labsystems). Results are calculated as ΔOD_{492}/min/equivalent (1 equivalent = 50 μl hemolymph) and expressed as the mean ± standard deviation. Pairwise statistical analysis of experimental and control samples was done based on non-parametric statistics, using the Wilcoxon–Mann–Whitney two-sample test (Fig. 1).

In some cases, the absolute values of (pro)PO-activity were found to vary between experiments with different batches of animals; PO-activity in plasma incubated with laminarin-treated hemocytes ranged from 1.3 to 2.0 ΔOD_{492}/min/equivalent. Nevertheless, the relative differences observed between experimental and control conditions were always reproducible. To compare these experiments, the PO-activity for each of the experimental conditions was expressed as the percentage (%Max) of the reference condition, i.e. plasma incubated with laminarin-treated hemocytes. All these experiments were done at least twice and representative results are shown. Data are expressed as the mean (of four identical replicates) ± standard deviation (Fig. 2).

2.4. *Quantitative real-time PCR analysis*

2.4.1. *Immune challenge and sample processing*

Locusts were reared as described above. Laminarin was purchased from Sigma and dissolved in *S. gregaria* Ringer's at a concentration of 10 mg/ml. In a first experiment, different groups of female locusts (10 days old) were pooled and the control group ($n = 6$) was injected with 10 μl Ringer's solution and the experimental groups ($n = 6$) were injected with 10 μl containing 10 and 100 μg laminarin. Injections were carried out with a calibrated Hamilton syringe. At 3 and 20 h after immune challenge, tissues (brain, fat body, foregut, hindgut and gonads) from adult locusts were microdissected under a binocular microscope and immediately transferred to a tube containing RNA*later* (Ambion) solution to prevent degradation. Until further use, these pooled tissue samples were stored at −20 °C. RNA-extraction and cDNA-synthesis of these samples was done as described previously by Simonet et al. [17].

Based on the outcome of this experiment, two additional independent assays were performed; control and experimental animals were injected with Ringer's solution and 100 μg laminarin, respectively, and fat body tissue was collected 20 h after injection. Further processing of these samples was done as described above. One representative assay is shown and the data are expressed as the mean (of three identical replicates) ± standard deviation.

2.4.2. *Primer design, PCR amplification and cDNA quantification*

Primers for the SGPP-1 and SGPP-2 target sequences, as well as for the endogenous β-actin control, were designed previously by means of the 'Primer Express' software (Applied Biosystems) [17]. For the quantification of the SGPP-transcripts relative standard curves were generated with serial (10×) dilutions of fat body cDNA from control animals. Reaction

conditions are the same as described previously [17]. In order to compensate for differences in loading and RT-efficiency, a locust β-actin transcript was used as an endogenous control. Previous studies clearly indicated that the analyzed β-actin mRNA levels remain quite constant in locust tissues [5,6,17,22], regardless of their developmental or physiological condition. Thus, for both SGPP-transcripts, values were normalized relative to β-actin transcript levels.

3. Results

3.1. PO-activity in plasma or HLS

As shown in Fig. 1, control samples of HLS and plasma from naive adult male locusts, contain very low PO-activity. On the other hand, incubation of hemocytes with laminarin results in a significant induction of PO-activity as compared to the control samples. Furthermore, the response elicited by laminarin in terms of PO-activity equals the effect of the proteolytic activation of proPO in HLS by chymotrypsin. Incubation of plasma samples with LPS or laminarin seems to have a minor effect on the PO-activity. However, this induction is only marginal when compared to the PO-activity in plasma after treatment with chymotrypsin. Qualitatively similar results were obtained with samples from female instead of male locusts (data not shown).

3.2. The effect of HLS on the PO-activity in plasma

As shown in Fig. 2, adding a small volume of an extract of laminarin- or (to a lesser extent) LPS-treated hemocytes to plasma elicits a strong response in terms of PO-activity. Furthermore, when this induction of PO-activity in plasma by an extract of laminarin-treated hemocytes is expressed in absolute values (ΔOD_{495}/min/equivalent), it parallels the strong effect of the proteolytic activation of proPO in plasma by chymotrypsin (see Fig. 1). On the other hand, when hemocytes are incubated with Ringer's solution and HLS is added to plasma, the PO-activity is negligible and comparable to the control plasma samples shown in Fig. 1.

Finally, when SGPI-1, SGPI-2 or SBTI are co-incubated with laminarin-treated hemocytes, no inhibitory effect on the induction of plasma PO-activity is recorded. In contrast, SBBI proved to be an inhibitor of the PAPs in HLS. Similar results were obtained with samples from female instead of male locusts (data not shown).

3.3. Dose–response relationship for laminarin

Next, we incubated hemocytes with different amounts of laminarin and assessed the dose–response relationship for the PO-activity in plasma. Increasing the doses of laminarin to hemocytes from 100 to 750 μg led to a higher activation of PO-activity in plasma (Fig. 3).

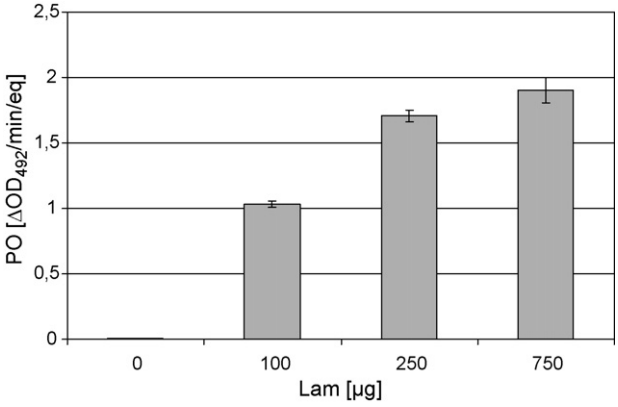

Fig. 3 – Dose–response relationship for the effect of laminarin-treated hemocytes on the PO-activity in plasma. The diluted hemolymph of 40 locusts was divided over four tubes (5 ml/tube) and plasma and HLS were prepared as described in Section 2. Hemocytes were pre-incubated with 1.5 ml Ringer's solution containing respectively 0, 100, 250 and 750 μg laminarin. Then, a mixture of 50 μl plasma, 10 μl hemocyte lysate and 40 μl Ringer's solution was incubated for 1 h at 37 °C. Then, PO-activity in plasma was measured. The bars represent the mean of three repeated measurements for each condition (ΔOD_{492}/min/equivalent ± standard deviation).

3.4. Quantitative real-time PCR analysis

In order to study the influence of laminarin on the relative abundance of the SGPP-1 and SGPP-2 transcripts in locusts, a real-time RT-PCR analysis was undertaken on samples from different tissues of adult female locusts that were injected either with Ringer's solution or with laminarin. Analysis of the dissociation curves of the experimental and the β-actin control samples showed a single melting peak, which indicates a specific signal, corresponding to both SGPP target sequences and the endogenous control. In all negative control samples, no amplification of the fluorescent signal was detected, indicating that the extraction procedure, including the DNase treatment, efficiently removed genomic DNA of the RNA samples.

An initial experiment (data not shown) showed that from all tissues tested, fat body contained the highest amount of SGPP-1 and SGPP-2 transcript. Furthermore, only in fat body tissue was laminarin found to induce SGPP-1 and SGPP-2 transcription (20 h after injection), as compared to control samples. Therefore two additional independent experiments focusing on SGPP-1 and SGPP-2 transcript levels in fat body were performed. Analysis of these data showed a similar trend for both assays: normalized SGPP-1 and SGPP-2 transcript levels are higher in the fat body of laminarin-injected animals as compared to control samples (Fig. 4).

4. Discussion

Previous *in vivo* studies of Goldsworthy et al. [8] showed that laminarin and LPS induce the PO-activity in the migratory locust, *L. migratoria*. To obtain more information about the mechanisms, underlying PO-activation, we have studied the *in vitro* effect of two different microbial cell wall compounds

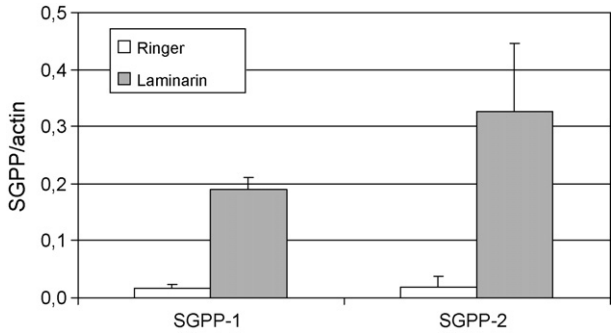

Fig. 4 – Quantitative real-time PCR analysis of SGPP-1 and SGPP-2 transcripts in female locusts after immune challenge with laminarin. SGPP-1 and SGPP-2 transcripts were measured in fat body from adult female locusts at 20 h after injection with Ringer's solution or laminarin (100 μg). All data were taken from a representative assay and were normalized relative to β-actin, representing the mean (n = 3) of three identical replicates ± standard deviation.

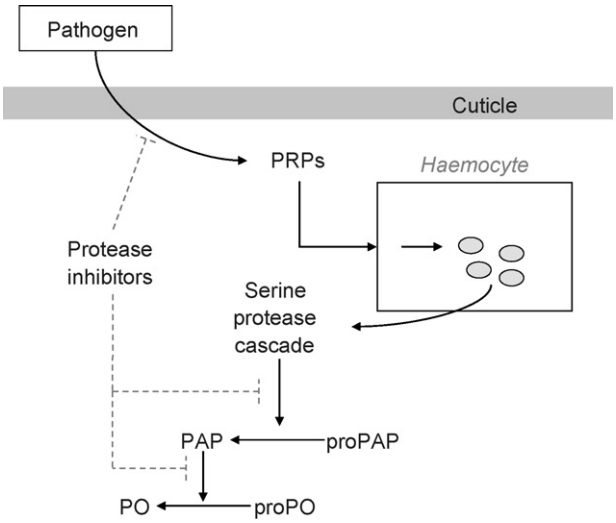

Fig. 5 – Schematic representation of proPO-activation in *S. gregaria*. Pathogen-associated molecular markers (e.g. LPS) are recognized by PRPs in locust hemolymph and trigger the activation of a proteolytic cascade which is contained within the hemocytes. Activation of the proteolytic cascade leads to the activation of the proPO-activating protease. This enzyme in turn catalyzes the activation of the zymogenic proPO to PO, leading to melanization. The putative regulation of proteases involved in the activation of proPO by hemolymph protease inhibitors is indicated by dashed lines. In addition, hemolymph protease inhibitors might inhibit pathogen-secreted proteases and thus prevent the degradation of the insect cuticle and/or internal tissues.

(laminarin and LPS) on the PO-activity in plasma and HLS. While injection of laminarin (and to a lesser extent LPS), results in a significant induction of the PO-activity in cell-free plasma (unpublished results), addition of the same elicitors to plasma *in vitro* fails to trigger a PO-response (Fig. 1). On the other hand, when chymotrypsin, which is known to be a potent catalyst of the proteolytic activation of the zymogenic proPO to PO in locusts, is supplemented to plasma, high levels of PO-activity are recorded. Interestingly, when laminarin or chymotrypsin is added to hemocytes, both result in the same effect, i.e. a moderate but significant increase of PO-activity, whereas, incubation of hemocytes with LPS had no effect on the PO-activity in HLS (Fig. 1). Altogether, these data suggest that while the majority of the proPO precursor is present in cell-free plasma, the proPO activation cascade, triggered by laminarin, is contained and/or initiated within the hemocytes.

Therefore, a novel approach to verify this hypothesis was designed. Hemocytes from naive animals were pre-incubated with laminarin or LPS and a small volume (10 μl) of HLS was added to plasma. In parallel two control reactions were performed. First, hemocytes were incubated with Ringer's solution and further processed as described above, and second, laminarin- or LPS-treated hemocytes were added to Ringer's solution instead of plasma. In both control reactions, only marginal PO-activity was detected (data not shown). In contrast, challenging intact hemocytes with one of both immunogens resulted in a strong PO-response (Fig. 2). This response was higher with the laminarin-treated hemocytes as compared to hemocytes pre-incubated with LPS. This observation is in line with *in vivo* studies, showing that injection of laminarin in both *S. gregaria* (Simonet et al., unpublished results) and *L. migratoria* results in higher PO-activity than injection with LPS [7]. However, since previous studies have shown that LPS from different bacterial strains vary in their capacity to induce PO-activity in *S. gregaria* [16], generalized interpretations are to be avoided. Nevertheless, for further experiments laminarin was used and it was shown that increasing the dose of laminarin in the pre-incubation medium from 100 to 750 μg, resulted in a dose-dependent increase in the PO-activity (Fig. 3).

Altogether these observations reveal the crucial role of hemocytes in the regulation and activation of proPO in adult locusts as schematically summarized in Fig. 5. When pathogens invade the hemocoel, associated cell wall components are recognized by PRPs. These PRPs are produced by hemocytes, although other sites of synthesis cannot be excluded. As a result, the proPO activation cascade, which is contained in hemocytes, probably in specialized vesicles, is activated and secreted. In contrast, if hemocytes are pre-incubated with Ringer's solution, no activation of proPO in plasma is measured, showing that an immune stimulus is indispensable both for the secretion as for the activation of the proPO proteolytic cascade. The above-described experiments were carried out with adult male locusts. However, experiments with female locusts resulted in qualitatively similar results (data not shown), indicating that the general mechanisms as presented in Fig. 5 account for both male and female locusts.

In several reports, the possible role of hemolymph serine PI in arthropod immunity has been discussed [12]. However, only few studies have reported on a direct interaction between PI and proPO-activating enzymes [11,20,21,23]. In crayfish, pacifastin has been shown to inhibit the proPO-activating

enzyme [1]. From locust hemolymph, pacifastine-related PI have been characterized [9,19] and Boigegrain et al. [2,3] claimed that two pacifastin-related PI, LMPI-1 and LMPI-2, are inhibitors of the proPO activating cascade in hemocytes. These findings inspired us to evaluate the effect of the orthologous *S. gregaria* serine PI, SGPI-1 and SGPI-2 on the regulation of proPO activation. In parallel, two plant-derived PI were tested. Based on our previous experiments showing that the proPO activation cascade is mediated by hemocytes, a variation of the same assay was performed; hemocytes were incubated with laminarin or with laminarin in combination with a PI. As shown in Fig. 2, SGPI-1 and SGPI-2 did not inhibit the limited proteolytic activation of proPO by laminarin-treated hemocytes. On the other hand, when hemocytes are pre-incubated with laminarin and SBBI, the activation of proPO is reduced by more than 50%. Although this observation has little physiological relevance, it does confirm that a proteolytic cascade is present in hemocytes and that the design of the assay is suitable to evaluate the effect of PI on the proPO-activating enzymes.

The apparent discrepancy between our data with the previous results on LMPI-1 and LMPI-2 can probably be attributed to the assay conditions. Boigegrain et al. [2,3] used mammalian trypsin to induce the PO-activity in an extract of hemocytes. Therefore, it cannot be excluded that the observed inhibition of proPO activation is due to the effect of LMPI-1 and/or LMPI-2 on trypsin rather than on the endogenous proPO-activating enzymes. Indeed, it has been shown that LMPI-1 and LMPI-2 are (relatively weak) inhibitors of mammalian trypsin [14]. In this study however, hemocytes were challenged by laminarin. Next, an extract was used to activate the proPO precursor in plasma, ruling out any indirect effect of SGPI peptides on exogenous mammalian trypsin.

Despite the fact that SGPI-1 and SGPI-2 failed to inhibit the locust proPO-AS *in vitro*, the encoding transcript, SGPP-1, was induced in fat body after laminarin injection. As shown in Fig. 4, also the mRNA level of SGPP-2, which codes for SGPI-3, was up-regulated 20 h after laminarin injection. Interestingly, although both transcripts are present in other tissues as well, this effect is restricted to the fat body (data not shown). This is not entirely surprising, considering that the fat body has an important role in innate immune responses and is particularly involved in the synthesis and secretion of antimicrobial peptides. In line with this and based on the fact that SGPI-1–3 have been purified from hemolymph, it is not unlikely to assume that after immune challenge, these peptides are secreted into the hemolymph where they might play a role in immunity, different from the proPO-AS. In this context it is interesting to consider that the *L. migratoria* serine PI, LMPI-1 (and HI), are very potent inhibitors of fungal trypsins [14] and that entomopathogenic fungi rely on proteases to degrade the insect cuticle. Therefore, it can be speculated that pacifastin-related inhibitors in locust hemolymph help to prevent penetration of fungi (Fig. 5). Analogously, higher endogenous PI levels upon recognition of microbial cell wall patterns in the hemocoel, would be advantageous to avoid excessive spreading of the pathogen, without compromising other immune responses, such as the proPO-activating cascade.

In conclusion, our findings show that while proPO is abundant in plasma, hemocytes are crucial for the proteolytic activation of proPO. Upon immune challenge, the proPO-AS is activated in hemocytes, leading to the proteolytic conversion of proPO in plasma. Furthermore, we found no direct effect of SGPI-1 and SGPI-2, respectively a trypsin and a chymotrypsin inhibitor, on the proPO activating proteases. However, expression of these peptides was shown to be regulated at the transcriptional level by laminarin injection, suggesting a yet unknown role in the immune response.

Acknowledgments

The authors especially thank R. Jonckers for taking care of the insects. The authors gratefully acknowledge the IWT, Belgian 'Interuniversity Attraction Poles Programme' (IUAP/PAI P6/14, Belgian Science Policy), the K.U.Leuven Research Foundation (GOA 2005/06) and the 'FWO-Vlaanderen' for financial support.

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