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

Vanessa Franssens [1], *Gert Simonet* [1], *Bert Breugelmans* [1], *Sofie Van Soest*, *Vincent Van Hoef*, *Jozef Vanden Broeck* [*]

Laboratory of Developmental Physiology, Genomics and Proteomics, Department of Animal Physiology and Neurobiology, Zoological Institute, K.U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium

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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

[*] *Corresponding author*. Tel.: +32 16 324260/323978; fax: +32 16 323902.
E-mail address: Jozef.VandenBroeck@bio.kuleuven.be (J. Vanden Broeck).

[1] These authors contributed equally to this work.

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.

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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