Journal of Insect Physiology 59 (2013) 795–803

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

The role of serine- and metalloproteases in *Nasonia vitripennis* venom in cell death related processes towards a *Spodoptera frugiperda* Sf21 cell line

Ellen M. Formesyn [a,*], Karen Heyninck [b], Dirk C. de Graaf [a]

[a] Laboratory of Zoophysiology, Ghent University, B-9000 Ghent, Belgium
[b] Laboratory of Eukaryotic Gene Expression and Signal Transduction (LEGEST), Ghent University, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 18 March 2013
Received in revised form 8 May 2013
Accepted 9 May 2013
Available online 15 May 2013

Keywords:
Nasonia vitripennis
Venom
Apoptosis
Spodoptera frugiperda

ABSTRACT

Proteases are predominant venom components of the ectoparasitoid *Nasonia vitripennis*. Two protease families, serine proteases and metalloproteases were examined for their possible cytotoxic functions in the *Spodoptera frugiperda* (Sf21) cell line using protease inhibitors that inactivate one or both protease families. Viability assays on adherent cells indicated that both protease families are among the main cytotoxic compounds of *N. vitripennis* venom. However, viability assays and flow cytometry performed on suspension cells 24 h after envenomation revealed that inactivation of metalloproteases did not improve cell survival. These results indicate that both protease families may have tissue specific functions. Time course experiments indicate that serine proteases of *N. vitripennis* venom are responsible for inducing apoptosis in the Sf21 cell line. However, other venom compounds could also be involved in this process and different cell death pathways may take over when a specific type of cell death is inhibited. During parasitation of their natural hosts, both protease families may possibly function in immune related processes and tissue destruction, enabling venom distribution. Overall, this study provides important insights into the functions of serine and metalloproteases in the venom of *N. vitripennis*.

1. Introduction

Parasitoid wasps are known to subdue their hosts by venom injection, enabling successful progeny development which eventually ends in killing the host. The ectoparasitoid wasp *Nasonia vitripennis* parasitizes pupae and pharate adults belonging to the Sarcophagidae and Calliphoridae families. The venom of *N. vitripennis* females is able to elicit several responses in the host. The most profound consequence for an envenomated host is the appearance of a developmental arrest and might be the result of several metabolic changes combined with apoptosis mediated cell death (Rivers and Denlinger, 1994, 1995; Rivers et al., 1999, 2006). Furthermore, the venom also plays a major role in suppression of host innate immunity by inhibition of hemolymph prophenoloxidase (PO) activity and encapsulation processes. The latter is affected by targeting granulocytes and plasmatocytes during parasitization of *Sarcophaga bullata* pupae (Rivers et al., 2002). The same responses were observed during parasitization of *Pseudaletia separata* by *Meteorus pulchricornis* (Suzuki et al., 2008) and *Galleria mellonella* exposed to *Pimpla turionellae* (Er et al., 2010, 2011). In many parasitoid-host relationships, the total number of hemocytes declined due to cell death, particularly by apoptosis, a process that is largely tolerogenic (Suzuki and Tanaka, 2006; Teramoto and Tanaka, 2004). This type of programmed cell death was also found to be one of the dominant mechanisms in BTI-TN-5B1 cells (Lepidoptera: Noctuidae) exposed to venom of *N. vitripennis* (Rivers et al., 2010).

Venoms of parasitoid wasps have been extensively studied in the past, in order to unravel the venom composition and their functionality in parasitoid–host interactions (Formesyn et al., 2011).Parasitoid venoms are known for their complex composition and may be accompanied with additional components like calyx fluid, polydnaviruses, virus-like particles and teratocytes to assure the successful development of the progeny (Asgari, 2011). Most of the attempts to unravel the venom composition of parasitoid wasps, were directed towards endoparasitoids. Previous analyses revealed the proteinacious character of *N. vitripennis* venom, which is known to lack those additional components such as polydnaviruses and virus-like particles (Rivers et al., 2006). Nevertheless, *N. vitripennis* is the only ectoparasitoid wasp whose complete genome and venom composition are known (Werren et al., 2010). The screening of its venom resulted in the identification of 79 venom proteins, of which proteases were the predominant venom components (de Graaf et al., 2010). Sixteen serine proteases (±20% of the protein ID's) and two metalloproteases were discovered in *N. vitripennis* venom. Members of these protease protein families were also identified in *Pteromalus puparum* (Zhu et al., 2010), *Chelonus*

* Corresponding author. Address: Laboratory of Zoophysiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium. Tel.: +32 9264 5151; fax: +32 9264 5242.
E-mail address: Ellen.Formesyn@UGent.be (E.M. Formesyn).

0022-1910/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jinsphys.2013.05.004

inanitus (Vincent et al., 2010) and *Pimpla hypochondriaca* (Parkinson et al., 2002a,b). In *Cotesia rubecula* a serine protease homolog venom protein was found to inhibit melanization in its host *Pieris rapae* (Asgari et al., 2003). A reprolysin-like metalloprotease in *Eulophus pennicornis* venom showed toxic activity towards *Lacanobia oleracae* (Price et al., 2009). These protease families in *N. vitripennis* venom may also play a profound role in the cytotoxic effects observed after envenomation.

In this study, the *Spodoptera frugiperda* 21 cell line (Lepidoptera: Noctuidae) was used for in vitro MTT assays, analyzing cytotoxicity induced by *N. vitripennis* venom. Previous research revealed the susceptibility of the Sf21 cell line to this venom (Rivers et al., 1999). Although not all Lepidopteran species tested so far are equally susceptible, they are considered as a suitable model for wasp venom research (Rivers and Denlinger, 1995; Rivers et al., 1993, 1999, 2005; Zhang et al., 2005). The involvement of both serine- and metalloprotease protein families present in the venom towards cytotoxicity was determined in adherent and suspension cells by treating venom with protease inhibitors. The type of cell death caused by complete venom or venom treated with protease inhibitors was characterized using flow cytometry, which enables the distinction between early, late apoptosis and necrotic cell death combined with DNA fragmentation assays.

2. Materials and methods

2.1. Chemicals

Insect saline solution (ISB, 150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid, pH 7) was prepared in distilled water (dH_2O). Pefabloc (Roche), an irreversible serine protease inhibitor, was dissolved in ISB at a final concentration of 100 mM. Complete protease inhibitor tablets (Roche), an inhibitor for serine, cysteine and metalloproteases, were dissolved in dH_2O in order to obtain a 25 × stock solution (9.25 mg/ml). Ethylene glycol tetra acetic acid 99% (EGTA), a metalloproteases inhibitor (Acros Organics) was dissolved in dH_2O to obtain a final concentration of 100 mM (pH 7.5). Thiazolyl Blue Tetrazolium Bromide (Sigma–Aldrich USA) was dissolved in ISB to obtain a concentration of 5 mg/ml. Hank's buffered salt solution (HBSS, 5 mM D-(+)-Glucose monohydrate, 5.4 mM KCl, 0.137 mM NaCl, 0.44 mM KH_2PO_4, 0.338 mM Na_2HPO_4, 4.16 mM $NaHCO_3$, pH 7.2) was prepared in dH_2O. All solutions were sterilized using a 0.22 μm filter. The Pierce® BCA Protein Assay Kit was purchased at Thermo Scientific.

2.2. Insect cell line and cultures

S. frugiperda 21 cells (Sf21, Invitrogen) were cultured as adherent monolayers in 25 cm^2 flasks at 28 °C in BacVector® Insect Cell Medium (Novagen), supplemented with or without 10% fetal bovine serum (FBS, Biochrom AG). This cell line originates from immature ovaries of *S. frugiperda* pupae and was established by Vaughn et al. (1977). Suspension cultures of Sf21 (20 ml) were maintained in BacVector® Insect Cell Medium using Erlenmeyer flasks, supplemented with 10% FBS, under continuous shaking at 100 rpm and 28 °C. All insect cell cultures were supplemented with 45 U/ml Penicillin and 0.045 mg/ml Streptomycin in 0.9% NaCl (Sigma–Aldrich USA).

2.3. Insect rearing

N. vitripennis Asym C, kindly provided by Prof. Dr. L.W. Beukeboom (Evolutionary Genetics, Centre for Ecological and Evolutionary Studies in The Netherlands), were reared on pupae of the flesh fly *Sarcophaga crassipalpis* under a light–dark cycle (LD 15:9 h) at 25 °C (Van den Assem and Jachmann, 1999). Flesh flies (*S. crassipalpis*) were provided by Dr. Hahn, University of Florida, and cultured in the laboratory as described by Denlinger et al. (1972).

2.4. Venom collection and protein estimation

Crude venom from *N. vitripennis* was collected according to Formesyn et al., (2011). Briefly, *N. vitripennis* females (age: 2–3 days) were allowed to feed on a sugar solution (10%) for 24 h at 25 °C under a long-day cycle (LD 15:9 h). Subsequently venom reservoirs were isolated by dissection at ISB and centrifuged to liberate the venom from the reservoirs. All venom samples were stored in −80 °C until further use. Before use, venom samples were pooled and the protein concentration of crude venom samples was estimated with the BCA assay using a BSA standard (Pierce).

2.5. Venom treatments

To test the protease families, venom was partially inactivated by pretreatment with protease inhibitors. Venom samples were independently treated with Pefabloc (15 mM), EGTA (20 mM) and Complete protease inhibitor mix (5× conc.) and incubated for 2 h at 28 °C. Furthermore, venom was treated to denaturate and inactivate venom proteins by heating the samples for 1 h at 100 °C, while other venom samples were depleted of larger proteins by filtering using a 10.000 MWCO filter (Sartorius Stedim Biotech). Subsequently, the filtered venom samples were examined on SDS–PAGE gels (15%) and stained with Coomassie Brilliant Blue R-250 to confirm depletion.

2.6. Viability assay with MTT

Effect of venom on the viability of Sf21 cells was determined by MTT bioassays. In this assay yellow tetrazolium bromide (MTT) is used as a substrate and is reduced into purple formazan by mitochondrial succinate-dehydrogenase in viable cells only. Adherent Sf21 cells (4×10^5 cells/ml) were seeded into 96-well plates (100 μl/well). After 24 h, spent medium was replaced by fresh medium and cells were incubated for another 24 h. Subsequently, cells were treated with venom in serial dilutions (0.31, 0.62, 1.25, 2.5, 5, 10 and 25 μg/ml[1]) for 6, 12, 24, 36 and 48 h in order to determine the inhibitory concentration inducing 50% loss of cell viability (IC_{50}). Furthermore, adherent Sf21 cells were exposed to venom (10 μg/ml) that was pretreated with protease inhibitors (see Section 2.5). Additionally venom proteins (25 μg/ml) were inactivated by heating or depleted by filtration. All adherent cultures were incubated for 24 h at 28 °C. In order to test whether protease inhibitors were acting directly on the venom proteins and not the Sf21 cells and the influence of FBS, a supplementary MTT assay was performed using adherent cells with or without 10% FBS. Here, cells were immediately treated with venom and protease inhibitors without any preincubation period. Alternatively, suspension Sf21 cells (5×10^5 to 1×10^6 cells/ml) were seeded in 125 ml Erlenmeyer flasks (Corning) containing 20 ml cells and cultured for 24 h at 28 °C and continuous shaking at 100 rpm. Next, the Sf21 cell cultures were induced with venom (2.5 μg/ml), venom + protease inhibitor or ISB. In all experiments, ISB treated cells were included as the control group. After 24 h, samples were taken and diluted (1/5) with medium in 96-well plates. Subsequently, 20 μl of Thiazolyl Blue Tetrazolium Bromide (MTT) solution (5 mg/ml) was added to the wells and cells were incubated for 5 h. To solubilize the precipitate, 80 μl SDS/HCl solution was added to each well and incubated over night at room tem-

[1] A concentration of 25 mg/ml venom was only used for the 24 h measuring point.