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Identification of a small pacifastin protease inhibitor from *Nasonia vitripennis* venom that inhibits humoral immunity of host (*Musca domestica*)

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ABSTRACT

Nasonia vitripennis is an important natural enemy of many flies. Pacifastin protease inhibitors (PPIs) play an important role in development and innate immunity of insects. In this study, cDNA sequences of a small pacifastin protease inhibitor in *N. vitripennis* (*NvSPPI*) was characterized and its open reading frame (ORF) contains 243bp. Real-time quantitative PCR (RT-qPCR) results revealed that *NvSPPI* mRNA were detected specifically in the venom apparatus, while they were expressed at low levels in other tissues tested. In the venom apparatus, *NvSPPI* transcript was highly expressed on the fourth day post eclosion and then declined gradually. The *NvSPPI* gene was recombinantly expressed utilizing a pGEX-4T-2 vector, and the recombinant products fused with glutathione S-transferase were purified. Inhibition of recombinant GST-NvSPPI to three serine protease inhibitors (trypsin, chymotrypsin, and protease K) were tested and results showed that recombinant NvSPPI could inhibit the activity of trypsin. Meanwhile, we evaluated the influence of the recombinant GST-NvSPPI on the phenoloxidase (PO) activity and prophenoloxidase (PPO) activation of hemolymph from a host pupa, *Musca domestica*. Results showed PPO activation in host hemolymph was inhibited by recombinant NvSPPI; however, there was no significant inhibition on the PO activity. Our results suggested that NvSPPI could inhibit PPO activation in host hemolymph and trypsin activity in vitro.

1. Introduction

The pacifastin protease inhibitors (PPIs), characterized by multiple pacifastin light chain domains (PLDs), comprise a large family of protease inhibitors. The current knowledge on the function of PPIs is mainly on their inhibition of proteolytic enzymes, especially serine protease (Breugelmans et al., 2009a,b; Gai et al., 2008; Simonet et al., 2003). The first member of the pacifastin family was found in the crustacean species, *Pacifastacus leniusculus* (Crustacea: Decapoda) (Hergenhahn et al., 1987). Subsequently, PPIs were found in *Eriocheir sinensis* (Crustacea: Decapoda), *Penaeus monodon* (Crustacea: Decapoda) *Schistocerca gregaria* (Orthoptera: Acrididae), *Bombyx mori* (Lepidoptera, Bombycidae) and other arthropods (Breugelmans et al., 2009a,b; Gai et al., 2008; Liu et al., 2015a,b; Sangsuriya et al., 2016; Simonet et al., 2002, 2004). All PLDs of PPIs are characterized by a conserved pattern of six cysteine residues (Cys1 – Xaa9-12 – Cys2 – Asn –Xaa – Cys3 – Xaa – Cys4 – Xaa2-3 – Gly – Xaa3-6 – Cys5 – Thr –Xaa3 – Cys6) (Breugelmans et al., 2009a,b). The 3-D structure analysis shows that these six residues form three disulfide bridges through Cys1–Cys4, Cys2–Cys6 and Cys3–Cys5 models, which give PPIs a typical fold and remarkable stability (Breugelmans et al., 2009a,b; Gáspári et al., 2002; Kellenberger et al., 2003; Mer et al., 1996; Roussel et al., 2001).

Although many PPIs were discovered in arthropods, the functional analyses were carried out mainly on the crustacean species and the locust species. In crayfish, the transcript levels of PPI in hemocytes could be up-regulated through immune challenge. RNA interference (RNAi) of crayfish PPI could not only induce a higher phenoloxidase activity after immune challenge but give rise to the enhancement of nodules and phagocytosis. These results indicated that PPIs in the crayfish were involved in both humoral and cellular

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immune responses (Hergenhahn et al., 1987; Liu et al., 2007). In swimming crab, *Portunus trituberculatus* (Crustacea: Decapoda), PPIs were involved in antibacterial defense and prophenoloxidase cascade (Sangsuriya et al., 2016; Sivakamavalli et al., 2016). In *S. gregaria*, the transcript levels of PPIs were more abundant in the fat body of immune challenged individuals, which suggested PPIs In *S. gregaria* might participate in the immune response (Franssens et al., 2008). In *B. mori*, the PPI could inhibit a fungal trypsin-like cuticle degrading enzyme, suggesting its protective function of defensing against entomopathogenic fungi (Breugelmans et al., 2009a,b). In other insects, no direct evidence is available on the possible functions of PPIs.

Nasonia vitripennis (Hymenoptera: Pteromalidae) is a model parasitic wasps (Shuker et al., 2003; Werren et al., 2010). Parasitic wasps are natural enemy insects that play an important role in biological control. They lay eggs into hosts or on the surface of hosts along with maternal and embryonic factors such as venom, polydnavirus (PDV), virus-like particles (VLP), ovarian proteins, teratocytes, and proteins secreted from larvae to interfere with the host immune responses for successful parasitization (Dong et al., 2007, 2009; Zhu et al., 2008). Unlike ichneumonid and braconid parasitoids, *N. vitripennis* only injects venom into its host (flies) after oviposition. So it must interfere with the host immune response through venom proteins. Using proteomics methods, De Graaf et al. (2010) previously identified 79 venom proteins from *N. vitripennis*, one of which is small serine protease inhibitor-like venom protein (NCBI accession number NP_001155083). Our group also determined the transcriptome and proteome of venom gland and other residual tissues in *N. vitripennis* by RNA-seq (RNA sequencing) and LC-MS/MS (liquid chromatography–tandem mass spectrometry) methods (Qian, 2013), and we also detected a small protease inhibitor protein with a pacifastin domain in venom (NvSPPI), as described by de Graaf et al.

The first venom PPI, cysteine-rich venom protein 4 (CVP4), was found in the venom of an endoparasitoid wasp (*Pimpla hypochondriaca*), that is known to influence the immune system of the host organism (Parkinson et al., 2004). But no direct evidence is available on the possible immune functions of the venom PPIs. Here, we molecularly characterized a small pacifastin protease inhibitor in *N. vitripennis* venom (NvSPPI) and determined its tissue and developmental expression patterns. We also tested the role of NvSPPI in inhibition of host (*Musca domestica*, Diptera: Muscidae) immune response, such as the inhibition of recombinant NvSPPI on three serine protease inhibitors' in vitro and PO activity and PPO activation of host hemocytes. These results will provide further insight into the role of PPIs in insects' immune responses and offer a new ideal for the biological control of insect pests.

2. Materials and methods

2.1. Insect rearing

Cultures of *Musca domestica* and *N. vitripennis* were collected from the experimental field of Zhejiang University, Hangzhou, China. In brief, the host larvae were fed on an artificial diet composed of 15% milk powder, 35% wheat bran, and 50% water, in 500 mL glass canning jars (at 25 ± 1 °C, light:dark = 10 h:14 h, relative humidity (R. H.) = 75%) until eclosion. *M. domestica* adults were maintained on a mixture of sugar and milk powder (10:1) and water, within a stainless steel-mesh cage (55 cm × 55 cm × 55 cm) under the same conditions just described. Freshly pupated hosts were exposed to mated female wasps (pupae:wasps = 1:10) in a 500 mL glass canning jar for 24 h. The parasitized pupae were maintained under the conditions just described. After emerging, the female wasps were collected and held in glass containers (also under the conditions just described) and fed ad lib on 20% (v/v) honey solution to lengthen the life span for 3–4 days until dissection of the venom reservoir and gland.

2.2. Sample preparation

Female *N. vitripennis* from 0 to 7 days post eclosion were collected and paralyzed for 5 min at −70 °C. The head, thorax, gut, ovary, remaining abdomen carcass (the rest of the abdomen after dissection), and venom apparatus (containing venom reservoir and gland) were then collected on ice under a stereoscope (Lecia, Frankfurt, Germany). Samples were stored at −70 °C.

2.3. RNA extraction and cDNA cloning

The collected tissues were homogenized in liquid nitrogen, and total RNA were extracted using the TRizol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. RNA integrity was confirmed by ethidium bromide gel staining and RNA quantity was determined spectrophotometrically at $A_{260}/_{280}$. Single-stranded cDNAs were synthesized by using PrimeScript™ One Step RT-PCR Kit (Takara, Dalian, China). Oligonucleotide primers (Table 1) were designed based on cDNA sequences of *N. vitripennis* SPPIs (GenBank accession numbers is NM_001161611). PCR conditions were as follows: an initial delay at 95 °C for 5 min; 34 cycles of denaturation at 95 °C for 30 min; annealing at 55 °C for 30s; and extending at 72 °C for 1 min. PCR products were fractionated in a 1% agarose gel by electrophoresis and purified with the DNA gel extraction kit (Aidlab, Beijing, China), and then cloned into the pGEM®-T easy cloning vector (Promega, Madison, Wisconsin, USA). Positive clones were selected by PCR and confirmed by sequencing at Invitrogen, Shanghai, China.

2.4. Sequence analysis

The cDNAs and deduced amino acid sequences were analyzed by DNAStar software (version 5.02, DNAStar, Madison, Wisconsin, USA) and online Blast. The signal peptides were analyzed by Signal P. Multiple sequence alignments were carried out with DNAman software (Lynnon Biosoft, Quebec, QC, Canada) and Clustal W2. The phylogenetic tree was constructed by using MEGA 5.1 (Tokyo Metropolitan University, Tokyo, Japan) with the neighbor-joining (NJ) method. The predicted tertiary structure of the protein was predicted by phyre2 online (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and Raswin software.

2.5. Protein expression, purification, and antibody preparation

The primers (Table 1) containing *Bam*H I and *Xho* I restriction enzyme sites were designed to amplify the ORF cDNA sequences of NvSPPI by PCR. PCR products and the pGEX-4T-2 vector were ligated after they were double digested by *Bam*H I and *Xho* I (Takara, Dalian, China). The recombinant plasmids were confirmed by sequencing, transformed into *Escherichia coli* BL21 (DE3) cells (AxyGen, Shanghai, China), and induced by 1 mM isopropyl thiogalactoside (IPTG) for protein expression. The recombinant proteins were analyzed by 12% SDS-PAGE and then purified using GST·Bind™ Resin Kit (Novagen, Hilden, Germany) according to the protocols. Protein concentrations were determined by using the BCA Protein Assay Kit (Novagen, Hilden, Germany).

2.6. Real-time quantitative PCR (RT-qPCR)

RT-qPCR was used to determine the transcription profiles of NvSPPI in different tissues and ages of *N. vitripennis* females. The