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Two c-type lysozymes boost the innate immune system of the invasive ladybird *Harmonia axyridis*

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ABSTRACT

The invasive ladybird beetle *Harmonia axyridis* has a two-layered immune system, featuring the constitutive production of the low-molecular-mass antimicrobial compound harmonine and the inducible production of a broad range of antimicrobial peptides (AMPs). Here we show that the immune system also features two c-type lysozymes, the acidic c-lys3 (p*I* = 5.46) and the basic c-lys4 (p*I* = 8.18). The injection of bacteria into *H. axyridis* boosted *c-lys4* gene expression 8-fold in the gut, whereas the *c-lys3* gene was expressed at comparable levels in both naïve and challenged beetles. Both c-lys3 and c-lys4 were expressed in *Pichia pastoris* and the bacteriolytic activity of the recombinant proteins was found to be calcium-dependent with pH maxima of 6.0 and 6.5, respectively. In a *Bacillus subtilis* growth inhibition assay, the antimicrobial activity of harmonine and two highly-inducible *H. axyridis* AMPs (coleoptericins) was potentiated in the presence of c-lys4 but not c-lys3, resulting in 4-fold (harmonine) and up to 16-fold (AMP) lower minimum inhibitory concentrations. Our results suggest that two structurally and functionally distinct lysozymes contribute to innate immune responses of *H. axyridis* and augment the harmonine and AMP components of the immune response.

1. Introduction

The harlequin ladybird beetle *Harmonia axyridis* (also known as the multi-colored Asian ladybird) has emerged as a model species in invasion biology (Roy and Wajnberg, 2008). It is indigenous in central and eastern Asia but has been released in Europe and Northern America as a biological control agent against aphids and other scale insects (Koch, 2003). Over the last few decades, *H. axyridis* has spread beyond the introduced areas and can now be found even in South Africa and South America. Population genetics approaches have recently been used to reconstruct the invasion routes as well as the origin of the invasive populations (Lombaert et al., 2011). A prominent feature of *H. axyridis* is its ability to outcompete native ladybird species successfully in newly colonized habitats.

A number of adaptations have recently been proposed to enhance the invasive success of this species, including a superior immune system which provides greater resistance against fungal parasites and microsporidia compared with native ladybird beetles such as *Adalia bipunctata* and *Coccinella septempunctata* (Roy et al., 2008; Vilcinskas et al., 2014). Indeed, recent studies suggest that *H. axyridis* has a two-layered innate immune system featuring the constitutively synthesized low-molecular-mass antimicrobial compound harmonine [(17*R*,9*Z*)-1,17-diaminooctadec-9-ene] and a broad spectrum of inducible antimicrobial peptides (AMPs) (Röhrich et al., 2012; Schmidtberg et al., 2013).

Following an experimental immune challenge involving the injection of bacteria, the harmonine concentration in the hemolymph declines but this is compensated by the induced synthesis of AMPs. This switch between constitutive and inducible defense mechanisms may reflect a trade-off resulting from fitness-related costs associated with the simultaneous synthesis of harmonine and AMPs (Schmidtberg et al., 2013; Vilcinskas et al., 2013a). Sequencing the *H. axyridis* transcriptome on the Roche 454 FLX platform resulted in the identification of more than 50 AMPs, the highest number thus far reported in a multicellular organism. The diverse spectrum of AMPs includes genes encoding for 10 attacins, 15 coleoptericins, four coleoptericin-like peptides, 19 defensins and four thaumatins (Vilcinskas et al., 2013a). In addition, genes encoding 10 different lysozymes were identified, four representing the chicken/conventional (c-type) subfamily and six representing the invertebrate (i-type) subfamily.

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Lysozymes are an evolutionarily-conserved group of enzymes that mediate innate immunity (Wiesner and Vilcinskas, 2010). Their expansion by gene duplication and sequence divergence has also translated into functional diversity (Irwin, 2014; Schulenburg and Boehnisch, 2008). The c-type lysozymes are structurally related to the well-characterized lysozyme found in the albumin of hens' eggs and typically possess muramidase activity, enabling them to break down bacterial cell walls (Callewaert and Michiels, 2010). Much less is known about i-type lysozymes, but they typically comprise separate domains with muramidase and isopeptidase activity, although i-type lysozymes in insects are thought to lack the former domain while the presence of isopeptidase activity is unclear (Van Herreweghe and Michiels, 2012). More recent studies suggest that c-type lysozymes also possess biological functions unrelated to muramidase activity, e.g. mammalian α-lactalbumin (which phylogenetically clusters with the c-type lysozymes but lacks the catalytic residues) regulates lactose synthesis (Irwin et al., 2011). Complexes of partially unfolded α-lactalbumin or equine milk lysozyme with oleic acid are cytotoxic and may help to prevent cancer (Ho et al., 2013; Nielsen et al., 2010). Furthermore, the non-bacteriolytic c-type lysozyme-like protein SLLP1 is located in the acrosomal matrix of the sperm head and is thought to play a role in sperm–egg binding (Mandal et al., 2003).

In foregut fermenters including ruminants, leaf-eating colobine monkeys and hoatzins (the only known foregut-fermenting bird) digestive c-type lysozymes with distinct physicochemical characteristics have evolved, enabling the usage of nutrients assimilated by symbiotic bacteria (Kornegay et al., 1994; Stewart et al., 1987). Functionally similar digestive c-type lysozymes in the fruit fly *Drosophila melanogaster* and the housefly *Musca domestica* disrupt bacteria taken up when the insects feed on decomposing material (Cançado et al., 2008; Daffre et al., 1994; Regel et al., 1998). The intestines of blood-sucking triatomine bugs such as *Rhodnius prolixus, Triatoma brasiliensis* and *Triatoma infestans* also contain c-type lysozymes which may digest symbionts that multiply rapidly in the anterior midgut after a blood meal (Araújo et al., 2006; Kollien et al., 2003; Ursic-Bedoya et al., 2008). A c-type lysozyme in the gut of the malaria mosquito *Anopheles gambiae* was found to bind the oocysts formed after infection with *Plasmodium berghei* or *Plasmodium falciparum* and, paradoxically, to promote the development of the parasites (Kajla et al., 2011).

Here we demonstrate that two *H. axyridis* c-type lysozymes possess muramidase activity and thus are likely to play an important role in the innate immune system. One of the two structurally distinct lysozymes is produced constitutively, whereas the other is induced during an immune response, particularly in the gut. The induced lysozyme (but not its constitutive relative) displayed synergy with harmonine and, to an even greater extent, with two coleoptericins representing the most strongly induced AMPs in this species.

2. Materials and methods

2.1. Insect collection, rearing, challenge experiments and sample preparation

H. axyridis adults were collected in and around Giessen, Germany, and were subsequently bred in captivity in cages at 26 °C and 60% humidity with a 16-h photoperiod. Bean plants (*Phaseolus vulgaris*) infested by aphids (*Acyrthosiphon pisum*) were provided as food. The beetles were challenged by injecting 10 µl of a suspension containing 4.75×10^8 cfu of *Micrococcus luteus* (DSM 20030) and *Escherichia coli* D31. The bacterial suspension was diluted with anticoagulant saline (100 mM glucose, 30 mM tripotasium citrate, 29 mM KCl, 27 mM NaCl, 26 mM citric acid, 10 mM Na_2-EDTA, 2 mM $NaHCO_3$, pH 4.6) and injected using a Nanoliter 2000 microinjector with a SYS-Micro4 controller (World Precision Instruments). Non-injected beetles kept under the same conditions were used as controls. After 24 h, hemolymph samples were pooled from 10 insects by cutting the legs at their coxal bases and drawing drops of hemolymph into ice-cold anti-coagulant saline (final dilution ~1:2). Guts were dissected from adult beetles in sterile water for gene expression analysis after 12 h or for muramidase activity assays after 24 h. For preparing the samples for the muramidase activity assays, the guts from 5 beetles were submerged in 50 µl water in a 1.5-ml microcentrifuge tube and homogenized with a conical pestle. All samples were stored at –20 °C.

2.2. RNA preparation and quantitative real-time RT-PCR analysis

Total RNA was isolated from naïve and challenged beetles using the NucleoSpin RNA kit (Macherey-Nagel). RNA was also isolated from dissected guts, which were pooled in groups of five to obtain comparable amounts of tissue. The quality of the RNA was confirmed by agarose gel electrophoresis and the concentration determined by measuring the OD_{260} with an Eon microplate spectrophotometer equipped with a Take3 micro-volume plate (BioTek Instruments, Bad Friedrichshall, Germany). Contamination with genomic DNA was excluded by PCR analysis. Aliquots of 2 µg RNA were reverse transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and oligo$(dT)_{18}$ primers. The resulting cDNA was diluted to a concentration of 400 pg/µl.

Primers were designed using Oligo Explorer v1.1.2 (http://www.softpedia.com/get/Science-CAD/Oligo-Explorer.shtml) to yield amplification products of 50–200 bp with T_m values of ~60 °C. We used the lysozyme gene-specific primers Haxy_c-lys3_for (5′-AAC AGC AAC ACT CCT GGT AAA GG-3′), Haxy_c-lys3_rev (5′-ATC GTC GTT GAT GTC ATC GTT C-3′), Haxy_c-lys4_for (5′-AAT CTG GGA GTG CCA AGA AAC C-3′) and Haxy_c-lys4_rev (5′-TGA ATC TCC AGC ATT CTG TGT TG-3′) as well as primers specific for the ribosomal protein gene *RPS3* (RPS3_for, 5′-GGC TAC CAG AAC CGA ACA GAG-3′ and RPS3_rev, 5′-GTG CTA TGG CGC ATA ATC CT-3′) and the translation elongation factor gene *EF1A* (EF1A_for, 5′-CGT TGG TGT CAA CAA GAT GG-3′ and EF1A_rev 5′-CAG AGA TTG GCA CAA AAG CA-3′) as reference for normalization (Vilcinskas et al., 2013a). The products were verified by dissociation curve analysis. Quantitative real-time PCR was carried out on a StepOnePlus system (Applied Biosystems) using optical 96-well plates in 10-µl reactions comprising 5 µl Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µl cDNA and 300 nM of each primer. The reactions were heated to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s and a melt curve stage with stepwise temperature increases from 60 °C to 95 °C in 0.5 °C increments. Three technical replicates were run and the results were averaged. Raw data and cycle thresholds were analyzed by the ΔΔCT method using fold change of relative expression level in immunized beetles compared with non-injected control beetles.

2.3. Heterologous production of H. axyridis lysozyme proteins in P. pastoris

The *H. axyridis* c-type lysozymes c-lys3 and c-lys4 were produced as recombinant proteins using the PichiaPink Expression System (Invitrogen). Synthetic genes encoding the predicted mature lysozyme sequences codon-adapted for *P. pastoris* were synthesized by Eurofins MWG Operon (Germany). Glutamic acid and alanine residues were added to the N-terminus to ensure efficient cleavage of the vector-encoded α-mating factor signal sequence by Kex2 protease, and a His_6 tag (GHHHHHH) was added to the C-terminus to facilitate downstream purification. The corresponding synthetic genes were flanked by MlyI and KpnI restriction sites and were ligated into the pPinkα-HC vector which had been