Published in final edited form as:

Insect Mol Biol. 2008 June ; 17(3): 217–225. doi:10.1111/j.1365-2583.2008.00797.x.

Cloning and molecular characterization of two invertebrate-type lysozymes from *Anopheles gambiae*

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Abstract

We sequenced and characterized two novel invertebrate-type lysozymes from the mosquito *Anopheles gambiae*. Alignment and phylogenetic analysis of these and a number of related insect proteins identified through bioinformatics strategies showed a high degree of conservation of this protein family throughout the Class Insecta. Expression profiles were examined for the two mosquito genes through semiquantitative and real-time PCR analysis. Lys i-1 transcripts were found in adult females in the fat body and Malpighian tubules, whereas Lys i-2 was detected only in fat bodies. Blood-feeding resulted in significantly increased transcript abundance for both genes in the midguts. Neither gene was upregulated following bacterial challenge.

Keywords

Blood feeding; i-type lysozyme; mosquito

Introduction

Mosquitoes have innate immune mechanisms that can be activated in response to invasion by prokaryotic and eukaryotic organisms. Understanding how these responses are triggered and regulated is an important goal in vector biology. Accordingly, substantial progress has been made in the identification of immune-related genes that are induced or repressed upon exposure to abiotic targets, bacteria or eukaryotic parasites in the malaria vector, *Anopheles gambiae* (Dimopoulos *et al.*, 2000; Oduol *et al.*, 2000; Christophides *et al.*, 2002; Dong *et al.*, 2006) and in assigning functional roles to some of these proteins (Levashina *et al.*, 2002; Osta *et al.*, 2004; Frolet *et al.*, 2006; Paskewitz *et al.*, 2006; Shiao *et al.*, 2006).

Lysozymes constitute a large and diverse family of antibacterial peptidoglycan-hydrolyzing enzymes. Several distinct groups of lysozymes have been identified in invertebrates. One group, the chicken-type (c-type) lysozymes, is well known from the insects (Hultmark, 1996) and has been characterized in *An. gambiae* (Kang *et al.*, 1996; Li *et al.*, 2005, 2006; Li & Paskewitz, 2006) and other mosquitoes (Moreira-Ferreto *et al.*, 1998; Gao & Fallon, 2000; Hernandez *et al.*, 2003; Ursic Bedoya *et al.*, 2005). In addition, the widespread existence of a second lysozyme family, the invertebrate or i-type lysozymes, is now evident (Ito *et al.*, 1999; Nilsen *et al.*, 1999; Zavalova *et al.*, 2000; Bachali *et al.*, 2002). At least 20 i-type lysozymes have been identified by sequence mining or protein purification in the invertebrate phyla Mollusca, Nematoda, Annelida and Arthropoda (Ito *et al.*, 1999; Bachali

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et al., 2002). Insects are the only metazoans known to date which have both c-type and i-type proteins.

Functional roles for some invertebrate i-type lysozymes have been investigated. Muramidase (peptidoglycan-hydrolyzing) activity has been demonstrated for many of the bivalve i-type lysozymes (Nilsen *et al.*, 1999; Bachali *et al.*, 2002) and for the starfish protein (Jollès and Jollès, 1975). Chlamysin, an i-type lysozyme from Icelandic scallops, displays broad antibacterial activity with strong growth inhibiting effects on both Gram-positive and Gram-negative bacteria (Nilsen *et al.*, 1999). These observations argue for a role for i-type lysozymes in antibacterial immunity. However, leech destabilase, an annelid member of this lysozyme family, was named for its ability to break down or destabilize fibrin in blood clots (Zavalova *et al.*, 2000). Taken together, these observations suggest that possible functional roles for mosquito i-type lysozymes might be related to blood-feeding or immunity. In this report, we provide the results of initial characterization of the two i-type lysozyme genes from *An. gambiae* with special attention to expression analyses that might suggest functional roles for these arthropod proteins.

Results

Identification and sequence analysis of i-type lysozyme genes in the *An. gambiae* genome

Blast searches of the *An. gambiae* genome database using the leech destabilase sequence revealed two i-type lysozyme genes. We named these genes i-1 and i-2 to distinguish them from the eight chicken type lysozymes we also found in *An. gambiae* (Li *et al.*, 2005). We sequenced cDNAs for each gene and compared these sequences with the genome. Accession numbers are: Lys i-1, AY659931 and Lys i-2, EF492429. The positions of introns, exons and untranslated regions are shown in Fig. 1. Lys i-1 contains three introns, including a large 4.249 kB intron beginning at nt 202. This intron/exon structure is similar to that of chlamysin (Nilsen and Myrnes, 2001). Lys i-2 contains only one small (70 bp) intron. Lys i-1 is located on the left arm of chromosome 3 at division 41C and Lys i-2 is on the X chromosome in division 3D.

Alignment and features of mature *An. gambiae* i-type lysozymes

We used the SignalP 3.0 (Jannick *et al.*, 2004) program to predict the location of signal peptides and the Protean program (DNASTar, Lasergene, Madison, WI, USA) to estimate the pIs and molecular weights for each of the predicted mature proteins. Both of the i-type lysozyme gene products have a predicted signal peptide (Fig. 2). For Lys i-1, cleavage of the signal peptide occurs between amino acids 21 and 22. The mature Lys i-1 peptide is 146 aa, with a molecular weight of 15.88 kDa and an isoelectric point of 4.71. There are no predicted glycosylation sites in this protein. For Lys i-2, cleavage of the signal peptide is predicted to occur between amino acids 20 and 21. The mature Lys i-2 peptide is 136 aa, with a molecular weight of 14.86 Da and an isoelectric point of 4.83. There is one potential N-linked glycosylation site in Lys i-2 at position 7 of the mature peptide. The Lys i-1 and i-2 peptides are 32.9% identical over the full length and 55% identical over the domain shown in Fig. 3.

Alignment and analysis of *An. gambiae* and other invertebrate i-type lysozyme proteins

We identified additional arthropod i-type lysozymes from translated expressed sequence tag (EST) and genomic databases. These included novel sequences from exopterygote insects in the Orders Homoptera (Aphididae; *Acyrtosiphon pisum*; Pseudococcidae; *Maconellicoccus hirsutus*) and Orthoptera (Locustidae; *Locusta migratoria*) as well as endopterygote insects from the Coleoptera (Curculionidae; *Curculio glandium*), Lepidoptera (Bombycidae, *Bombyx mori*; Sphingidae, *Manduca sexta*) Hymenoptera (Apidae, *Apis mellifera*;

Pteromalidae, *Nasonia giraulti*) and Diptera (Culicidae, *Aedes aegypti*; Psychodidae, *Lutzomyia longipalpis*; Glossinidae; *Glossina morsitans*). Accession numbers for all these sequences are provided in Fig. 3. We also included sequences from annelids (leech destabilase from *Hirudo medicinalis*), crustaceans (*Penaeus setiferus*, *Carcinus maenas*), nematodes (*Caenorhabditis elegans*, *Strongyloides stercoralis*), bivalve molluscs (*Bathymodiolus azoricus*, *Calyptogena* sp., *Chlamys islandica*, *Mytilus edulis*, *Tapes japonicus*) and from *Drosophila melanogaster*, most of which had been previously identified (summarized in Bachali *et al.*, 2002).

The alignment (Fig. 3) reveals highly conserved residues, insect-specific features and variation in the hypothesized active site amino acids (Bachali *et al.*, 2002). Ten cysteines are conserved in all proteins from Annelida, Mollusca, Nematoda and Arthropoda (numbered 1–10 in Fig. 3). Also universally conserved are a glycine, an aspartic acid, a tyrosine and a histidine residue (G26, D37, Y71 and H94, according to the numbering of *An. gambiae* i-1 in Fig. 3; marked with * in Fig. 3). Two residues which were hypothesized to form part of the active site (E11 and D25; our numbering; marked with arrows in Fig. 3; Bachali *et al.*, 2002) are not conserved in this alignment. D25 is found in all non-arthropod sequences in a conserved motif of ten amino acids. The first part of this motif does not align well in the insects and there is a 4–5 aa deletion in this area. E11 is absent from all arthropods, perhaps replaced functionally by E/D at position 9. Arthropods also lack cysteines at positions 38 and 68 that are conserved in all other invertebrates.

The phylogenetic analysis suggested that the current assemblage of i-type sequences from the insects is paraphyletic (data not shown). Examination of results for *D. melanogaster*, *Ae. aegypti* and *An. gambiae* (for which complete genomes are available), suggests that *An. gambiae* i-1 is the orthologue of *Ae. aegypti* EB101347 and that i-2 is the orthologue of *Ae. aegypti* AAEL001485 (Fig. 4). None of the *D. melanogaster* sequences were clear orthologues of the mosquito proteins. *D. melanogaster* NP611163 clustered strongly with the two *Glossina morsitans* proteins (bootstrap proportion 91%). The two lepidopteran proteins also formed a cluster (BP 100%) as did the Hymenoptera (BP 60%) but the homopteran proteins did not. Clustering of bivalve proteins and of arthropod proteins was as previously reported (Bachali *et al.*, 2002).

Developmental and tissue profiles of transcript levels

Semiquantitative RT-PCR was used to examine *An. gambiae* i-type lysozyme transcripts at different developmental stages and in different tissues from adult female mosquitoes. Lys i-1 and i-2 were expressed in all developmental stages. Lys i-1 was easily detectable in all stages while Lys i-2 appeared to be more abundant in adults (Fig. 5A). There were also differences in the expression profiles for individual adult female tissues (Fig. 5B). Notably high levels of Lys i-1 transcripts were observed in ovaries following a bloodmeal and in Malpighian tubules and fat bodies. Lys i-2 transcripts were seen only in the sample prepared from fat bodies. Neither transcript was detected in the salivary glands or the midguts by semiquantitative analysis.

To further examine the effects of blood-feeding on transcript abundance, real-time PCR was used to investigate Lys i-1 and i-2 expression in female midguts at 24 h after feeding. We measured transcript abundance relative to nonblood-fed controls. Samples of cDNA were produced from five independent trials. Two reference genes (actin and RPS7) were used for comparisons. These reference genes were generally stable in the midgut after feeding, with thresholds remaining within 0.5 cycles of the nonblood-fed control for the 24 h samples. Lys i-1 was barely detectable prior to blood-feeding ($C_T = 30$ cycles) but was upregulated at least fourfold in midguts at 24 h postblood-feeding in four of five trials (Fig. 6; $P < 0.01$). Lys i-2 expression in midguts after blood-feeding also showed significant upregulation 24 h

after blood-feeding in all five cDNA samples (Fig. 6; $P < 0.05$). We also tested midguts at 48 and 72 h postblood-feeding but these samples yielded highly variable results (data not shown).

To examine expression in the ovaries, we used cDNA produced during two independent trials from ovaries dissected at 0, 24, 48 and 72 h after feeding. For these samples, neither actin nor RPS7 was stable. Both reference genes exhibited large changes after blood-feeding, with thresholds varying by as much as five cycles from the 0 h control. No consistent patterns were apparent for the target genes in relation to these reference genes.

***An. gambiae* i-type lysozyme immune challenge profiles**

Work in other invertebrates raised the possibility that the mosquito i-type lysozymes might be antibacterial and that transcripts could be immune-responsive. Although immune associated genes do not necessarily demonstrate changes in transcript levels during an immune response, such changes are consistent with a role in immunity. Transcript levels of the *An. gambiae* i-type lysozyme genes were monitored by quantitative real time PCR during responses to wounding and to injection of *Micrococcus luteus*. We tested three samples of cDNA produced from three independent replicates of inoculated mosquitoes and used three reference genes for comparison. RPS7 was more stable than actin or tropomyosin under these experimental conditions. RPS7 varied by no more than 0.5 threshold cycles between uninjected and inoculated samples in all experiments, whereas the threshold for actin ranged between 0 and 1.5 cycles higher in the inoculated samples. Therefore, we used RPS7 to normalize the samples. In comparison with uninoculated controls, Lys i-1 transcripts were unaffected by wounding (Fig. 7A). Mosquitoes that were injected with *M. luteus* also did not show increased transcript levels in two of the three trials, although transcripts were 3.2-fold higher at 24 h post-inoculation in trial 2 (Fig. 7). Overall, these results were not statistically different from the wounding experiments. Lys i-2 was downregulated (0.4–0.8 $\times$) at 24 h post-inoculation in all samples ($P < 0.05$) regardless of whether the challenge was wounding or *M. luteus* injection.

Discussion

Lysozymes are proteins that have been defined by their muramidase activity, meaning the ability to cleave the glycosidic bond between N-acetylmuramic acid and N-acetyl glucosamine (GlcNAc) in the peptidoglycan layer of bacterial cell walls. This action results in the loss of cell wall integrity and the lysis of susceptible bacteria. Many studies have shown bacteriolytic activity against Gram-positive bacteria in insects (Mohrig & Messner, 1968a,b; Ribeiro & Pereira, 1984; Zachary & Hoffmann, 1984; Schneider, 1985; Fujita *et al.*, 2001). Some investigators have purified the active proteins and definitively identified them as c-type lysozymes by protein sequence analysis (eg Rosenthal & Dahlman, 1991; Abraham *et al.*, 1995; Yu *et al.*, 2002), but many have relied on activity assays as the only evidence for assignment as a c-type lysozyme. Herein, we show that a distinct family of proteins, the invertebrate-type (i-type) lysozymes, is represented in both exopterygote and endopterygote insects. I-type lysozymes are similar in size to c-type lysozymes but differ from them in both primary sequence and in charge (acidic/neutral vs. basic for c-type proteins). The peptidoglycan-hydrolyzing activity of the insect i-type lysozymes is yet to be determined but such activity has been shown for other members of this family. The existence of the invertebrate-type lysozymes means that further investigations are required as to the source of Gram-positive bacteriolytic activity in insects.

We investigated two i-type lysozymes from the mosquito, *An. gambiae*, in more detail. Sequence analysis shows that these proteins are missing amino acids that are hypothesized to be critical for muramidase activity in other invertebrates (Bachali *et al.*, 2002). Loss of

the active site requires confirmation but if true, may be related to the fact that insects are the only invertebrates to have both i and c-type lysozymes. Perhaps, the c-type proteins assumed the muramidase function while the i-type proteins diverged to fill other functional roles.

Expression analyses identified a striking difference in the developmental profiles of these two proteins. Lys i-1 is constitutively expressed in all stages, whereas Lys i-2 transcripts are abundant only in adults and late instar larvae. This pattern is similar to that seen for members of the c-type lysozyme family, which consists of eight genes in *An. gambiae* (Li *et al.*, 2005). Several of the c-type lysozyme transcripts are found primarily in larval stages, whereas others are more much more abundant in adult stages. The pattern may reflect differences in the diet or environmental exposure of adult and larval mosquitoes to bacteria.

Expression analyses did not support a role for the i-type lysozymes in the response of adult female mosquitoes to inoculation of the haemocoel with bacteria. Real-time PCR estimates of relative transcript abundance demonstrated a significant increase 24 h after bacterial inoculation in only one of three trials for Lys i-1. This interassay variation may reflect differences specific to the cohorts of mosquitoes used for the three independent experiments. Semiquantitative RT-PCR analysis also failed to identify upregulation in a fourth experiment. Lys i-2 transcripts were consistently downregulated in response to wounding, regardless of whether bacterial were deliberately introduced.

Bacteria also occur within the midgut of adult females. Bacterial populations increase significantly within mosquito midguts after blood-feeding (DeMaio *et al.*, 1996; Pumpuni *et al.*, 1996) and we found that Lys i-1 and Lys i-2 expression in midguts also increased consistently after blood-feeding. In other Diptera (*D. melanogaster*, *Musca domestica*) c-type lysozymes are found in the midgut and are involved in digestion of bacteria ingested after feeding on decaying fruit and plant materials (Lemos *et al.*, 1993; Daffre *et al.*, 1994). Lys i-1 or i-2 might play this type of digestive role or might function in immune defence against the large bacterial populations in the gut.

Alternatively, increases in transcripts of the i-type lysozymes after blood-feeding are suggestive of a functional role in breaking down the blood clot. Other i-type lysozymes have this ability. For example, leech destabilases are endoeptase(epsilon-Glu)-Lys isopeptidases, which cleave isopeptide bonds formed by Factor XIIIa action during clotting (Zavalova *et al.*, 2003). Leech destabilases are clearly members of the i-type lysozyme family (Fig. 3) and isopeptidase activity has been identified for other i-type lysozymes from bivalves (Takeshita *et al.*, 2004). Takeshita *et al.* (2004) determined that the active sites for muramidase and isopeptidase activity were independent of each other in the bivalve protein, a fact which may be relevant because the amino acids said to be needed for muramidase activity are missing in arthropod sequences (Bachali *et al.*, 2002). In mosquitoes, the bloodmeal becomes a firm mass within 24 h and the blood mass consists of erythrocytes bound in a meshwork of fibrin strands, like a typical blood clot (Clements, 1992).

In summary, insect i-type lysozymes may play an unrecognized role in immune defence, may function in digestion of blood or bacteria or may have evolved to serve other roles in these organisms. Further analysis of both isopeptidase and muramidase activity using purified or recombinant proteins is needed. Gene silencing via injection of dsRNA may also provide insights into the functional roles of the i-type lysozymes in mosquito biology.

Experimental procedures

Identification of *An. gambiae* i-type lysozyme gene family

tBlastn searches were performed on the *An. gambiae* genome project (Holt *et al.*, 2002). A two-step screening strategy was employed. First, leech destabilase (GenBank accession no. AAA96144) was used as a probe to search for similar genes. Next, secondary tBlastn searches were run using the candidate genes identified in the first step, but no new genes were identified. Chromosomal positions for the two genes were determined using the search engine provided on the Ensembl website (www.ensembl.org/Anopheles_gambiae).

Phylogenetic analysis

We used the *Anopheles* sequences to identify additional arthropod i-type lysozymes from translated EST and genomic databases. The sequences were aligned using ClustalW, truncated at the amino and carboxy termini to remove unaligned amino acids, and then adjusted by eye to minimize insertion/deletion events (Fig. 3). For phylogenetic analysis, we used the ClustalX neighbour-joining program, with the 'delete positions with gaps' and 'correct for multiple substitutions' settings on. The tree was bootstrapped 1000 times.

Mosquito strains, bacteria

All analyses of transcript levels were performed with the G3 strain of *An. gambiae* and repeated with the L3-5 or 4arr strains (Gorman *et al.*, 1996). The G3 strain was used for sequencing cDNAs. The bacterial stock used for immune challenge was the 2007 strain of *M. luteus* (= *lysodeikticus*). The *M. luteus* strain was a gift from John Lindquist (University of Wisconsin-Madison).

cDNA library screening and sequencing of full length clones

A λ ZAPII cDNA library made from larvae and pupae of the G3 strain of *An. gambiae* (Besansky *et al.*, 1995) was used in conjunction with PCR primers to obtain sequences for Lys i-1 and i-2. The largest PCR amplicons were cloned and cycle sequenced. Clones were sequenced in both directions. Full-length sequences were assembled from overlapping clones and analysed by DNASTAR (Lasergene, Madison, WI, USA).

Immune challenge and controls

For all experiments, mosquitoes were placed into small humidified cages supplied with 10% sucrose and allowed to recover in an incubator at 70–80% relative humidity and 25–26 °C. As controls, adult female mosquitoes were anaesthetized by brief chilling on ice without further treatment. For wounding tests, a fine needle was used to pierce the thorax. For immune activation tests, overnight cultures of *M. luteus* were centrifuged. A fine needle was dipped into the bacterial pellet and used to inoculate the anesthetized mosquitoes by puncturing the thorax.

Semiquantitative RT-PCR

Semiquantitative RT-PCR was carried out to analyse transcripts in whole organisms at various stages and in adult female tissues. Total RNA was isolated using the AquaPure RNA extraction kit (BioRad, Hercules, CA, USA). Genomic DNA was removed by treatment with DNase I (Invitrogen, Carlsbad, CA, USA). The SuperScript Preamplification System for First Strand cDNA Synthesis kit (Invitrogen) was used to make cDNA from 5 μ g total RNA [using the Oligo (dT) primer]. Gene-specific primer sets were as follows: Lys i-1 [forward (F) 5'-CAGTTTGTCTGCGTGTGTATAG-3' and reverse (R) 5'-GGAGAGCCTTACGTGATGTGT-3'] and Lys i-2 (F 5'-GCTGCTGCTGCTGCTATCATT-3' and R 5'-CTTTGGGGCTTTCGTACAGTT-3'). The

S7 ribosomal protein was used to normalize the samples. Expression of the ribosomal protein S7 gene (RPS7; Salazar *et al.*, 1993) was assayed in parallel reactions by using sequence-specific primers: S7 (5'-TGCTGCAAACCTTCGGCTAT-3' and 5'-CGCTATGGTGTTCGGTTCC-3').

Promega (Madison, WI, USA) reagents were used for PCR reactions. Final concentrations of reagents were 1× reaction buffer, 200 µM dNTPs, 0.5 µM each primer, 1 unit Taq polymerase and 2 mM MgCl₂. Cycle conditions were: an initial denaturation at 94 °C for 1 min; then repeated cycles of 92 °C for 30 s, 57 °C for 30 s, and 72 °C for 90 s, followed by a final step of 72 °C for 7 min. For developmental stages and tissues, 30 cycles were used for both lysozymes. For semiquantitative analysis of transcript levels following wounding and immune challenge, the number of cycles required to produce faint but visible bands (to avoid saturation) was determined empirically. All PCR reactions generated a single major DNA fragment of the expected size and fragments amplified from genomic DNA were not detected. All experiments were replicated at least twice.

Real-time PCR

Whole mosquitoes were used for RNA extraction following immune challenge while ovaries and midguts were dissected to analyse the effects of blood-feeding. Total RNA was isolated using the Aqua Pure RNA isolation kit (BioRad) according to the supplied instructions. Total RNA was treated with RQ1 DNase (Promega) to remove contaminating genomic DNA. RNA concentration was measured with micro-spectrophotometry (Nanodrop NT1000, Thermo Fisher Scientific, Waltham, MA, USA). Next, removal of DNA from the RNA samples was confirmed by real-time PCR using housekeeping gene primer sets (no cDNA control). Samples that yielded threshold cycle (C_T) values larger than 33 were deemed acceptable. One hundred nanogrammes of high-quality total RNA was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The resulting cDNA was diluted 1:5 and stored at -20 °C until use.

Primers for internal reference genes (ribosomal protein S7, tropomyosin and actin) and the two target genes (Lys i-1 and i-2) were designed using the Beacon designer software (Premier Biosoft International, Palo Alto, CA, USA; Table 1). The program setting 'avoid template structure' was chosen to limit primer sequences to regions of little secondary template structure. The primers were synthesized by IDT Technologies (Coralville, IA, USA). The performance of these primers (PCR efficiency and standard curves) was tested on five dilutions of cDNA prepared as described above. All standard curves were generated from triplicate reactions of the fivefold dilution series of cDNA. Suitable internal reference gene primer sets were chosen on the basis of (1) primer efficiencies that were close to 100%, and (2) threshold stability before and after experimental treatment. Both reference and target primers exhibited similar efficiencies as determined using a dilution series of cDNA derived from *An. gambiae* total RNA (uninoculated adult female control).

Real-time PCR was carried out using an iCycler machine (BioRad) and analysed using the iQ software package (BioRad). All reactions were performed in triplicate 25 µl volumes using iQ SYBRGreen Supermix (BioRad). A master mix was prepared for each primer set containing SYBRGreen and an appropriate volume of each primer to yield a final primer concentration of 200 nM. The reaction conditions were enzyme activation and well factor determination at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s (denaturation) and 58 °C for 45 s (annealing and elongation); the melt curve protocol began immediately after amplification and consisted of 95 °C for 1 min, followed by 55 °C for 1 min and then 80–10 s steps of 0.5 °C increases at each step. Threshold values for threshold cycle (C_T)

determination were generated automatically by the iCycler software. The absence of primer artefacts was determined from the melt curve profile of the PCR products.

The stability of the reference genes in comparison to each other during the treatments was analysed using the BestKeeper program (Pfaffl *et al.*, 2004; MS-Excel based Q-PCR data analysis program). To demonstrate a statistically significant difference among the different groups, the *P*-value of paired Student's *t*-test was calculated based on the normalized data.

Acknowledgments

We thank Professor Kyle Willis, University of Wisconsin, Department of Plant Pathology, for his help in establishing and interpreting quantitative PCR protocols. We thank Jun Wang for sharing her expertise in this area as well. We also acknowledge the work of Beth Schadd, who reared all mosquitoes used in these experiments. This grant was supported by NIH RO1AI037083 to SMP.

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Figure 1. Genomic organization of two invertebrate (i)-type *Anopheles gambiae* lysozymes. The length (in nt) and relationships of introns (lines) and exons (boxes) are shown in the diagrams for Lys i-1 and Lys i-2.

Figure 2.

Deduced amino acid sequence of *Anopheles gambiae* Lys i-1 and Lys i-2. The predicted signal peptides are shaded. The predicted N-linked glycosylation site in Lys i-2 is marked with an arrow. Conserved residues are marked with asterisks.

Figure 3.

Alignment of selected invertebrate (i)-type lysozymes from insects and other invertebrates. Accession numbers are provided for each sequence as an extension of the species name. Ten conserved cysteines are numbered. Other conserved amino acids are marked with asterisks. Putative active site amino acids are marked with arrows. A cysteine conserved within the insects is marked with a caret (^). Residues that are identical to the column consensus are printed in white and shaded black. Groups of similar, but not identical residues, are printed on grey backgrounds.

Figure 4. Phylogenetic tree of the invertebrate (i)-type lysozyme proteins from *Aedes aegypti*, *Drosophila melanogaster* and *Anopheles gambiae*. The alignment is presented in Fig. 3. An unrooted distance tree (neighbour joining) is presented. Numbers at the nodes represent bootstrap proportions (BP) on 1000 replicates. Only BPs over 75% are shown.

Figure 5.

Expression of invertebrate (i)-type lysozymes in tissues and by stage, measured by semiquantitative real-time PCR. (A) Stage-specific expression. E, embryo; L4, first instar; L9, fourth instar; P, pupae; F0, newly emerged adult females; M0, newly emerged adult males; F4, 4-day-old adult females; M4, 4-day-old adult males. (B) Tissue expression. Hd, head; Th, thorax; Ab, abdomen; FB, fat body; Ov, ovary; OvB, ovary 24 h after blood-feeding; Mg, midgut; Mt, Malpighian tubule; Sg, salivary gland.

Figure 6.

Analysis of expression of invertebrate (i)-type lysozymes from *Anopheles gambiae* in midguts following blood-feeding. Aliquots of five independent cDNA samples were used as templates for real-time PCR reactions. Three replicates of each reaction were performed for each cDNA sample. Expression of the i-type lysozymes are reported at 24 h postblood-feeding relative to time 0 (preblood-feeding). Results are reported using the ribosomal protein S7 as the reference gene.

Figure 7.

Analysis of expression of invertebrate (i)-type lysozymes from *Anopheles gambiae* following immune challenge and wounding. (A) Transcript abundance at 24 h following wounding is reported relative to uninjected controls. (B) Transcript abundance at 24 h following bacterial inoculation with *Micrococcus luteus* is reported relative to uninjected controls. For both experiments (wounding and bacterial inoculations), aliquots of three independent cDNA samples (prepared from entire carcasses) at 0 and 24 h after treatment were used as templates for real-time PCR reactions. Three replicates of each reaction were performed for each cDNA sample. Results are reported using the ribosomal protein S7 as the reference gene.

Table 1

Sequences of primers used for real-time PCR analysis

Primer name	Forward primer (5'–3')	Reverse primer (5'–3')
Actin	CAGTCCAAGCGTGGTATC	GTTAGCCTTCGGGTTTCAG
S7	CCTATGGTGTTTCGGTTCC	GATCGCCTTCTTGTTGTG
Tropomyosin	GAACGGATTCAGCAGGTG	TTCTCAGCATCTTCAAGCC
Lys i-1	TGTATAGAGCAAGCCAAAATG	GACGAAGCCTCACAGATG
Lys i-2	CGAGGGTAAGCGTGATTC	ACAGAACAGGTTTCCAATTATAC