Chapter 4
Role of Lysozymes of *Anopheles Mosquitoes* in *Plasmodium* Development

Clelia Oliva*, Luca Facchinelli*, Nicoletta Basilico, and Roberta Spaccapelo

1 Introduction

The hydrolytic enzyme lysozymes are widely found in all living organisms. They are important participants of the antibacterial defense but may also show a digestive function (Callewaert and Michiels 2010). Three major distinct lysozyme types have been identified (c-type, g-type, and i-type) with a common ability to hydrolyze the glycosidic bond between *N*-acetylmuramic acid and *N*-acetyl glucosamine in the peptidoglycan layer of bacterial cell walls.

The antibacterial activity of lysozymes has been demonstrated in most organisms. Bacterial challenge or wounding induces a higher expression of lysozyme genes. The muramidase activity results in the loss of cell wall integrity and the lysis of susceptible bacteria or inhibition of cell growth (Nakimbugave et al. 2006). However, the existence of nonenzymatic bactericidal pathways has been put forward. This may act through the activation of bacterial autolysins or induction of membrane leakage following direct interaction with the cell membrane (During et al. 1996; Ibrahim et al. 2001; Masschalck and Michiels 2003). Lysozyme strongly affects numerous Gram-positive bacteria species and to a lesser extent Gram-negative ones (in insects: Abraham et al. 1995; Yu et al. 2002; Skerrett 2004; Mai and Hu 2009) in which the peptidoglycan layer is shielded by the outer layer of lipopolysaccharide and protein (Masschalck and Michiels 2003). Besides their direct bactericidal activity, lysozymes may be important regulators of the overall

*Author contributed equally with all other contributors.

C. Oliva (✉) • L. Facchinelli • R. Spaccapelo
Dipartimento di Medicina Sperimentale, Università degli Studi di Perugia, Perugia, Italy
e-mail: cleliaoliva@gmail.com

N. Basilico
Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche, Università degli Studi di Milano, Milan, Italy

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M. Prato (ed.), *Human and Mosquito Lysozymes*,
DOI 10.1007/978-3-319-09432-8_4

response to bacteria. The interaction of peptidoglycan recognition proteins with the lysozyme-digested peptidoglycans activates the prophenoloxidase cascade leading to melanization (Christensen et al. 2005; Park et al. 2007; Kim et al. 2008).

Insects are the only invertebrates to possess both c- and i-type lysozymes, which suggests that each type might have evolved to fill diverse functional roles (Paskewitz et al. 2008). C-type lysozymes (Hultmark 1996) are the most studied group and have a 35–40 % sequence homology and share a common three-dimensional fold to alpha-lactalbumin. The i-types differ from the c-types in their primary sequence and in electric charge (acidic/neutral vs. basic, respectively) and are shown to have antibacterial activity (Ito et al. 1999; Nilsen et al. 1999; Zavalova et al. 2000; Bachali et al. 2002), although they miss potentially critical amino acids for the muramidase activity (Bachali et al. 2002).

In the mosquito *Anopheles gambiae*, lysozymes are present in different tissues and developmental stage expression profiles (Li et al. 2005; Paskewitz et al. 2008), which probably enhance the response to the bacteria population corresponding to different diet and/or environments. Recently, lysozymes have been shown to play an important role in the development of *Plasmodium* parasite in *Anopheles* species, protecting the oocytes from melanization. Their potential importance for the development of malaria control tools is discussed.

2 Lysozymes in *Anopheles* Mosquito

The first isolation and characterization of a gene encoding a lysozyme (now known as *LysC1*) in *An. gambiae* was reported in 1996 by Kang et al. They showed a strong expression of the transcript in sugar-fed females and low levels of proteins after blood feeding (Kang et al. 1996). It is suggested that lysozyme could be involved in the digestion process of the bacteria and fungi present in the nectar, similarly to *Lys P* in Drosophila (Kylsten et al. 1992). In the higher flies, *Musca domestica* and *Drosophila melanogaster*, lysozymes occurring in the gut exhibit isoelectric points that are adaptive for a digestive function under acidic conditions (Lemos et al. 1993; Daffre et al. 1994). The presence of these enzymes in the salivary glands also suggests a role in the prevention of bacterial infection of the mouthparts (Rossignol and Lueders 1986; Moreira-Ferro et al. 1999).

In *An. gambiae*, eight different lysozymes belonging to the c-type have been discovered (Kajla et al. 2010). Their functional roles are still not completely understood, but they probably possess diverse function and target diverse tissues (Li et al. 2005). *LysC1* and *LysC2* are the most documented proteins and are involved in the innate immunity. The gene expression profiles and the analyses of the predicted proteins suggest that the remaining six genes might be involved in novel functions in immunity or other biological processes. *LysC4*, *LysC5* and *LysC7*, and several of the domains of *LysC*6 are lacking critical amino acids for muramidase activity. However, these proteins might still possess an antibacterial activity, which could derive from their ability to bind to *N*-acetyl glucosamine or other oligosaccharides (Li et al. 2005). *LysC4* and *LysC7* transcripts did not increase following bacterial

infection or wounding, which makes them unlikely to be involved in immunity. The function of *LysC3* and *LysC8* has not yet been unrevealed, but the presence of a potential calcium binding site suggests that they could be involved in the digestion of bacteria (Li et al. 2005).

I-type lysozymes have been little studied yet and their functional roles in mosquito biology are still unclear. Two genes belonging to the i-type have been discovered in *Anopheles* (Paskewitz et al. 2008). *LysI1* and *LysI2* are expressed in all developmental stages of *An. gambiae* females but not in the salivary glands and in the midgut of non-blood-fed females (Paskewitz et al. 2008). Blood feeding strongly increases the transcript levels of *LysI1* in the ovaries, Malpighian tubules, and fat bodies. *LysI1* and *LysI2* are both upregulated in the mosquito midgut after blood feeding. The expression of i-type lysozymes in the gut could suggest a digestive rather than an immune function. The involvement of these proteins in the immunity has not yet been demonstrated, and wounding or injection of *Micrococcus luteus* did not affect the transcription of *LysI1* in *An. gambiae* but consistently downregulates *LysI2* transcripts (Paskewitz et al. 2008). These enzymes could be involved in the digestion of bacteria present in the blood or in the breaking down of the blood clots (Zavalova et al. 2000; Paskewitz et al. 2008).

3 Lysozyme C1 and *Anopheles* Immune System

Kajla et al. (2010) stated that lysozyme C1 is constitutively expressed in the midgut and in the salivary glands of *An. gambiae* but the same researchers failed to detect it in the midgut in a later study (Kajla et al. 2011). Bacterial challenge upregulates the expression of *LysC1* gene at least up to 72 h posttreatment, induces a strong increase of the protein in the hemolymph and a higher muramidase activity from 15 to 120 h posttreatment (Li et al. 2005; Dong et al. 2006, 2009; Kajla et al. 2010). However LysC1 directly kills only a few bacteria species but seems to play an important indirect role in the immune response. Indeed, the knocking down of the gene increased the mosquito mortality after infection with the Gram-negative *E. coli* although the bacteria were not killed in vitro by the enzyme (Kajla et al. 2010). Kajla et al. (2010) showed that the knocking down of *LysC1* does not affect the transcription of other genes involved in the immune response. It is therefore hypothesized that the production of small peptidoglycan fragments by *LysC*1 might upregulate the signaling cascades that result in the production of antimicrobial peptides.

4 Interaction of Lysozyme and *Plasmodium*

The sporogonic development of malaria parasites depends on a complex interaction with their mosquito hosts. In *An. gambiae*, LysC1 binds to and can protect an abiotic target (CM-Sephadex beads) from melanization (Li and Paskewitz 2006).

Kajla et al. (2011) showed through immunohistochemical analyses and gene silencing that physical interaction of LysC1 with the parasite surface following the critical period of midgut invasion was associated with parasite persistence. The injection of dsRNA into the thorax of female *An. gambiae* G3 mosquitoes significantly reduced the expression of *LysC*1. Four days after dsRNA injection, mosquitoes were allowed to feed on mice infected with GFP-expressing *Plasmodium berghei.* Three days post-infection the number of oocysts per midgut were scored showing that knockdown of *LysC*1 significantly reduced prevalence and intensity of *P. berghei* infections (Kajla et al. 2011). Similar results were obtained in a different study where the knockdown of *AdLys C1* gene in *Anopheles dirus* showed the agonistic role of *LysC1* in the response of mosquitoes during *P. berghei* infection (Kajla et al. 2010).

Knockdown of *LysC*1 in *An. gambiae* did not result in changes in numbers of viable *P. berghei* parasites until 3 days post-infection (Kajla et al. 2011). Similar numbers of fluorescing parasites were seen in control and knockdown mosquitoes at 24 h post-infection (Kajla et al. 2011). This suggested that formation of ookinetes and invasion of the midgut were similar in treated and control mosquitoes and that the block occurred after oocysts formation (Kajla et al. 2011). The transition to oocysts occurs once the ookinetes move between the epithelial cells and the midgut basal lamina (BL). The rapidly expanding oocysts stretch the overlying layer of the BL at the hemocelic surface while a new BL is generated between the oocysts and the epithelial cells (Meis et al. 1989). At the same time, mosquito-derived collagen and laminin are incorporated into oocyst capsules (Dessens et al. 2003; Osta et al. 2004; Adini and Warburg 1999; Castillo et al. 2006). Knockdown of laminin mRNA led to a substantial reduction in the number of successfully developed oocysts (Arrighi et al. 2005). Laminin has been shown to bind to at least five *P. berghei* proteins (P25, P28, SOAP, circumsporozoite, and TRAP related) in yeast two hybrid assays (Meis et al. 1989; Dessens et al. 2003; Vlachou et al. 2001). Nacer et al. (2008) showed that mosquito-produced laminin indeed becomes part of the parasite capsule during its passage through the gut. The acquisition of the basal lamina proteins is likely to help protect the developing oocysts from the mosquito immune system and, therefore, may facilitate their prolonged extracellular development in the mosquito body cavity (Castillo et al. 2006). Vertebrate lysozymes bind to glycosaminoglycans in extracellular matrices (Mahairaki et al. 2005) and insect basal laminae are negatively charged (Moss et al. 1997), which could promote interaction with the basic LysC1. Lysozymes have also been shown to bind and prevent the proteolytic degradation of the elastin component of elastic fibers in the basal lamina, indicating that lysozyme interaction can protect elastic fibers at the sites of injury (Park et al. 1996). Arrighi et al. (2005) suggested that the production of new basal lamina around the midgut may be a normal process following blood feeding, a process that has been co-opted by the parasite. Kajla et al. (2011) hypothesize that LysC1 might associate with components of the midgut BL and become incorporated during formation of the BL-related capsule around the parasite. Immunohistochemistry data on the interaction of LysC1 and malaria oocysts support a direct LysC1 association with the parasite (Kajla et al. 2011). Since the detection of LysC1 in Western

blots failed and after extended incubation periods of midgut extracts muramidase activity could not be detected, Kajla et al. (2011) speculated that the protein may not originate from the midgut cells. Ahmed et al. (2002) also failed to detect muramidase activity in midgut extracts following blood feeding. By contrast Kajla et al. (2011) detected LysC1 in mosquito hemolymph through Western blotting (Li and Paskewitz 2006; Kajla et al. 2010) and Ahmed et al. (2002) determined that muramidase activity in the hemolymph increased following blood feeding. Castillo et al. (2006) also described the occurrence of LysC1 in hemocytes. Together, these observations suggest that LysC1 associated with parasites is derived from the hemolymph. In studies of the transport of molecules from the hemolymph across the basal lamina to the intercellular spaces of the midgut epithelium, other researchers have shown that cytochrome-*c* can make this passage (Reddy and Locke 1990). Cytochrome-*c* is nearly identical to LysC1 in both size and charge. Thus, it seems likely that LysC1 can also move in this direction.

Rao et al. (2010) suggested that the trade-off between lysozyme activity and phenoloxydase activity (PO) (Cotter et al. 2008; Povey et al. 2009) might result in the lysozyme inhibiting the melanization. They showed that direct protein interaction between lysozyme and pro-PO inhibited its cleavage and therefore the activation pathway; however, lysozyme had no effect on active PO. *Plasmodium* apparently evolved to avoid attacks from *Anopheles* immune system taking advantage of lysozyme interaction.

Kajla et al. (2011) considered the possibility that the regulation of parasite development might offer new target for malaria control. Although this research field may open the possibility to develop malaria control tools, there is not a neat picture of *Plasmodium–Anopheles* interactions yet. The role of lysozymes in the regulation of oocysts development and the mechanism of action are still unclear.

References

Abraham EG, Nagaraju J, Salunke D et al (1995) Purification and partial characterization of an induced antibacterial protein in the silkworm, Bombyx mori. J Invertebr Pathol 65:17–24

Adini A, Warburg A (1999) Interaction of *Plasmodium gallinaceum* ookinetes and oocysts with extracellular matrix proteins. Parasitology 119:331–336

Ahmed AM, Maingon BR, Hurd H (2002) The cost of mounting of an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. Oikos 97:371–377

Arrighi RBG, Lycett G, Mahairaki V et al (2005) Laminin and the malaria parasite's journey through the mosquito midgut. J Exp Biol 208:2497–2502

Bachali S, Jager M, Hassanin A et al (2002) Phylogenetic analysis of invertebrate lysozymes and the evolution of lysozyme function. J Mol Evol 54:652–664

Callewaert L, Michiels CW (2010) Lysozymes in the animal kingdom. J Biosci 35:127–160

Castillo JC, Robertson AE, Strand MR (2006) Characterization of hemocytes from the mosquito *Anopheles gambiae* and *Aedes aegypti*. Insect Biochem Mol Biol 36:891–903

Christensen BM, Li J, Chen CC, Nappi AJ (2005) Melanization immune responses in mosquito vectors. Trends Parasitol 21:192–199

Cotter SC, Myatt JP, Benskin CM, Wilson K (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. J Evol Biol 21:1744–1754

Daffre S, Kylsten P, Samakovlis C, Hultmark D (1994) The lysozyme locus in *Drosophila melanogaster*: an expanded gene family adapted for expression in the digestive tract. Mol Gen Genet 242:152–162

Dessens JT, Siden-Kiamos I, Mendoza J et al (2003) SOAP, a novel malaria ookinetes protein involved in mosquito midgut invasion and oocyst development. Mol Microbiol 49:319–329

Dong Y, Aguilar R, Xi Z et al (2006) *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. PLoS Pathog 2:e52

Dong Y, Manfredini F, Dimopoulos G (2009) Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog 5:e1000423

During K, Porsh P, Mahn A (1996) The non-enzymatic microbicidal activity of lysozymes. FEBS Lett 449:93–100

Hultmark D (1996) Insect lysozymes. EXS 75:87–102

Ibrahim HR, Thomas U, Pellegrini A (2001) A helix–loop–helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J Biol Chem 276:43767–43774

Ito Y, Yoshikawa A, Hotani T et al (1999) Amino acid sequences of lysozymes newly purified from invertebrates imply wide distribution of a novel class in the lysozyme family. Eur J Biochem 259:456–461

Kajla MK, Andreeva O, Gilbreath TM et al (2010) Characterization of expression, activity and role in antibacterial immunity of *Anopheles gambiae* lysozyme c-1. Comp Biochem Physiol B Biochem Mol Biol 155:201–209

Kajla MK, Shi L, Li B et al (2011) A new role for an old antimicrobial: lysozyme c-1 can function to protect malaria parasites in *Anopheles* mosquitoes. PLoS One 6:e19649

Kang D, Romans P, Lee JY (1996) Analysis of a lysozyme gene from the malaria vector mosquito, *Anopheles gambiae*. Gene 174:239–244

Kim CH, Park J-W, Ha N-C et al (2008) Innate immune response in insects: recognition of bacterial peptidoglycan and amplification of its recognition signal. BMB Rep 41:93–101

Kylsten P, Kimbrell DA, Daffre S, Samakovlis C, Hultmark D (1992) The lysozyme locus in *Drosophila melanogaster*: different genes are expressed in gut and salivary glands. Mol Gen Genet 232:335–343

Lemos FJA, Ribeiro AF, Terra WR (1993) A bacteria-digesting midgut-lysozyme from *Musca domestica* (diptera) larvae. Purification, properties and secretory mechanism. Insect Biochem Mol Biol 23:533–541

Li B, Calvo E, Marinotti O et al (2005) Characterization of the c-type lysozyme gene family in *Anopheles gambiae*. Gene 360:131–139

Li B, Paskewitz SM (2006) A role for lysozyme in melanization of Sephadex beads in *Anopheles gambiae*. J Insect Physiol 52:936–942

Mahairaki V, Voyatzi T, Siden-Kiamos I, Louis C (2005) The *Anopheles gambiae* gamma 1 laminin directly binds the *Plasmodium berghei* circumsporozoite and TRAP-related protein (CTRP). Mol Biochem Parasitol 140:119–121

Mai W, Hu C (2009) cDNA cloning, expression and antibacterial activity of lysozyme C in the blue shrimp (*Litopanaeus stylirostris*). Prog Nat Sci 19:837–844

Masschalck B, Michiels CW (2003) Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. Crit Rev Microbiol 29:191–214

Moss JM, Van Damme MPI, Murphy WH, Preston BN (1997) Dependence of salt concentration on glycosaminoglycan-lysozyme interactions in cartilage. Arch Biochem Biophys 348:49–55

Meis JF, Pool G, van Gemert GJ et al (1989) *Plasmodium falciparum* ookinetes migrate intercellularly through *Anopheles stephensi* midgut epithelium. Parasitol Res 76:13–19

Moreira-Ferro CK, Marinotti O, Bijovsky AT (1999) Morphological and biochemical analyses of the salivary glands of the malaria vector, *Anopheles darlingi*. Tissue Cell 31:264–273

Nacer A, Walker K, Hurd H (2008) Localisation of laminin within *Plasmodium berghei* oocysts and the midgut epithelial cells of *Anopheles stephensi*. Parasit Vectors 1:33

Nakimbugave D, Masschalck B, Atanassova M et al (2006) Comparison of bactericidal activity of six lysozymes at atmospheric pressure and under high hydrostatic pressure. Int J Food Microbiol 108:355–363

Nilsen IW, Overbo K, Sandsdalen E et al (1999) Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity. FEBS Lett 464: 153–158

Osta M, Christophides GK, Kafatos FC (2004) Effects of mosquito genes on *Plasmodium* development. Science 303:2030–2032

Park PW, Biedermann K, Mecham L et al (1996) Lysozyme binds to elastin and protects elastin from elastase-mediated degradation. J Invest Dermatol 106:1075–1080

Park JW, Kim CH, Kim JH et al (2007) Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. Proc Natl Acad Sci U S A 104: 6602–6607

Paskewitz SM, Li B, Kajla MK (2008) Cloning and molecular characterization of two invertebrate-type lysozymes from *Anopheles gambiae*. Insect Mol Biol 17:217–225

Povey S, Cotter SC, Simpson SJ et al (2009) Can the protein costs of bacterial resistance be offset by altered feeding behaviour? J Anim Ecol 78:437–446

Rao X-J, Ling E, Yu X-Q (2010) The role of lysozyme in the prophenoloxidase activation system of *Manduca sexta*: an in vitro approach. Dev Comp Immunol 34:264–271

Reddy JT, Locke M (1990) The size limited penetration of gold particles through insect basal laminae. J Insect Physiol 36:397–408

Rossignol PA, Lueders AM (1986) Bacteriolytic factor in the salivary glands of the *Aedes aegypti*. Comp Biochem Physiol 83:819–822

Skerrett SJ (2004) Lysozyme in pulmonary host defense. Am J Respir Crit Care Med 169: 435–436

Vlachou D, Lycett G, Siden-Kiamos I et al (2001) *Anopheles gambiae* laminin interacts with the P25 surface protein of *Plasmodium berghei* ookinetes. Mol Biochem Parasitol 112:229–237

Yu KH, Kim KN, Lee JH et al (2002) Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, *Galleria mellonella*, *Bombyx mori*, *Agrius convolvuli*. Dev Comp Immunol 26:707–713

Zavalova LL, Baskova IP, Lukyanov SA et al (2000) Destabilase from the medicinal leech is a representative of a novel family of lysozymes. Biochim Biophys Acta 1478:69–77