Chapter 6

Serine Protease and Pro-phenoloxidase in
P. vivax Refractory and Susceptible Mosquitoes
6. SERINE PROTEASE AND PRO-PHENOLOXIDASE IN P. vivax REFRACTORY AND SUSCEPTIBLE MOSQUITOES.

6.1. Results

6.1.1. Expression of Serine Protease (AcSP30) and Pro-phenoloxidase (AcPPO6A) in Refractory and Susceptible strains.

The expression levels of serine protease (AcSp30-Accession Number AY995188) and pro-phenoloxidase (AcPPO6A-Accession Number AF466196) transcripts in 4-5 day old naïve adult female mosquitoes of the refractory and susceptible phenotypes were analyzed by real-time PCR using β-actin as an internal control for normalizing the amount of RNA in each sample (Figures 25a and 25b). Both the genes were expressed constitutively in refractory and susceptible strains but with a remarkable difference in their expression levels. Transcript level of AcSp30 was ~40 folds higher in the refractory strain compared to the susceptible strain. Similarly, ~100 folds higher levels of AcPP06A transcript were observed in the refractory strain than in the susceptible strain.

6.1.2. Plasmodium Infection Induced Changes in Serine Protease and Pro-phenoloxidase.

Both, refractory and susceptible female mosquitoes (4-6 day old) were separately fed on blood of Plasmodium vinckei petteri infected Balb/c mice. The P. vivax-refractory strain of Anopheles culicifacies is partially refractory to the rodent malaria parasite. Mosquitoes fed on un-infected mice served as blood-fed controls. Temporal expression of both the genes was monitored after blood meal at regular intervals over a period of 24 hours by real-time PCR (Figures 26 and 27). In the refractory strain, although ~68 folds increase in AcSp30 transcript was observed after 24 hours of normal blood meal, however, the induced level increased by ~300 folds when fed on Plasmodium infected blood (Figure 26b). Similarly, a significant up-regulation of AcPPO6A transcript levels was observed in response to parasite invasion. Approximately 22 folds increase was observed in the expression levels of AcPPO6A after 6 hours of infected blood feeding as compared to normal blood feeding alone (~16 folds). These levels almost doubled after
18 hours of infected blood meal thereby demonstrating the induction of \textit{AcPPO6A} in response to parasite (Figure 27b). This result is in conjunction with the microscopic observation where-in melanotic encapsulation of \textit{Plasmodium} ookinetes was observed 16 to 24 hours post-infective blood feeding. On the other hand, in the susceptible strain, the expression levels of the \textit{AcSp30} gene remained at the basal level upon blood feeding and parasite-infected blood feeding (Figure 26a). Unlike the \textit{AcSp30} gene, the \textit{AcPPO6A} transcript levels in the susceptible strain were insignificantly up-regulated (~1.4 folds increase), in response to blood and parasite (Figure 27a). But these induction levels were substantially lower when compared to the refractory strain. These results demonstrate the specificity of coordinated up-regulation of \textit{AcPPO6A} and \textit{AcSp30} gene transcription in the refractory strain.

6.1.3. \textit{Micrococcus luteus} induced changes in Serine Protease and Pro-phenoloxidase.

The expression pattern of both the serine protease (\textit{AcSP30}) and pro-phenoloxidase (\textit{AcPPO6A}) genes in adult naïve female refractory mosquitoes was also studied temporally following injury and bacterial infection (Figure 28). The mosquitoes were injured using a fine entomological needle and infected with Gram-positive bacteria, \textit{Micrococcus luteus} as described in the Materials and Methods. The mosquitoes were collected at different time intervals and the levels were analyzed by real-time PCR.

\textit{AcSp30} transcript increased by ~2.5 folds upon injury in contrast to ~4 folds increase in \textit{AcPPO6A} transcript under identical conditions (Figures 28a and 28b respectively). This level of the \textit{AcSp30} was maintained throughout the 24 hour post-injury. A marginal increase in \textit{AcSp30} transcript was observed upon challenge with \textit{Micrococcus luteus}. The transcript levels of \textit{AcSp30} were much lower than those compared to the levels obtained after sterile injury indicating that this serine protease is not involved in triggering the cascade for bacterial elimination. Upon injury, an increase in the \textit{AcPPO6A} transcript level was observed after 2 hours (~2 folds) and continued to increase up to 12 hours (~4 folds) and finally declined to the basal level in 24 hours. The modulation of PPO upon injury is suggestive of its role in wound healing. A temporally
Figure 25a. Relative abundance of serine protease transcript in refractory and susceptible strains in freshly emerged females (4-6 day old).

Figure 25b. Relative abundance of pro-phenoloxidase in refractory and susceptible strains in freshly emerged females (4-6 day old).
Figure 26a. Relative level of serine protease of 4-6 day old susceptible strain of *Anopheles culicifacies* to *Plasmodium vinckei petteri* challenge.

Figure 26b. Relative level of serine protease of 4-6 day old refractory strain of *Anopheles culicifacies* to *Plasmodium vinckei petteri* challenge.
Figure 27a. Relative level of pro-phenoloxidase of 4-6 day old susceptible strain of *Anopheles culicifacies* to *Plasmodium vinckei petteri* challenge.

Figure 27b. Relative level of pro-phenoloxidase of 4-6 day old refractory strain of *Anopheles culicifacies* to *Plasmodium vinckei petteri* challenge.
Figure 28a. Relative level of serine protease of 4-6 day old refractory strain of *Anopheles culicifacies* to *Micrococcus leutus* challenge.

Figure 28b. Relative level of pro-phenoloxidase of 4-6 day old refractory strain of *Anopheles culicifacies* to *Micrococcus leutus* challenge.
divergent regulation of PPO transcription was observed in mosquitoes challenged with Gram-positive bacteria *M. luteus*. Interestingly, ~5 folds increase in *AcPPO6A* transcript was observed immediately after two hours of infection and maximum level was attained 6 hours post-challenge (~9 folds increase). Such a rapid induction of the *AcPPO6A* gene in response to bacterial infection suggests its role in combating bacterial invasion.

6.2. Discussion

Melanization requires the proteolytic activation of the inactive PPO zymogens, which are present in the hemolymph, to the active phenoloxidases (PO) by the action of the serine proteases (Soderhall and Cerenius, 1998). Therefore, we explored the molecular components of the melanization cascade in an attempt to determine the events, which correlate with the refractoriness of this mosquito strain.

Both the serine protease and pro-phenoloxidase genes were expressed at very high levels in refractory strain as compared to susceptible strain signifying their role in conferring refractory phenotype to the mosquito strain. We speculate that the inherent high levels of such genes which are involved in melanization cascade would prepare the refractory mosquito strain to mount an immediate and effective immune response against any microbial challenge which would otherwise require longer time for transcription and *de novo* synthesis of these proteins. Furthermore, it is proposed that further *AcSP30* and *AcPPO6A* could be developed as molecular markers for differentiating refractory and susceptible strains of malaria vector, *An. culicifacies*.

The response of both refractory and susceptible strains upon blood feeding and parasite invasion was specifically studied in terms of expression levels of *AcSp30* and *AcPPO6A*. In the refractory strain, the expression levels of both *AcSp30* and *AcPPO6A* were significantly increased upon blood feeding. Such an induction of either of the genes in response to blood feeding was not observed in the susceptible strain of *An. culicifacies*, thereby making this a refractory strain-specific response. The presence of malaria parasites in the ingested blood further up-regulated the transcription of *AcSp30* and *AcPPO6A* in the refractory mosquito. This shows that inherent high levels of these genes
were not sufficient to combat the invading parasite and a tremendous induction was required to block the development of parasite. Noticeably, the up-regulation of AcPPO6A and AcSp30 transcript levels at 18 hours and 24 hours post-parasite feeding respectively coincided with the appearance of melanotic capsules in the gut of refractory strain of An. culicifacies. Such a coordinated response suggests that AcSP30 and AcPPO6A enzymes are a part of the melanization cascade that is triggered in response to Plasmodium. A further validation of this fact is that the non-melanizing susceptible strain showed no induction of transcript levels when fed on Plasmodium-infected blood. In general, blood feeding has a tremendous impact on mosquito physiology. It stimulates the production of digestive enzymes and the arthropod principal steroid hormone 20-hydroxyecdysone (20-HOE) by the ovaries, which is responsible for a wide variety of developmental processes such as molting, metamorphosis and reproduction (Muller et al., 1999). Muller and co-workers (1999) showed that 20-HOE is able to modulate PPO gene expression in An. gambiae Sua4a-3B cell line. In An. gambiae, PPO2, PPO3 and PPO9 genes, were induced following blood feeding (Osta et al., 2004b). The coordinated up-regulation of AcSp30 and AcPPO6A upon Plasmodium-challenge is indicative of the possibility of these enzymes being part of a single cascade leading to phenoloxidase activation.

The serine protease (AcSP30A) and pro-phenoloxidase (AcPPO6A) gene of the refractory strain seems to be involved in the melanotic encapsulation response. Further the immune-inducibility of these genes was also studied in the refractory strain against bacterial infection and sterile injury. The refractory mosquito responded to injury by rapid generation of AcSp30 and AcPPO6A transcripts, which coincided with the deposition of brown pigment at the site of damage. It has been observed that the abrasion of cuticle or invasion of a microbe triggers the release of PPOs into the hemolymph from hemocytes, which is transported to the site of abrasion or invasion, through the underlying cuticular epithelium to facilitate the defense mechanism (Muller et al., 1999, Ashida, 1971, Durrant et al., 1993, Asano and Ashida, 2001, Ashida and Brey, 1995). The refractory strain of An. culicifacies was challenged with a Gram-positive bacterium, Micrococcus luteus by injecting the bacteria directly into the hemolymph. An immediate (within 2 hours of injection) and significant (~9 folds) induction of AcPPO6A transcript
levels was observed which is indicative of the role of AcPPO6A in combating bacterial infection. *M. luteus* has been reported to be melanized in *Aedes aegypti* and *Armigera subalbatus* within minutes of inoculation and the melanization reaction occurred independent of the Gram-character of the invading microorganism (Hillyer et al., 2003a, 2003b, 2004). Although a dose-dependent regulation of AcSp30 and AcPPO6A was not studied, we could demonstrate that invasion of Gram-positive bacteria had absolutely no effect on the expression of AcSp30 thus showing that *Micrococcus luteus* does not trigger the cascade involving AcSp30.

High induction of AcSp30 gene upon Plasmodium infection and no induction upon bacterial infection show the specificity of the gene for combating the malaria parasite. Mosquitoes are known to contain multiple phenoloxidases, which may function in different physiological processes like wound healing and immunity and consequently it is probable that multiple PPO-activators or PPAEs also exist. One important feature of PO cascade is the up-regulation of PPO-activating protease. In lepidopteran insects like *Bombyx mori*, it has been well established that a pattern recognition protein of the invading organism interacts specifically with a serine protease and triggers the cascade (Yoshida et al., 1996). In *Anopheles gambiae* seven putative peptidoglycan recognition proteins (PGRPs), six putative Gram-negative bacteria-binding proteins (GNBPs), thioester-containing proteins (TEPs), Leucine-rich immune proteins (LRIMs) and C-type lectins (CTLs) have been identified and found to be differently regulated upon bacterial and Plasmodium challenge (Christophides et al., 2002, Blandin et al., 2004, Osta et al., 2004a). Apparently in the present context, pattern recognition protein is divergent in *Micrococcus* and *Plasmodium* or alternatively another isoform of the serine protease is up-regulated. In conclusion, AcPPO6A plays an important role in the immunity of *An. culicifacies* and depending upon the nature of invading organism different serine proteases may be activated that converge on immune responsive phenoloxidase.