J. Entomol. Res. Soc., 22(1): 41-51, 2020
Print ISSN:1302-0250

Research Article
Online ISSN:2651-3579

The First Identification of a Serine Protease Inhibitor (Serpin) Encoding Gene in the Mosquito Species *Aedes cretinus* (Edwards, 1921)

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ABSTRACT

Mosquitoes are vectors for many infectious diseases including malaria, yellow fewer, dengue, encephalitis, lymphatic filariasis and others. The mosquito's immune system is critical in fight with the pathogens before infecting a human host. Serine protease inhibitors (serpins, SRPNs) have been shown in many biological processes in insects, such as reproductive and developmental processes, hematophagy, cellular secretion and immunity. Mosquito genome annotations have allowed identification of serpins in various mosquito species but there is little information on their role in mosquito immunity. In this study, a 534 bp nucleotide sequence of a putative serpin gene from the mosquito species *Aedes cretinus* has been identified for the first time through conventional molecular techniques. The amplified gene product encoded a 159 amino acid peptide sequence. The cDNA and peptide sequence analysis have indicated that the identified serpin gene has the highest identity to *Aedes aegypti Srpn2 a*nd *Aedes albopictus Srpn2* genes. Thus, the gene was named as *AcSrpn2* in *Ae. cretinus* due to its possible orthology with other *Aedes* mosquito *Srpn2* genes. Conservation among other mosquito species indicates that SRPN2 may have an important and common role in the mosquito immunity and physiology. The identification of serpin genes in different mosquito species is an important step to determine their biological functions and understand the mosquito immune system.

Key words: Aedes cretinus, mosquito, mosquito immune system, pathogen, serpin, orthologous gene.

Şengül Demirak, M.Ş. (2020). The first identification of a serine protease inhibitor (Serpin) encoding gene in the Mosquito species *Aedes cretinus* (Edwards, 1921). *Journal of the Entomological Research Society,* 22(1), 41-51.

INTRODUCTION

The vectoral capacity of the mosquitoes is determined by the complex interplay between the pathogen and the effectiveness of the vector's innate immune system. In insects, innate immune reactions are developed in response to viral, bacterial, parasitic and fungal infections which include melanization, phagocytosis, autophagy, cellular encapsulation, nodulation, apoptosis, RNA-mediated virus destruction and lysis mechanisms that involve different effector genes (reviewed in Hillyer, 2016). The pathogen is recognized when pathogen-associated molecular patterns (PAMPs) bind to pattern recognition receptors (PRRs) of the host. Following recognition, some PRRs elicit immune reactions such as melanization and phagocytosis while others activate intracellular signaling pathways that include transcriptional activation of immunity genes (Levashina et al, 2001; Choe, Werner, Stöven, Hultmark, & Anderson, 2002; Nakhleh, El Moussawi, & Osta, 2017).

PRR initiates proteolytic cascades that involve serine proteases and serine protease inhibitors (serpins, SRPNs). Serine proteases play critical roles in insect immunity by processing the signal and triggerring the response while their inhibitors diminish the signal and participate in a suicide inhibition. Serpins are usually 350-400 amino acid residues long with a reactive center loop (RCL) that is located 30-40 residues from the C-terminal end that occupies the proteinase active site (Huber & Carrell, 1989). Once the specific bond (scissile bond) at the loop is cleaved, serpin undergoes a conformational change that traps the target proteinase and causes its inactivation (Huntington, Read, & Carrell, 2000; Whisstock & Bottomley, 2006; Dunstone & Whisstock, 2011).

Serpins are structurally conserved but functionally distinct proteins that are present in all higher eukaryotes. The first arthropod serpins were isolated from the hemolymph of the silkworm *Bomyx mori* (Sasaki & Kobayashi, 1984). Similar peptides were also cloned in *Manduca sexta* genome (Kanost, Prasad, & Wells, 1989). With the availability of insect genome sequences, serpin genes have been identified in many insects, including 34 serpin genes in *B. mori* (Zou, Picheng, Weng, Mita, & Jiang, 2009), 32 in *M. sexta* (Li et al, 2018), 7 in *Apis mellifera* (Evans et al, 2006), 31 in *Tribolium castaneum* (Zou et al, 2007) and 35 in *Drosophila melanogaster* (Reichhart, Gubb, & Leclerc, 2011). The genomes of the mosquito species *Anopheles gambiae*, *Aedes aegypti* and *Culex pipiens quinquefasciastus* are also sequenced and includes 18, 23 and 31 serpin genes, respectively (Christophides et al, 2002; Suwanchaichinda & Kanost, 2009; Gulley, Zhang, & Michel, 2013).

D. melanogaster has served as a model organism to understand invertebrate immunity. Through biochemical and genetic studies, some serpins are functionally characterized but some remain to be discovered. Serpins named Spn43Ac and Spn27A were reported to play roles in Toll and prophenolexidase pathways in innate immunity of *D. melanogaster* (Reichhart et al, 2011). Spn27A has been found to activate phenoloxidase (PO) in blood and stimulate melanization reactions (De Gregorio et al, 2002). Mutations in the Spn43Ac gene showed negative regulation of serine proteases as a result of constitutive activation of the Toll pathway which elicit

activation of the antimicrobial peptide Drosomycin (Levashina et al, 1999). Other *D. melanogaster* serpins, such as Spn28, Spn38, Spn42, Spn55 and Spn88 have been demonstrated to have roles in development (Reichhart, 2005).

Mosquito are vectors for devastating diseases and the interaction between the pathogen and the mosquito immune system determines the impact of disease transmission. Some serpins have been functionally characterized in different mosquito species. In *Ae. aegypti* and *An.gambiae* genomes, three serpins (SRPN1, SRPN2, SRPN3) are clustered and could be possible orthologs of *D. melanogaster* Spn27A (Michel, Budd, Pinto, Gibson, & Kafatos, 2005). In *An. gambiae*, SRPN2 was shown to be involved in melanization response through inhibition of prophenoloxidase activating proteinase (PAP) (An, Lovell, Kanost, Battaile, & Michel, 2011). In *Ae. aegypti*, SRPN1 and SRPN2 were also shown to be key regulators in melanization response (Zou, Shin, Alvarez, Kokoza, & Raikhel, 2010). SRPN6 in *An. gambiae* was determined to be involved in immunity against malaria parasites since knockdown of *SRPN6* resulted in increased malaria parasite number in the mosquito midgut and salivary glands (Abraham et al, 2005; Pinto, Kafatos, & Michel, 2008). While these studies indicates important roles of serpins in mosquito immunity, biological functions of many mosquito serpins are still unknown.

Aedes cretinus (Edwards, 1921) is a closely related mosquito species to *Ae. albopictus* and *Ae. aegypti* and has been found in Greece and other Mediterranean countries, including Turkey (Patsoula et al, 2006). This species lacks a sequenced genome. Additionally, no immunity related study has been performed with this pecies. *Aedes* mosquitoes are vectors for yellow fever virus, dengue viruses, Zika virus, and other disease agents. Thus, identification and comparison of immunity related genes, such as serpins, will provide insights into the mechanisms involved in *Aedes* mosquito immunity. This study demonstrates the first report of molecular cloning and identification of a serpin encoding gene in the mosquito species, *Ae. cretinus*. About 534 bp nucleotide sequence of a putative serpin gene sequence has been determined, named AcSrpn2, which showed more than 90% peptide identity with possible mosquito ortholog SRPN2. Its existence in the genome and high conservation among other mosquito species are indications that mosquito serpin2 has an important and a common role in mosquito innate immune system. Serpins that are well conserved within the mosquito lineage could help our understanding of their function in relation with mosquito-borne disease transmission. Genome-wide identification of mosquito serpins and comparative genomics will unravel serpin genes in the mosquito genomes and will enrich our knowledge on serpin roles in immune pathways. Therefore, cloning and identification of serpins through molecular analysis is an important step in understanding their roles to prevent mosquito disease transmission.

MATERIALS AND METHODS

Mosquitoes

Aedes cretinus (Edwards 1921) mosquitoes were reared at 25±2 ºC and 80-90% relative humidty with a photoperiod of 16:8 L:D at the Department of Entomology and Agricultural Zoology at Benaki Phytopathological Institute, Athens, Greece.

DNA extraction

Total DNA from a pool of male and female adult mosquitoes were isolated using a protocol described by Barik, Hazra, Prusty, Rath, & Kar (2013). The quantity and purity of the isolated DNA were checked using Multiskan GO (Thermo Scientific) and 0.8% agarose gel electrophoresis.

Primer design

D. melanogaster Spn27A, *An. gambiae* AgSrpn2, *Ae. aegypti* AaSrpn2 and *Ae. albopictus* Srpn2 orthologs were obtained from National Center for Biotechnology (NCBI, https://www.ncbi.nlm.nih.gov/) and Vectorbase (https://www.vectorbase.org/) databases with accession numbers NP652024.1, AGAP006911, AAEL014078 and AALF012780, respectively. Peptide sequences for these serpin genes were aligned using Clustal Omega programme (Sievers et al, 2011). Conserved regions were selected to design degenerate primers: SrpnF1: 5'-GARATTGCCACCAAGTTCTTC (Tm: 57.4 ºC) and Srpn2R1: 5'-CACTTCRGTTTCATCCATGTA (Tm: 55.5 ºC) which were used in polymerase chain reaction (PCR) amplifications.

PCR amplification and sequencing

DNA amplification was performed in a 30 µl volume mixture containing 15 µl 2X Dream Taq Master mix (Thermo Scientific), 5 µl *Ae. cretinus* genomic DNA, 3 µl degenerate primer pair (final concentration of 1 µM each) and 4 µl sterile ddH_2O. A negative control reaction was also run in parallel in the absence of genomic DNA, including sterile ddH_2O instead. PCR amplification conditions included 3 min initial denaturation at 95 ºC followed by 40 cycles of denaturation at 95 ºC for 30 sec, annealing at 51 ºC for 30 sec and extension at 72 ºC for 30 sec and a final extension for 10 min. PCR products were run in a 1 % agarose gel electrophoresis and positive PCR products were gel purified by using Wizard SV Gel and PCR Clean-up System (Promega) and sent for sequencing (BM Labosis Inc., Ankara). PCR products were sequenced from both ends using forward and reverse PCR primers.

Sequence analysis

Genomic sequences generated from this study were examined using Basic Local Alignment Search Tool (BLAST) programme (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and amino acid sequence alignment was performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al, 2011) using default settings. Previously identified serpin peptide sequences were retrieved from GenBank or Vectorbase

databases and used for phylogenetic analysis. An unrooted phylogenetic tree was constructed using Molecular Evolutionary Genetics Analysis software (MEGA 6.0; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) using the Neighbor-Joining, Minimum Evolution and Maximum Parsimony methods with bootstrap support of 1000 replicates and default parameters. The bootstrap support values >50% were marked on the branches of the tree.

RESULTS AND DISCUSSION

Identification of a putative *Srpn2* gene ortholog in *Aedes cretinus*

Serpins have not been previously described from the mosquito species, *Ae. cretinus*. A putative serpin gene, *Srpn2*, which was previously identified in other *Aedes* mosquito species, was used to determine its possible ortholog in *Ae. cretinus*. Since the genome sequence is unavailable for this species, PCR amplifications using degenerate primers were conducted which resulted in a partial segment of SRPN2 in *Ae. cretinus*. BLAST results have confirmed that *An. gambiae* serpin 2 (*AgSrpn2*), *Ae. aegypti* serpin 2 (*AaSrpn2*) and *Ae. albopictus* serpin 2 (*AalbSrpn2*) genes are possible orthologs of the putative serpin gene identified in *Ae. cretinus*. As a result of high sequence identity among mosquito SRPN2 and orthology (see phylogenetic analysis below), this gene was named as *Ae. cretinus* serpin 2, *AcSrpn2* (GenBank Accession Number: MK592887), in order to be in accordance with the nomenclature of serpin genes in other mosquito species. As shown in Fig. 1, a partial genomic sequence corresponding to a 534 bp region has been determined which also included a 57 bp intronic sequence. In vectorbase database, *AaSrpn2* gene structure contains a long first intron region, thus the first coding exon and the intron regions were unable to be determined in *Ae. cretinus*. The identified *AcSrpn2* gene sequence was corresponding to the exons 4 and 5 of the *AaSrpn2*.

In order to examine the conserved regions of SRPN2 among different mosquito species, AcSRPN2 partial peptide sequence identified in *Ae. cretinus* was aligned with the previously annotated SRPN2 full length peptides from *An. gambiae*, *Ae. aegypti*, *Ae. albopictus* and its possible ortholog in *D. melanogaster* (Fig. 2). Amino acid residues at several positions were identical among *Aedes* species which may indicate that those regions could be structurally important domains of the protein. Additionally, there is a lack of peptide sequence identity when compared with that of *An. gambiae* and *D. melanogaster* SRPN2. While a high degree of conservation (98 % identity) was observed among *Aedes* mosquitoes, about 55 % identity was observed between *Aedes* and *Anopheles* mosquito SRPN2. The genomes of *Aedes* and *Anopheles* mosquitoes are estimated to have diverged about ~ 145-200 MYA (Krzywinski, Grushko, & Besansky, 2006), while *Anopheles* mosquitoes and *D. melanogaster* have diverged approximately 250 MYA (Zdobnov et al, 2002). *D. melanogaster* and mosquitoes have adapted different ecological niches and preferences for their survival. These adaptations and differences may have resulted in significant divergence of their innate immune systems.

Fig. 1. Partial nucleotide sequence and the encoded amino acid sequence of the putative AcSRPN2. Bold sequences indicate the exon regions and deduced peptide sequences are shown benith the coding sequences. Underlined sequence is corresponding to the intronic region.

Mosquito *SRPN2* gene orthology

In order to understand the evolution of mosquito serpins, a phylogenetic tree was constructed using previously identified mosquito serpins from *Ae. aegypti*, *Ae. albopictus* and *An. gambiae* and their possible orthologs in *D. melanogaster*. Although many serpins were identified in these mosquito genomes, 6 different serpins that are more functionally related to SRPN2 were included in the phylogeny. Accordingly, the tree was constructed including mosquito serpins named SRPN1, 2, 3, 5, 8 and 9 and their *D. melanogaster* orthologs (Fig. 3). The tree showed five phylogenetic clusters which clearly indicates that orthologous serpin genes were grouped together. For example, AaSRPN1, AalbSRPN1 and AgSRPN1 serpins were clustered together while AcSRPN2 was clustered with AaSRPN2 and AalbSRPN2. Previously, it has been reported that mosquito serpins SRPN1, SRPN2 and SRPN3 were inhibitory serpins which regulate melanisation by inhibiting PPO-activating proteases (reviewed in Gulley et al, 2013). It appears that these serpins have retained common roles in the same immune pathways. Its ortholog in *D. melanogaster*, Spn27A also regulates the melanization pathway in the hemolymph. In addition to its function in immunity, Spn27A has been found to have a role in development during early embryogenesis. Although, mosquitoes and *D. melanogaster* have different adaptations for their innate immune systems, it is possible that mosquito SRPN2 and *D. melanogaster* Spn27A have retained conserved biological function which may indicate its role in insect-specific features. Additionally, our results also indicate that most mosquito serpins have one-to-one orthology which supports the idea that they had a common ancestral gene before the speciation event.

Fig. 2. Alignment of mosquito SRPN2 peptides and its possible ortholog in *D. melanogaster*. AcSRPN2 was identified in this study and shown in bold. Other mosquito SRPN2 peptide sequences were obtained from Vectorbase and NCBI databases. A minimum of six consecutive amino acid residues that are conserved among all mosquito species are highlighted. Abbreviations: Aalb: *Aedes albopictus*, Ac: *Aedes cretinus*, Aa: *Aedes aegypti*, Ag: *Anopheles gambiae*, Dm: *Drosophila melanogaster*.

Due to different physiological challenges, the expression patterns of mosquito serpins could be highly variable but may provide insight into their biological roles. It is shown that AgSRPNs (including AgSRPN2) are expressed at similar levels in all developmental stages of the *An. gambiae* (Suwanchaichinda & Kanost, 2009).It is also reported that some *AgSrpn* genes show higher expression profile in female mosquitoes compared to males (Baker et al, 2011). Microarray analyses indicated that mRNA levels of serpins changed after a blood meal of the female mosquitoes (Marinotti, Nguyen, Calvo, James, & Ribeiro, 2005). It is indicated that a decrease in expression level of serpins may trigger stronger immune responses within midgut and hemolymph after a blood meal (Gulley et al, 2013). Mosquitoes challenge with different pathogens also elicit serpin expressions. In *Ae. aegypti*, it is reported that AaSRPN2 and other serpin transcript levels are increased after infection with the fungal pathogen *Beauveria bassiana* and regulated by REL1 transcription factors of the Toll pathways (Shin et al, 2005). These results clearly demonstrate that mosquito *Srpn2* has retained essential functions in mosquito innate immune responses in the Toll pathway.

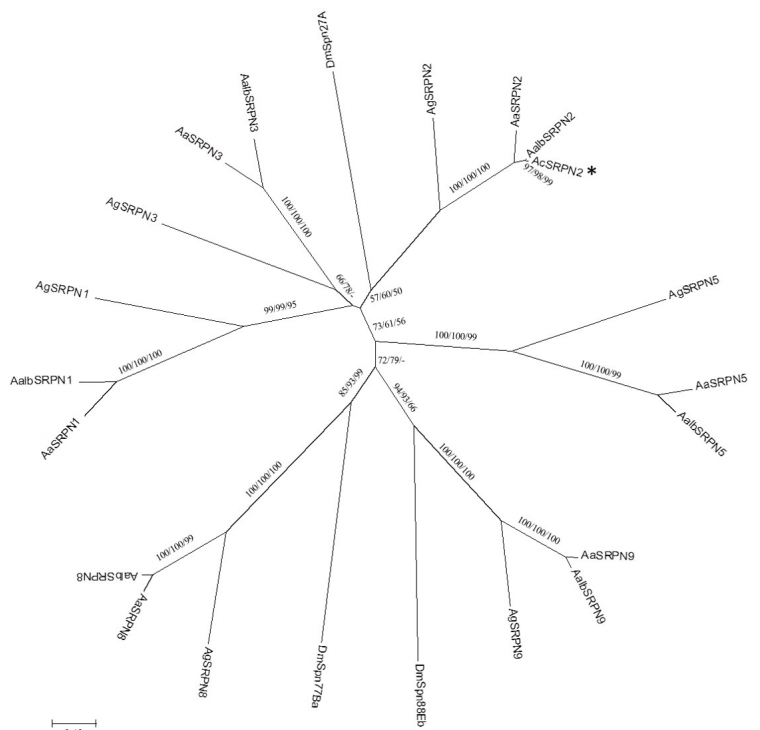

Fig. 3. Phylogenetic relationship between AcSRPN2 (indicated with asteriks) orthologs and representatives of other mosquito serpins. An unrooted tree was constructed using the neighbour-joining algorithm based on the alignment of serpin peptides with removed first exon region. Two additional methods, minimum evolution and maximum parsimony, were also used. Bootstrap values are based on 1000 replicates and represented as the first, second and the third numbers above the branches derived from neighbour-joining, minimum evolution and maximum parsimony analyses, respectively.

CONCLUSION

It is now clear that serpins are involved in a variety of functions in insect immunity. The mosquito genome projects have unravelled many serpin genes in different mosquito species. However, physiological functions of many serpins are still unknown. Studies conducted in *D. melanogaster* provide great wealth of information to understand their functions and thus, their orthologs found in other mosquito species will be helpful to better understand their biological roles in innate immunity.

This study shows the first molecular identification of a serpin encoding gene in the mosquito species, *Aedes cretinus*, in which the genome sequence is unavailable. The *AcSrpn2* gene sequence has been identified and the evolutionary relationship has been determined. The SRPN2 was well conserved among the different mosquito species. It is possible that SRPN2 could have conserved biological functions in the mosquito innate immune system. While bioinformatic analyses reveal existence and divergence of immunity genes in mosquito genomes, functional characterization of interspecies immune responses and immune competencies are still lacking. The biological functions of other mosquito serpins need to be determined in the future to undiscover potential roles in mosquitoes. Mosquito immune system can be manipulated through serpins. Among known serpins, SRPN2 is a good target to interfere with mosquito immune system and develop control strategies to cope with mosquito-borne diseases.

ACKNOWLEDGEMENTS

The author thanks to Dr. Antonios Michaelakis (Department of Entomology and Agricultural Zoology Benaki Phytopathological Institute, Athens, Greece) for providing *Ae. cretinus* adult mosquitoes and undergraduate student Ahmet Simsek for laboratory help. The author is grateful to Tokat Gaziosmanpasa University Research Fund (BAP Project No. 2018/63) for financial support.

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Received: April 19, 2019 *Accepted: September 23, 2019*