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Nidhi Verma
Department of Biotechnology, Chaudhary Bansi Lal University (CBLU), Bhiwani, Haryana, India

Vikas Kumar
Department of Biological Sciences, Birla Institute of Technology and Science (BITS) Pilani Campus, Pilani, India

Lalita Gupta
Department of Zoology, Chaudhary Bansi Lal University (CBLU), Bhiwani, Haryana, India

Sanjeev Kumar
Department of Biotechnology, Chaudhary Bansi Lal University (CBLU), Bhiwani, Haryana, India

Corresponding Author:
Sanjeev Kumar
Department of Biotechnology, Chaudhary Bansi Lal University (CBLU), Bhiwani, Haryana, India

Structural modeling and expression analysis of *Aedes aegypti* prophenoloxidase 5 (PPO5) gene

Nidhi Verma, Vikas Kumar, Lalita Gupta and Sanjeev Kumar

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Abstract
Aedes aegypti mosquitoes, the major intermediate host of infectious arthropod-borne viral diseases, possess prephenoloxidases (PPOs), enzymes involved in melanization, are crucial for mosquito immunity and development. RNA-seq analyses of PPO5 gene revealed stage-specific and sex-biased expressions, with notably higher levels in female pupae and adults. This gene showed significant upregulation in ovaries 12-24 h post-blood feeding, suggesting its involvement in blood meal-associated physiological changes. Physicochemical analysis indicated that PPO5 is hydrophilic, with a slightly acidic pI and exhibits moderate thermostability. Secondary-structure analyses of PPO5 protein revealed the dominance of alpha-helices and random coils, while homology modeling suggested a homodimeric structure with conserved copper-binding catalytic region. Functional characterization revealed that PPO5 is a secreted protein without any signal peptide and likely involved in immunity and development. The present research improves our understanding of PPO5 molecular features and highlights its possiblities as a target for novel mosquito vector control strategies.

Keywords: Mosquito, prophenoloxidase, immunity, development

1. Introduction
The most crucial feature connecting insects to public health is their role as infection vectors. Among such insects, mosquitoes of the family Culicidae transmit infectious diseases such as dengue, malaria, Zika, and chikungunya. Among them, *Aedes aegypti* is one of the primary vectors for most viral infections and thus, considered a serious threat throughout the world [1]. Vector-transmitted diseases responsible for over seventeen percent of all infectious diseases, causing approximately 0.7 million fatalities per year [2]. Vector-borne diseases are becoming more prevalent as we struggle with climate change and, in addition, exhibit significant economic impact due to the increased insecticide resistance in mosquitoes [3]. Knowledge of the biological processes of mosquitoes is essential to designing control methods that would be effective in curbing their spread.

In the mosquito life cycle, phenoloxidases (POs) are the key enzymes which have crucial functions in immunity and developmental processes. The primary role of POs is to facilitate the oxidation process of phenolic compounds, leading to the production of quinones, that subsequently polymerize to synthesize melanin. This melanin synthesis is vital for several physiological functions, including defense mechanisms against pathogens, wound healing, desiccation resistance, and cuticle hardening [4]. Interestingly, the inactive zymogen form of mosquito prophenoloxidase (PPO) is converted to an active phenoloxidase (PO) form under various conditions. This process involves a complex system and multiple factors, including pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) such as amphiphilic lipids, β-1, 3-glucans, peptidoglycans, and lipopolysaccharides [4,5]. Interactions of PRRs with PAMPs activate serine proteases called prophenoloxidase-activating proteinases (PAPs), which cleave PPO zymogens at the amino-terminal and convert them to an active PO. PAPs have been found in many organisms, including *Bombyx mori*, *Menduca sexta*, *Holotrichia diomphalia*, and *Pacifastacus leniusculus* [4, 6] PAPs activity is regulated by serpins (serine protease inhibitors). Some of the chemicals like detergents, fatty acids, and alcohols also activate PPOs.

The pH profile and relative activity of insect POs also differ. For example, the POs from *Ephestia kuehniella* are highly active at low pHs and their functions are accelerated by Cu^{2+} ions [7].

In general, the majority of insects have up to three prophenoloxidase genes while the genome of mosquitoes revealed the presence of nine to sixteen prophenoloxidase genes [8]. For example, ten prophenoloxidase genes are reported in the genome of *Aedes aegypti* [6]. The understanding of PPO genes expansion and their roles in mosquito biology will be subsequently helpful to regulate mosquito-borne diseases. However, there is limited information available regarding the phenoloxidases in disease vectors. In this study, we have characterized a prophenoloxidase 5 (PPO5) gene in *Aedes aegypti* using bioinformatics approaches and the microarray analysis of gene expression and discuss the importance of our findings in terms of targeting PPOs to reduce the impact of mosquito-transmitted diseases.

2. Materials and Methods

2.1 Characterization of ppo5 gene

Prophenoloxidase 5 (PPO5) gene sequence was obtained from NCBI Genome Browser as well as the VectorBase. Genomic details, including its location on chromosome 2 and orientation on the antisense strand was obtained from the data available from NCBI and VectorBase. The Gene Structure Display Server (GSDS2.0) tool was applied to analyze the exon-intron structure, 5' and 3' untranslated regions (UTRs), and transcripts for PPO5 gene.

2.2 RNA-seq data analysis of *Aedes aegypti* ppo5 gene

The RNA-seq library data was retrieved from the VectorBase and analyzed by R program to determine the expression of *Aedes aegypti* ppo5 gene in different developmental stages as described before [9, 10]. The "featureCounts" tool was used to extract the raw counts of ppo5 gene from the RNA-seq data [10]. The "countToFPKM" module was then applied to convert the raw counts to fragments per kilobase per million mapped reads (FPKM). By considering the gene length and the total amount of mapped reads, the FPKM values offered a normalized measure of gene expression. Each of the gathered FPKM values was log10-transformed by adding 1 to make additional analysis easier [10].

2.3 Analyses of primary structure and physical parameters of ppo5 gene

Primary structure and physical parameters i.e. amino acid numbers, molecular weight, theoretical isoelectric point, instability index, aliphatic index, and grand average of hydrophobicity (GRAVY) for PPO5 protein were evaluated using ExPasy-ProtParam software [11]. The secondary structure of PPO5 protein was analyzed by ExPasy-SOPMA and PSIPRED tools [12]. SOPMA tool predicted the percentage of different types of secondary structures *viz*. alpha-helix, beta-sheet, random coils, turns, etc. in the protein. PSIPRED 4 was used for the graphical presentation of PPO5 secondary structure [13]. The SWISS model software with default parameters was used for preparing the homology model of PPO5 protein [14]. The structure was confirmed with the help of SAVESv6.1 server ERRAT, PROCHECK, and Verify 3D tools, as well as QMEAN and QMEANDisCo from the SWISS tools [15, 16, 17].

2.4 Functional analysis of the PPO5 protein

The TMHMM2.0 tool was used to determine the localization (membrane-spanning or extracellular) of PPO5 protein [18]. The SignalP 6.0 tool predicted signal peptides aiding protein secretion [19]. Subcellular localization, an essential step for the analysis of protein function, was predicted using the LocTree3 tool [20].

3. Results and Discussion

3.1 Genomic organization of PPO5 gene

We retrieved the general information including genomic location, number of exons, ESTs, and transcripts, for PPO5 gene (accession no. AAEL013492) from the VectorBase and NCBI databases. The PPO5 gene was found to be located on antisense strand (-strand) of chromosome 2 between nucleotide positions 199,300,303 and 199,314,209 (total length 13,906 nucleotides) and has seven exons. Eight ESTs and two transcripts were retrieved for ppo5 gene [21]. The PPO5 gene sequence was subjected to GSDS2.0 program to display the coding sequences (CDS) and introns graphically (Fig. 1).

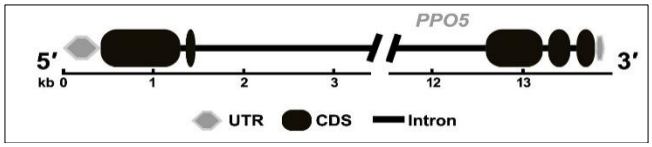

Fig 1: Schematic diagram of *Aedes aegypti* ppo5 gene representing the position of exons and introns.

3.2 Expression profile of ppo5 gene in various development stages of *Aedes aegypti*

The RNA-seq data was processed as described in Materials and Methods [9,10] to determine the expression of *Aedes aegypti* ppo5 gene in different stages of mosquito development (e.g., eggs, larvae, pupae, or adults).

The RNA-seq data for ppo5 gene was also analyzed during the early stages of embryo development (0-72 h after egg laying) as shown in Figure 2. The relative mRNA levels revealed a dual behavior of ppo5 gene expression during embryo development. Relatively higher levels ppo5 transcript during the initial (0-8 h) and mid (40-44 h) stages of development after the eggs laying demonstrate its relevance during embryonic development. Interestingly, these time points corroborate with the melanization process in fertilized eggs which in turn supports embryo development [22]. These findings revealed the importance of ppo5 in early- and mid-stage of embryo development (Fig. 2).

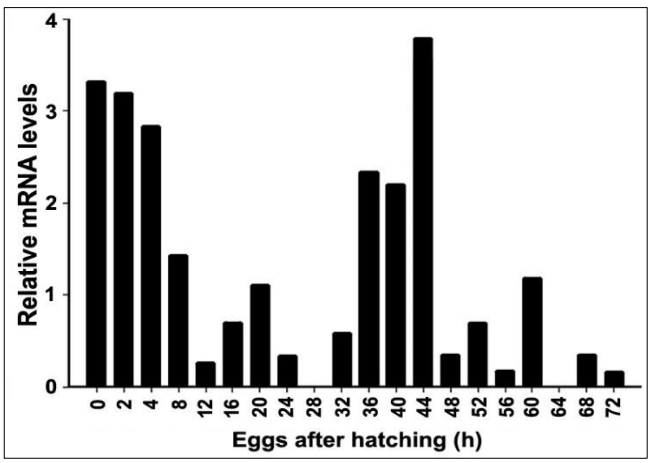

Fig 2: Transcript levels of ppo5 gene in *Aedes aegypti* fertilized eggs at different time intervals after laying.

Figure 3 represents the relative mRNA levels of ppo5 gene in different development stages of mosquito. The mRNA levels of ppo5 gene were found to be higher in female pupae as well as adult females when compared to other stages of development. These observations revealed that ppo5 might be playing an important role in female pupae and adults and thus, it seems to be a female-biased gene (Fig. 3). These findings are in corroboration with other female-biased genes such as hexamerin 1.2, which play an important role in reproduction by acting as amino acid reserve for oogenesis in *Aedes* sp. [23, 24].

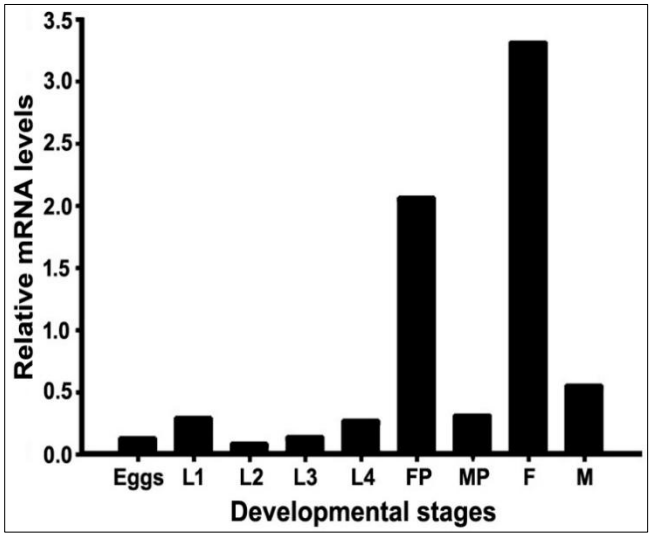

Fig 3: Transcript levels of ppo5 gene at different development stages of *Aedes aegypti*. Eggs, L1= 1st instars larvae, L2= 2nd instars larvae, L3=3rd instars larvae, L4= 4th instars larvae, FP= female pupae, MP= male pupae, F= non blood fed adult females and M= adult males.

The RNAseq data of reproductive organs of blood fed *Aedes aegypti* mosquitoes was also analyzed to determine the transcript levels of ppo5 gene as shown in Figure 4. The transcript levels of ppo5 gene increased dramatically at 12 h and 24 h post blood feeding, with a peak at 24 h. Furthermore, these transcript levels decreased from 36 h to 72 h post blood feeding (Fig. 4). In male reproductive organs, the mRNA levels of ppo5 gene were rather modest, comparable to 48 h to 72 h post-blood-fed females ovaries. Notably, in-silico transcription factor binding site by JASPAR analysis revealed the presence of a putative ecdysone receptor (EcR)/ultraspiracle (USP) binding site within the ppo5 gene 5' UTR locus, supporting the possibility of direct hormonal regulation (data not shown). This temporal expression pattern, combined with the presence of an EcR/USP response element, suggests that *ppo5* gene may be responsive to the post-blood-meal surge of ecdysone. It is of note that ecdysone activates transcriptional cascades required for early oogenesis, vitellogenesis, and follicular growth. Thus, the synchronized rise of ppo5 gene expression with peak ecdysone levels strongly indicates that this gene is regulated by ecdysone signaling during the early stages of ovary development [25, 26, 27].

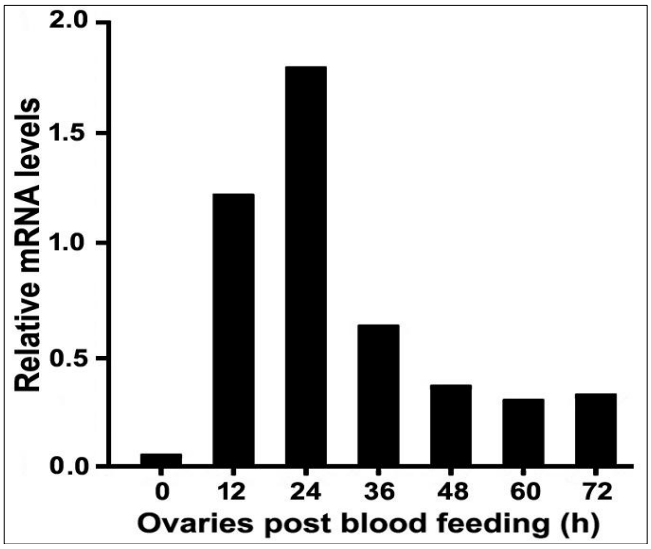

Fig 4: The expression kinetics of ppo5 gene in the ovaries of blood fed mosquitoes at different time intervals.

3.3 Physiochemical properties and levels of PPO5 protein organization

Proteins are complex molecules with distinguished physicochemical features. The Expasy-Protparam software was used to analyze the isoelectric point (pI), molecular weight, extinction coefficient, instability index, aliphatic index, and hydrophobicity of ppo5 protein as described elsewhere [11]. The isoelectric point (pI) value, the pH where surface charge of a protein is neutral, for PPO5 was theoretically found to be 5.98. This indicated that that PPO5 is mildly acidic as described in case of other proteins [28].

The average molecular weight of PPO5 protein was determined to be 79.03 kDa. A protein instability index measures its stability, with values less than 40 suggesting stability and greater than 40 showing instability [11]. The instability index for PPO5 protein was found to be 46.80, suggesting that it is unstable in nature. The aliphatic index of a protein is the relative amount of space occupied by aliphatic amino acids, such as alanine, in the side chain of protein. A larger aliphatic index range of 42.08 to 90.68 indicated a broader range of thermostability [28]. PPO5 protein has an aliphatic index of around 82.34, which revealed its thermostable nature. The hydrophobicity or hydrophilicity of the amino acids in a protein is generally measured by the GRAVY (Grand Average of Hydrophobicity) value. A negative GRAVY value indicates that the protein is hydrophilic [28]. Interestingly, the hydrophilicity of the PPO5 protein was found to be -0.338 by its GRAVY value.

The secondary structure of PPO5 protein was analyzed using SOPMA and PSIPRED (PSI-blast-based secondary structure PREDiction) tools [13] as mentioned in the Materials and Methods. The SOPMA analysis revealed that the random coil (49.05%) and alpha-helix (30.07%) are the most prominent components of PPO5 protein (Table 1). The secondary structure of PPO5 protein was predicted by PSIPRED 4.0 as shown in Figure 5. The observed confidence in prediction was high, indicating that this prediction was very reliable as reported earlier [29].

Table 1: Secondary structure elements of PPO5 protein.

Components	% portion
Alpha Helix	30.07
3_{10} helix	0
Pi Helix	0
Beta Bridge	0
Extented strand	17.08
Beta turn	3.8
Bend region	0
Random coil	49.05
Ambiguous states	0
Other states	0

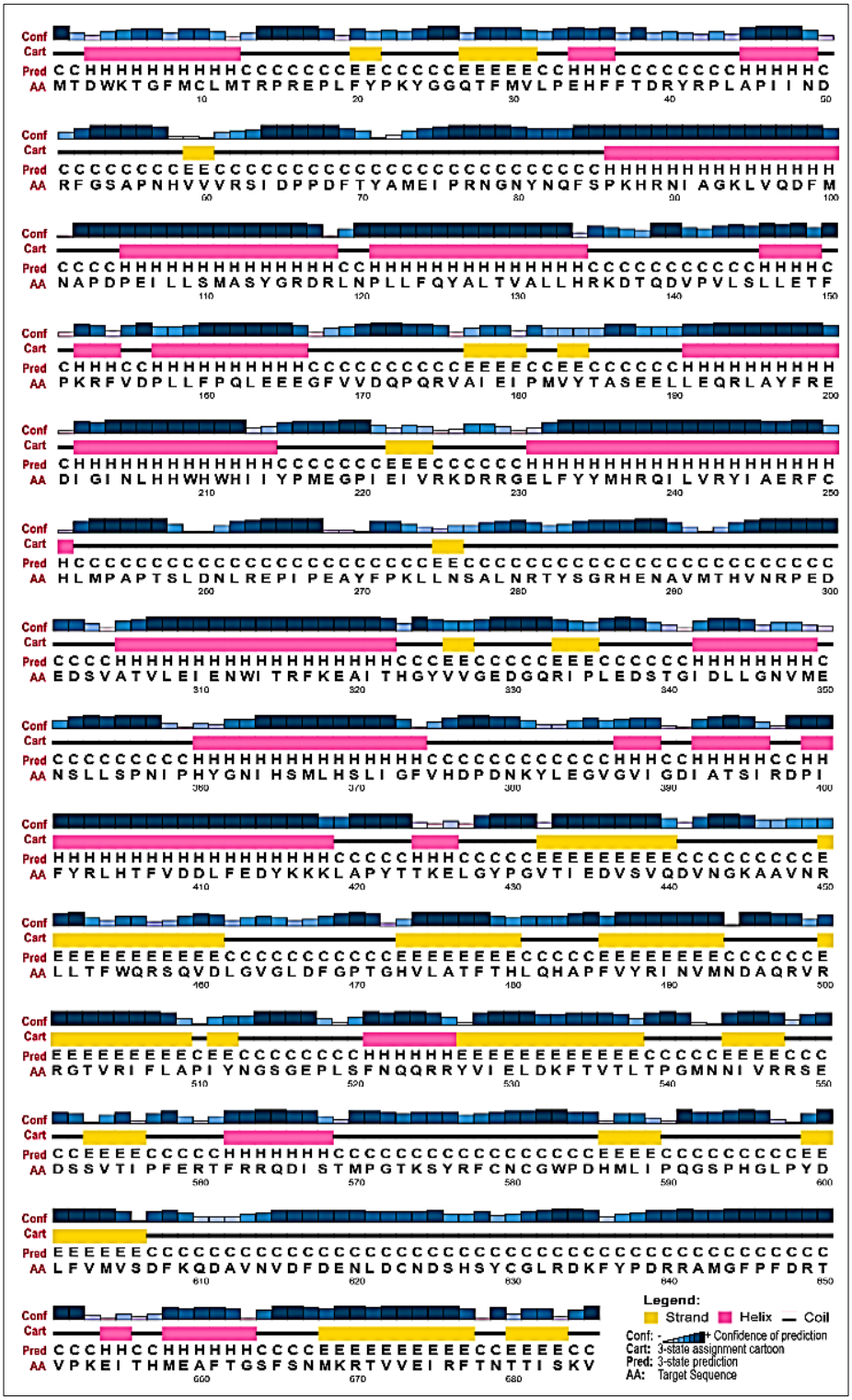

Fig 5: Secondary structure of PPO5 protein.

The SWISS modelling tool [30] was used to predict the tertiary structure of PPO5 protein using homology modelling and the best template as shown in Figure 6(A). Modelling was carried using *Anopheles gambiae* PPO8 crystal structure, which is represented by the template 4yzw, and exhibit 49.56% sequence identity with *Aedes aegypti* PPO5 protein. SWISS modelling analyses revealed that PPO5 is a homodimer. The histidine residues (208, 212, 237, 365, 369, and 405) were found to bind to the copper ions as depicted in Fig 6(B). The model was validated using a variety of tools, including the SAVES server and QMEAN as before [17].

The Verify 3D server deemed the model acceptable as at least 87.99% of amino acids scored > 0.1 in the 3D/1D profile of PPO5 protein (Fig. 7). The Ramachandran plot generated by the PROCHECK program on the SAVE server [31] revealed that 91.4% of residues are in the favoured zone (Fig. 8). The anticipated model seems to be good quality since more than 90% of the residues are in favourable regions [32]. The 3D structure of ppo5 protein deemed acceptable by the ERRAT program, with an overall quality factor of 95.15% (Fig. 9). In these analyses the error axis indicates the confidence to reject regions that exceed the error value and the overall quality factor is expressed as the percentage of protein for which the calculated error value falls below the 95% rejection limit. It is noteworthy to mention that high resolution structures generally produce values around 95% or higher, however, for lower resolutions (2.5 to 3 Angstrom) the average overall quality factor is around 91% (Fig. 9).

The graphical presentation of QMEANDisCO analysis of ppo5 protein, with a score of 0.78±05, indicated a good quality model (Fig. 10). QMEAN4 high-quality models are predicted to rank in the dark zone, scoring -1.76. Our model is in the black zone, shown by a red star (Fig. 10), suggesting it is of high quality.

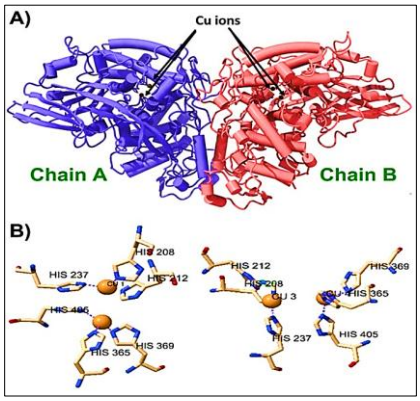

Fig 6: Analysis of PPO5 protein higher level structure. Tertiary structure of PPO5 protein using homology modelling (A) and association of PPO5 Histidine (His) residues with copper ions (B).

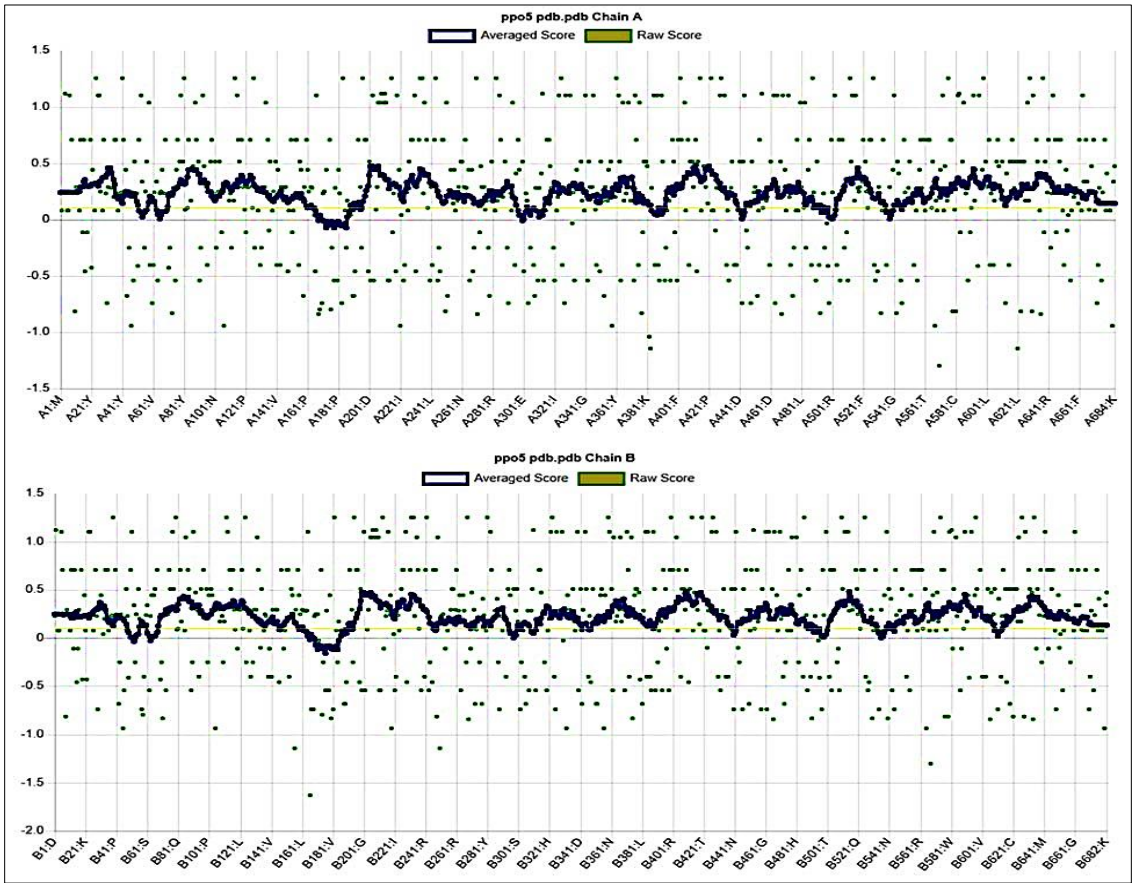

Fig 7: Verify3D graph representation for chain A and B in PPO5 homodimer.

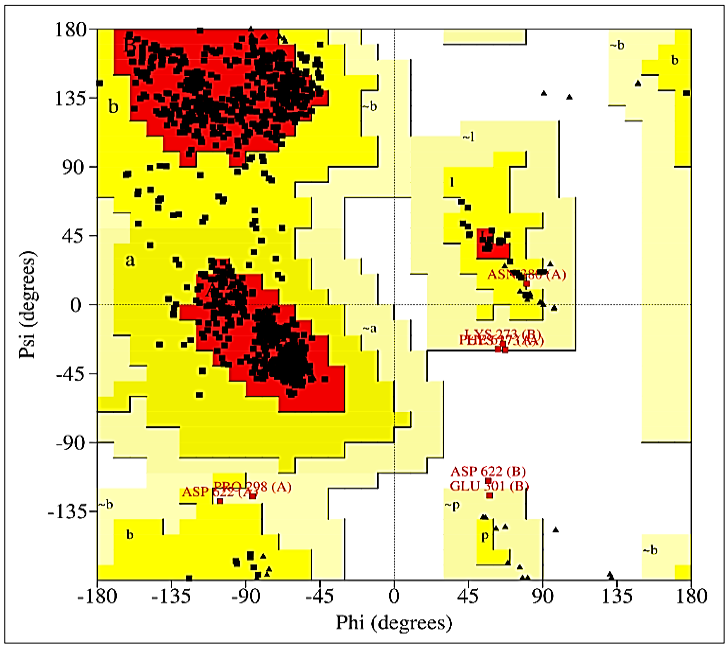

Fig 8: Ramachandran plot for PPO5 protein.

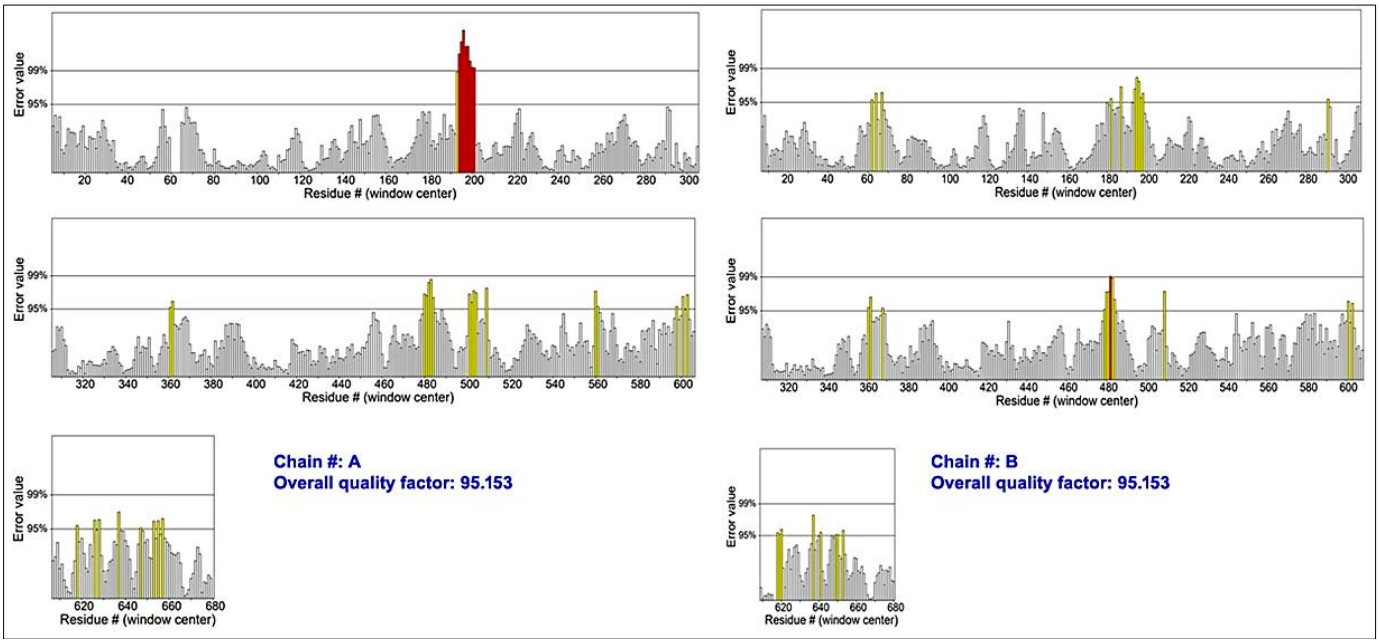

Fig 9: ERRAT analysis of PPO5 protein for chain A and chain B.

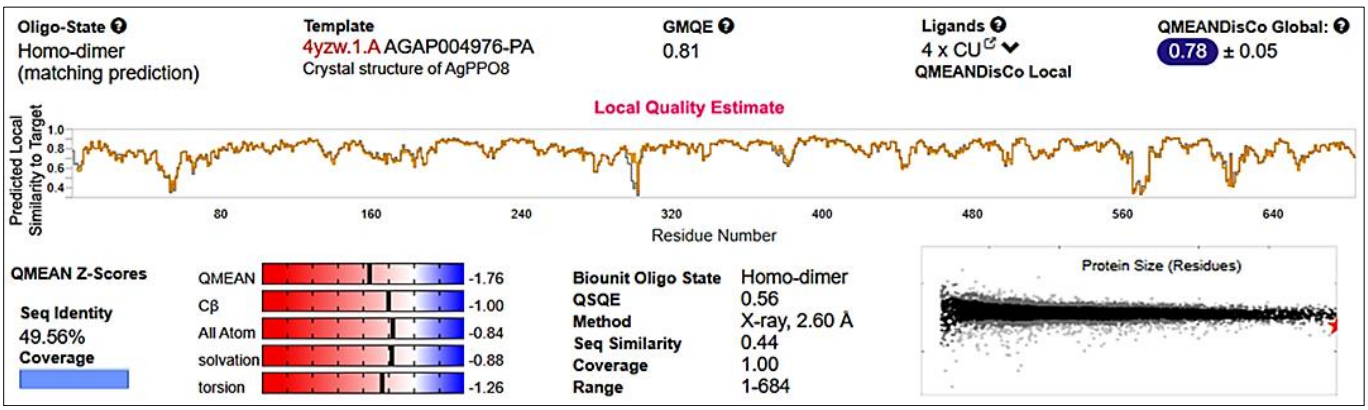

Fig 10: QMEAN DisCo and QMEAN analysis of PPO5 protein.

3.4 Functional analysis of PPO5 protein

Phenoloxidases belong to a type 3 copper protein family. The transmembrane helix prediction analyzed by TMHMM server 2.0 showed that no transmembrane helix is present in the protein (Fig.11). Predicting the sub-cellular localization of proteins is crucial for understanding protein function. SignalP6.0 analyses revealed the absence of signal peptide in PPO5 protein as shown in fig. 11. However, the subcellular location prediction by LocTree 3 revealed that ppo5 is an extracellular secreted protein. Therefore, PPO5 is a secreted protein without signal peptide, possibly due to unconventional/leaderless secretion pathway as reported in case of IL-1β and Acb1 proteins [33]. Conserved domain analysis by CDD software revealed that PPO5 protein is the member of hemocyanin/hexamerin family. The proteins from this family are copper containing and involved in oxygen transport or nutrient storage during non-feeding stages [34]. Interestingly, PPO5 contains three domains including Hemocyanin_N, Hemocyanin_M, and Hemocyanin_C. Hemocyanin_N and Hemocyanin_C are located at N and C terminal of the protein, respectively. Hemocyanin M domain contains the copper binding region and exhibits catalytic site (Fig. 12). These findings revealed that PPO5, being the member of hemocyanin/hexamerin family, might play an important role in immunity [35].

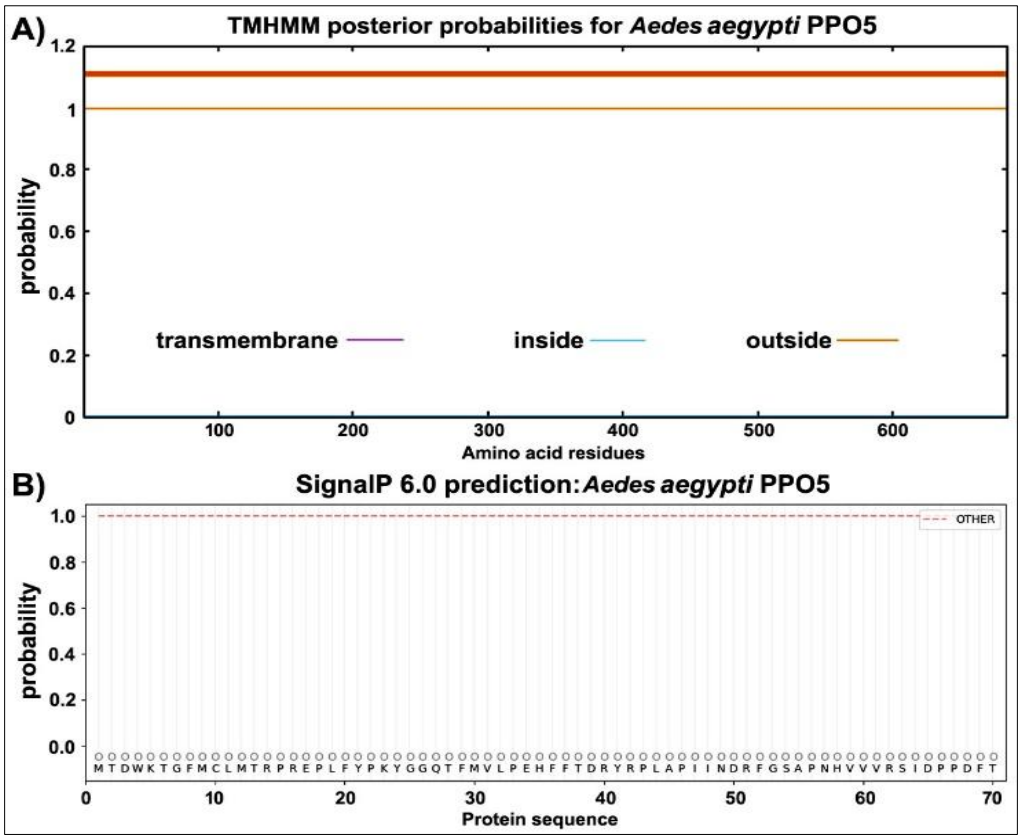

Fig 11: Graphical presentation of *Aedes aegypti* PPO5 protein based on TMHMM analysis (A) and SignalP 6.0 analysis (B).

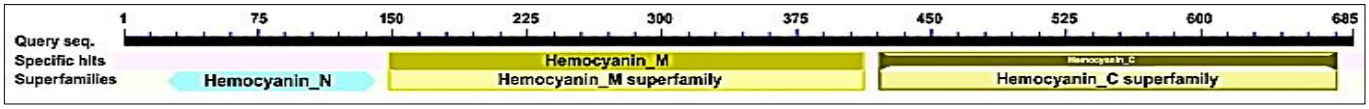

Fig 12: CDD analysis of PPO5 protein revealing three domains; Hemocyanin N, Hemocyanin M, and Hemocyanin C domain.

4. Conclusion

This study provides novel insights into the structural, biochemical, and expression characteristics of *Aedes aegypti* PPO5 gene, demonstrating its developmental and female sex-biased regulation. The upregulation of PPO5 gene post-blood feeding suggested its involvement in female reproductive physiology and immune responses. Structural analyses further confirmed its functional role as a secreted copper-binding enzyme with potential contributions to melanization and pathogen defence. Our findings contribute to the growing knowledge of phenoloxidase functions in mosquito biology, which may aid in the development of novel vector control strategies targeting mosquito immune pathways. Future studies focusing the functional validation, protein-protein interactions, and potential inhibitors of PPO5 to assess its feasibility as a target for disrupting mosquito survival and disease transmission will open new frontiers in mosquito transmitted diseases.

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