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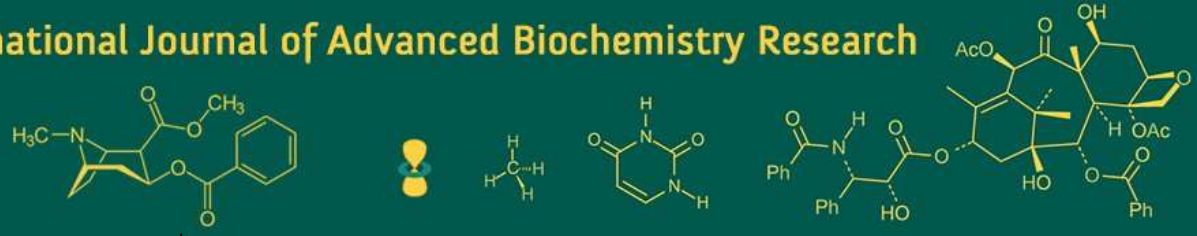

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Insect 14-3-3 proteins: Key players in immunity, signalling and disease control

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Abstract
This particular review explores the multifaceted roles of 14-3-3 proteins in various insect species, including mosquitos, silkworms and houseflies. The research findings have been keen in underscoring of how 14-3-3 proteins regulate the signal transduction, apoptosis and autophagy mitotic cell-check point, along with morphogenetic processes of plant insects. poly-sera raised for various manifested 14-3-3 proteins were noticed in different families (Culicidae and Bombycidae) of insect process with their distinct expression pattern indicative of global and spatio-temporal role in metabolic process. In consistent therein with higher eukaryotes, the 14-3-3 proteins have been known to play a pivotal role in insect cytoskeletal remodelling coupled with focal adhesion mechanisms and bacterial phagocytosis were shed light on the insect innate immunity by 14-3-3 proteins. In the holistic approach of this review on 14-3-3 proteins will address the dynamic roles in the insect species adaptation by modulating the fundamental biological processes such as apoptosis, immunity, and cellular signalling. Furthermore, the development of polyclonal antibodies against mosquito 14-3-3 proteins will offer a promising avenue for studying host-pathogen interactions, with implications for insect-borne disease management.

Keywords: 14-3-3 proteins, immunity, signalling, disease control

Introduction
The 14-3-3 proteins are well characterized in various organisms, focusing on their importance in critical cellular processes such as signal transduction, immunity and stress response mechanisms (Ajjappala *et al.*, 2009 & Jiao *et al.*, 2022) [1, 10]. 14-3-3 proteins have embarked as key players in a variety of biological pathways, from the inception of discovery in the bovine brain and hierarchical heriditament of homology and conservation in molecular structures out of prokaryotes to eukaryotes notwithstanding with class insecta the biggest order in kingdom animalia. 14-3-3 class proteins are known to regulate the metamorphosis, immune responses and host defence mechanisms in insects, including houseflies such as *Musca domestica* and prompted us to use as explicit model for studying insect immunity (Kong *et al.*, 2007) [12]. Keeping in view, distinctive studies have shed light with *Md14-3-3ζ* gene in *Musca domestica*, unique expression patterns and immune response mechanisms, of importance in precluding of bacterial pathogens by damaging their cell membranes. Based on the significant involvement of 14-3-3 proteins in plethora process of insect development would able to uncover the hallmarks in insect immunity and signalling pathways offering new insights into their evolutionary conservation and functional diversity across class insecta (Hinkle and Hogsette, 2021) [9].

Phagocytosis is an another key strategy, wherein insects use functional component of the insect innate immune system, allowing it to eliminate invading bacteria. This procedure entails the recognition of foreign particles, cytoskeletal remodelling and the formation of phagosomes, which mature into phagolysosomes for particle degradation (Mackintosh, 2004) [18]. The 14-3-3ζ protein, a conserved component in eukaryotic cells, has been implicated in bacterial engulfment and microbial resistance in insects, including *Drosophila* and *Aedes* mosquitos. These mosquitos transmit diseases like dengue and Zika, emphasising the importance of understanding their immune responses. This study used *Aedes aegypti* and *Aedes albopictus*-derived cell lines, Aag-2 and C6/36 HT, to investigate the role of 14-3-3 isoforms, specifically 14-3-3ε and 14-3-3ζ, in phagocytosis of both Gram-positive and Gram-negative bacteria (Chen and Yu, 2002) [2].

All these empirical evidences have enabled us to view in the direction of conserved functional roles of 14-3-3 proteins in insect immune responses, unarguably by providing valuable insights as potential therapeutic targets for vector control strategies against arboviruses transmitted by mosquitoes (Hillyer *et al.*, 2005) [8].

Unlike other eukaryotes, class Insecta, must adapt to hostile environments for survival, a process often regulated by intricate signalling pathways. Among the specialized scaffolding proteins involved in these pathways are the 14-3-3 family members, known phosphoserine/phosphor threonine binding proteins. These highly conserved 30 kDa acidic molecules play crucial roles in diverse cellular processes, including cell cycle regulation, development, and stress response, by modulating protein-protein interactions. They specifically recognize phosphorylated motifs but can also bind non-phosphorylated targets. While mammals possess seven isoforms, insects like *Drosophila* and *Bombyx mori* have only two: ε and ζ. These isoforms are implicated in various functions such as signalling pathways, development, and apoptosis. Despite their importance, 14-3-3 proteins in mosquito vectors, crucial for survival and immune functions, remain understudied. However, studies have shown their upregulation in response to infections and their presence in vital mosquito tissues. For instance, the genomic identification and phylogenetic analysis of the two 14-3-3 genes in *Aedes aegypti*, broaden the horizone on their expression patterns across developmental stages and tissues, contributing to a better understanding of their role in mosquito biology and immunity (Yano *et al*., 2006) [29].

The 14-3-3 proteins, highly conserved acidic proteins with monomer molecular weights ranging from 28 to 33 k Da, play critical roles in various eukaryotic cells, modulating protein-protein interactions and regulating cellular processes. While extensively studied in mammals, insects like the domesticated silkworm, *Bombyx mori*, have garnered attention as model organisms for basic research (Hermeking and Benzinger, 2006) [7]. With well-established genetic resources, including a draft genome sequence and extensive EST databases, the silkworm offers a valuable platform for gene discovery and functional analysis and identified the Bm14-3-3ζ and Bm14-3-3ε genes in *Bombyx mori* through cDNA sequencing of silkworm pupa. Phylogenetic analysis categorized these two silkworms 14-3-3 proteins and their expression patterns across various developmental stages. These genomic based tools have led us to explore 14-3-3 protein roles in plethora of insect adoptive phenomena in the area of silkworm biology (Porter and Khuri, 2006) [22].

The 14-3-3 proteins, ubiquitous in eukaryotic organisms, have been extensively studied in vertebrates, particularly in humans, where seven isoforms have been identified. However, in insects like *Drosophila melanogaster* and *Bombyx mori*, only two isoforms (ε and ζ) have been observed (Tabunoki *et al.*, 2008) [24]. While much of the insect 14-3-3 research has focused on *Drosophila*, of late the research findings have also significantly explored on Non-*Drosophila* based insect 14-3-3 proteins, including those from *Bombyx mori*. However, there has been no functional characterization of mosquito 14-3-3 proteins. Given the critical role of *anopheline mosquitoes* like *Anopheles stephensi* and *Anopheles sinensis* in malaria transmission, understanding the molecular mechanisms underlying the interaction between malaria parasites and vector mosquitoes is imperative. Intriguingly, given the explaination from theretofore, anti-polysera raised against mosquito 14-3-3ζ had showed cross-reactivity with other isoforms of 14-3-3 proteins especially from midgut cells of mosquitoes, thus providing new vistas to understand the potential involvement of 14-3-3ζ protein in Plasmodium-dependent apoptosis in mosquitoes (Thompson *et al.*, 1997) [25]. Furthermore, likelihood observations were also revealed through cross-reactivities of the mosquito 14-3-3ζ antibody to various 14-3-3 homologs from dipteran and lepidopteran insects, thus providing an explicit model to explore its specificity and utility for future studies (Rosenquist M, 2003) [23].

Functional roles of 14-3-3ζ protein of *Musca domestica* response to Bacterial infection.

The Md14-3-3ζ gene, derived from *M. domestica*, encodes a 257-amino acid protein characterized by conserved structural domains and predicted transmembrane regions (Feng *et al.*,2014). Phylogenetic analysis indicates close homology with related insect species, particularly *D. melanogaster* and *Stomoxys calcitrans*. Expression profiling reveals constitutive expression throughout housefly development, with heightened levels in adults and preferential expression in immune tissues (Fu *et al.*, 2000) [4]. Upon bacterial challenge, Md14-3-3ζ expression rapidly increases, suggesting a role in innate immunity (Gao *et al.*, 2015) [6]. Recombinant Md14-3-3ζ demonstrates bacteriostatic effects against E. coli and S. aureus, with evidence of cell membrane disruption. These findings underscore Md14-3-3ζ's significance in housefly immunity and its potential as a therapeutic target against bacterial pathogens (Li *et al*., 2015) [16].

Participation of 14-3-3ε and 14-3-3ζ proteins in the phagocytosis, component of cellular immune response, in *Aedes mosquito* cell lines

In the far reaching move, quite a few reports have explored and signified towards in the attribution towards 14-3-3 proteins of insects in the developmental regulation and cellular process involved in the embellishment of innate immune response. Research findings have indicated that the expression and function of two proteins, 14-3-3ε and 14-3-3ζ, within cell lines derived from *Aedes* mosquitoes. These proteins are found in high levels in the tested cell lines, namely C6/36 HT and Aag-2 cells. Through inhibition experiments, researchers observe that these proteins are involved in maintaining cell viability and have a significant impact on the cells' ability to engulf bacterial pathogens, a process known as phagocytosis. (Lalle *et al*., 2006) [14] Additionally, the study employs gene silencing techniques using DsiRNAs, which result in observable changes in cell morphology and a reduction in protein expression levels (Mortenson *et al*., 2015) [19]. This suggests that 14-3-3 proteins play crucial roles in various cellular functions. Overall, the findings contribute to understanding the regulatory mechanisms involved in innate immune responses and cellular processes, potentially offering insights for developing strategies to combat diseases transmitted by Aedes mosquitoes (Knetsch *et al.*1997) [11].

Identification and Expression Analysis of Two 14-3-3 Proteins in the Mosquito *Aedes Aegypti*, an Important Arboviruses Vector

14-3-3 protein isoforms are known share higher degree of homology nearly 97-98% at amino acid level in the higher eukaryotes has been well implicated by their inherent character of sticky-catalytic function from normal physiology to pathophysiological level. Furthermore research findings have focused on the identification, sequencing and characterization of two 14-3-3 genes in silkworms (Ganguly and Weller, 2005) [5]. One homologue, 14-3-3ζ, was identified and sequenced directly from a silkworm pupal cDNA library. Another homologue, 14-3-3ε, was identified by searching silkworm EST databases. Both genes were found to have conserved features similar to other 14-3-3 proteins. Sequence analysis revealed differences in gene structures between the two silkworm 14-3-3 genes and their counterparts in Drosophila (Yaffe *etal*., 1997) [28]. Furthermore, alignment of silkworm 14-3-3 proteins highlighted conserved residues involved in dimerization and interaction with ligands. The expression and purification of Bm14-3-3ζ in *E. coli* were successfully achieved, and immune assay confirmed its recognition by a specific polyclonal antibody (Waterman *et al.,* 1998) [27]. Expression analysis across different developmental stages and tissue distributions revealed differential expression patterns for Bm14-3-3ζ and Bm14-3-3ε. Bm14-3-3ζ showed higher expression levels compared to Bm14-3-3ε, particularly in silk glands. These findings were consistent with EST search results. Additionally, immunoblot assay has revealed an unidentified band in all tissue samples, indicating potential post-translational modifications or additional protein isoforms (Muslin *etal*. 1986) [20]. In line with evidential indicators, 14-3-3 proteins of insects are known to have multifaceted role in normal physiology and also with diseased situation like in any other eukaryotes.

Cross reactivity and distinct recognition of caricatured poly-sera from mosquito 14-3-3 ζ across other genera of insects

The present review attempted to explore the possible mechanism by which 14-3-3 regulates the cellular homeostasis. Research findings with protein modulation have revealed that the poly-sera developed distinctively for mosquito 14-3-3ζ protein had exhibited cross reactivity with other insect genera such as dipteran and lepidopteran insects. Since, 14-3-3

Leveraging the high conservation of 14-3-3 proteins across organisms, multiple alignments were conducted to identify conserved regions. MOTIF analysis identified two common 14-3-3 protein signatures, and a specific peptide region, DTQGDGDEPQEGGDN, was chosen for antibody production (Li W *etal.*,1997) [15]. This region was selected based on its uniqueness to 14-3-3ζ compared to 14-3-3∈. The synthesized peptide was conjugated to carrier proteins for immunization, and the resulting polyclonal antibody was tested via ELISA, demonstrating cross-reactivity with the target peptide. Zooblot analysis confirmed species specificity, showing cross-reactivity with dipteran and lepidopteran insects. Subcellular localization studies using immunostaining revealed cytoplasmic localization of 14-3-3ζ in mosquito midgut cells and various tissues in a butterfly species. Overall, the generated peptide antibody presents a valuable tool for studying the functional role of 14-3-3ζ in mosquito biology, particularly in interactions with pathogens like Plasmodium and baculoviruses (Kong *etal.*, 2007) [12].

Conclusion

In conclusion, the research elucidates the multifaceted roles of 14-3-3 proteins across various insect species, particularly in houseflies, mosquitoes, and silkworms. These proteins exhibit crucial functions in immune response modulation, cellular signaling, and developmental processes. The present review highlights the significance of 14-3-3ζ in pathogen defense mechanisms, with its expression patterns indicating a pivotal role in immune tissues and its ability to hinder bacterial growth by penetrating bacterial cell membranes. Furthermore, the investigation underscores the importance of 14-3-3 proteins in cellular processes such as phagocytosis, cytoskeletal remodelling, and protein complex organization. Insights into their involvement in adaptive signalling networks shed light on their potential therapeutic relevance. The development of peptide-based polyclonal antibodies against 14-3-3 proteins facilitates further exploration into their subcellular localization and functional implications, particularly in interactions with pathogens. These findings not only deepen our understanding of insect biology but also pave the way for potential applications in disease control strategies and therapeutic interventions. Continued research into the intricate mechanisms involving 14-3-3 proteins promises to uncover novel avenues for addressing various health challenges and advancing biomedical knowledge.

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