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INSECT CHITINASE: MOLECULAR BIOLOGY AND THE POTENTIAL ROLES IN INSECT PEST MANAGEMENT

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ABSTRACT: Insect chitinase and chitinase-like proteins play a significant regulatory role in degrading chitin in the exoskeletal and gut linings of insects. For several years, researches on insect chitinase have been carried out on their development as biopesticides or chemical defence proteins in transgenic plants. Targeting chitin degraded by insect chitinases may be an effective approach in integrated pest management programs because they are harmless for the plants and vertebrates, which do not have chitin in their tissues. The ability of chitinases to digest chitin in the peritrophic matrix or exoskeleton raises the possibility to use them as an insect pest control strategy. New technologies were continually applied for the studies on the structure, time and space expression, tissue specific expression and biological function of insect chitinases with the rapid development of molecular biology, and many innovative achievements were reached. The research advances of insect chitinases were reviewed in the paper.

KEYWORDS: Insect Chitinases, Molecular biology, Diversity, Functions, Pest Control

INTRODUCTION

Chitinase (EC 3.2.1.14) is an enzyme that breaks down β-1, 4-glycosidic bonds in chitin and chitooligosaccharides and hydrolyses chitin into monomers of N-acetylglucosamine. Chitinases are expressed in various organisms including those that lack chitin such as plants, bacteria, viruses and vertebrates, as well as those that contain chitin such as fungi, insects and crustaceans (Oyeleye & Normi, 2018). Insect chitinases belong to the class of hydrolytic enzymes with the potential to inhibit or degrade the chitin. In insects, chitin hydrolysis is essential for periodic shedding of the old cuticle ecdysis- it is the main component of the exoskeleton, peritrophic membrane, and cuticle, and it defends against external mechanical disruption and pathogen infection (W. Chen et al., 2018). Non-chitin-containing hosts like mammals and plants produce chitinases as specific pathogenesis-related proteins to recognize and degrade the chitin in chitin-containing pathogens such as fungi and nematodes (W. Chen et al., 2020).

Studies on inhibitors of chitinase have been substantially increased, which would help develop specific bioactive molecules with potential applications as drugs and agrochemicals (W. Chen et al., 2020). Also, chitinases and their inhibitors possess high potential as fungicides for the treatment of mycoses in animals and humans, therapeutic compounds against parasites, and

biopesticides for the control of insect pests (Merzendorfer, 2013). Insect chitinases belong to family 18 glycoside hydrolases (GH18) except a GH48 chitinase identified in the leaf beetle *Gastrophysa atrocyanea* (Nguyen et al., 2018). Insect chitinase has been studied in several insects which include the pea aphid *A. pisum*, African malaria mosquito *A. gambiae, silkworm B. mori,* rice brown planthopper *N. lugens, C. suppressalis* and cotton mealybug *P. solenopsis* (Nakabachi et al., 2010; Omar et al., 2019; Pan et al., 2012; Su et al., 2016; Xi et al., 2015; Zhang et al., 2011).

Chitinases hydrolyse chitin, which is present in different plant pest e.g. insects, fungi and nematodes either via directly targeting them (Cao et al., 2017; Su et al., 2016) or via manipulating their inhibitors as biopesticides (Arakane & Muthukrishnan, 2010) thus they represent promising biopesticides. Downregulating the expression level of insect chitinases results in severe phenotypes, including ecdysis disturbance, growth inhibition, pupation failure, and death, indicating that insect chitinases may be promising pesticide targets (Muthukrishnan et al., 2019). Chitinases have been an interesting research topic for biotechnological applications in the chemical and pharmaceutical industry because they can convert chitinous material from natural sources into usable components. This review mainly focuses on the potential of insect chitinases as an insecticidal target in insect pest management.

THE MOLECULAR STRUCTURE AND DIVERSITY OF INSECT CHITINASE

The Molecular Structure

They are usually composed of catalytic domains (GH18 domains), Cysteine-rich chitin-binding domains (CBN14 or peritrophic A domains) and serine/threonine-rich linker domains (STL). A catalytic domain with the β/α-barrel fold, whose function is to hydrolyse the β-1,4 linkage between N-acetyl-d-glucosamine residues of chitin (Malecki et al., 2020). Insect CBM14 domains have only six conserved cysteines, presumably forming three disulphide bridges (Merzendorfer, 2013). The common spacing between the conserved cysteines in the CBDs of GH18 chitinases appears to be as follows: 1Cx11-24-2Cx5-6-3Cx9-19-4Cx10-17-5Cx4-14-6C, where x is any other amino acid (Su et al., 2016). The GH18 and CBM14 domains are frequently but not always connected by serine/threonine-rich linker regions (STL), which are presumably modified by mucin-type O-glycosylation. The first step of the mucin-type O-glycosylation is catalysed by a polypeptide N-acetylgalactosaminyl transferase (GalNAc transferase). This enzyme transfers the sugar moiety from UDP-GalNAc to the serine and/or threonine residues of the acceptor polypeptide. GalNAc-transferases are absent in plants but present in insects, such as *D. melanogaster*, where specific isoforms appear to have unique functions in particular tissues, including epithelia known to synthesize and secrete chitinases (Tran et al., 2012). The STL region is predicted to be an unfolded polypeptide because it is not possible to model the structure of this region (Merzendorfer, 2013). Although insect chitinases are plentiful, only six insect chitinases have structural information (ChtI, ChtII, ChtIII, ChtIV and Chi-h from *Ostrinia furnacalis* and DmIDGF2 from *Drosophila melanogaster*) (W. Chen & Yang, 2020). Therefore, the research on insect chitinases should be strengthened in the future.

The Classification of Chitinases in Insects

Insects belonging to different orders vary in the number of groups, but many of them have representatives belonging to groups I through V (Noh et al., 2018). GH18 insect chitinases can

be further divided into eleven distinct subgroups (ChtI to ChtX, and Chi-h) based on sequence homology and domain architecture (Tetreau etq al., 2015). Group I chitinase is the most studied gene. They are characterized by the presence of an N-terminal catalytic domain joined to a CBM14 domain via a serine/threonine-rich linker. The linker region serves as a site for glycosylation which increases the stability of the enzyme against proteases. Group II chitinase has two or more catalytic domains at the C-terminal region that are presumed to have catalytic activity, with four conserved domains (Muthukrishnan et al., 2019). Group III chitinases differ from group I and II chitinases in that they are anchored to the plasma membrane by an N-terminal transmembrane helix. Group III chitinases consist of two catalytic domains and a single CBM and have morphogenetic roles in regulating abdominal contraction and wing expansion. Group IV chitinase constitutes of a highly divergent group. They are usually encoded by multiple genes in a single insect species (Merzendorfer, 2013). Group IV chitinases are usually composed of a signal peptide and one GH18 catalytic domain without a CBM and are proposed to be involved in the digestion of chitin containing substrates such as peritrophic matrix lining in the midgut. *Ostrinia furnacalis* ChtIV is structurally more similar to the plant antifungal chitinases than insect chitinolytic chitinases. It lacks the elements for crystalline chitin-binding and contains a few solvent-exposed aromatic residues in the substrate-binding cleft (W. Chen & Yang, 2020). Group V includes chitinase-like proteins such as the imaginal disk growth factors (IDGFs) that lack chitinase activity. Like group IV chitinases, they contain a signal peptide and one GH18 catalytic domain but no CBM. Group VI chitinases are characterized by the presence of a long C-terminal stretch in addition to a catalytic domain and a CBM. Group VII chitinases in turn resemble group IV chitinases in overall structure, but the phylogenetic analysis revealed that this group is an outlier of group II chitinases (Merzendorfer, 2013). They have an N-terminal signal peptide and a GH18 domain but they are devoid of a CMB14 domain. Group VIII chitinases have a GH18 domain but lack a signal peptide and a CBM14 domain. The domain architecture of group VIII chitinases includes a predicted transmembrane segment, one catalytic domain and no CBM. *Ostrinia furnacalis* Chi-h is thought to be horizontally transferred from bacteria. Similar to its bacterial homologue, *Ostrinia furnacalis* Chi-h is a processive exo-acting chitinase. It has a long and asymmetric substrate-binding cleft. There are 13 solvent exposed aromatic residues in the non-reducing end side but only two in the reducing end side. Two extra α-helices formed by a unique sequence are present in the wall of the *Ostrinia furnacalis* Chi-h substrate-binding cleft. This unique structural element narrows the substrate-binding cleft, which may increase the binding affinity of *O. furnacalis* Chi-h for chitin chains and, thus, favour *O. furnacalis* Chi-h in hydrolysing the crystalline substrate (Liu et al., 2017) .Other features such as transmembrane and signal regions may also be present in all groups. Almost all of them have signal peptides that get cleaved inside the ER and the rest of the protein packaged in secretory vesicles are transported into the extracellular space via a default secretory pathway. Exceptions are found in group III and group VIII chitinases, they have transmembrane segments and hence are predicted to remain on the plasma membrane with the catalytic domains facing the extracellular space. Overall insect chitinases have diverse domain organizations tissues and development patterns of expression and differences in their catalytic parameters and substrate preferences. This apparently leads to functional specialization among the chitinases, with some of them having roles in chitin degradation during moults, while others may have functions such as cuticle and peritrophic matrix remodelling digestion and defence or function as growth regulators (Muthukrishnan et al., 2019).

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THE FUNCTIONS AND CATALYTIC MECHANISM OF CHITINASES IN INSECTS

The Roles of Chitinases in Insects

Insect chitinase genes are expressed mainly in integument (groups I, II, and III) and gut (group IV), and expression (both transcripts and proteins) was also observed in the tracheal system, fat body, hemolymph, and reproductive system, suggesting their diverse physiological roles (Tetreau et al., 2015; K. Y. Zhu et al., 2016). Different types of insect chitinase exist in certain differences in function. Group I–II chitinases act in the degradation of the endocuticle during moulting with activities that have different effects on larval–larval, larval–pupal, and pupal–adult moults. Partially, these chitinases may have redundant functions (Mamta et al., 2016). The functions of the classic group I chitinases have received the most attention probably because they are highly abundant in the moulting fluid, developmentally regulated by insect hormones, and facilitate ecdysis (Noh et al., 2018). Group I is implicated in the turnover and shedding of the exuvium, remaining old cuticle especially at the pupal to the adult stage. Upon RNAi to knockdown specific chitinase genes involved in moulting, the insects are trapped in the pupal cuticle and cannot digest away the old cuticle after knockdown of specific chitinases genes involved in insect moulting (Li et al., 2015; Pesch et al., 2016; Su et al., 2016). The transcript for this group is expressed at all stages of development but is elevated during the pupal stages suggesting a critical role in adult eclosion (Muthukrishnan et al., 2019). Group I chitinases have also been found that they act synergistically with Chi-h to degrade cuticle chitin (Ma et al., 2017). Both groups I and II chitinases act in the degradation of the endocuticle during moulting with activities that have different effects on larval–larval, larval–pupal, and pupal–adult moults. Partially, these chitinases may have redundant functions (Mamta et al., 2016). Group I and II chitinases are likely complementary, possibly group II enzyme carries out the initial decrystallization of α-chitin followed by an endo-type of attack along with the group I chitinase (Muthukrishnan et al., 2019). Group III chitinases appear to be required for processes that occur immediately after pupation such as abdominal contraction and the extension of wings and elytra. In *T. castaneum* RNAi to silence group III genes resulted in several morphological abnormalities observed soon after pupation (Noh et al., 2018). The adults had significantly smaller appendages including the wing and legs, also the hindwings did not fold properly under the elytra. The movement of joints for adults was not affected but they fell on their backs frequently and had difficulties in up-righting their bodies. RNAi to silence expression of a group III chitinase gene in white-backed planthopper (*Sogatella furcifera*) suggested that the gene may also function in development in the early adult stage (C. Chen et al., 2017). More so group III chitinase from *O. furnacalis* (*Of*ChtIII) was observed to co-localize with chitin synthase in the epidermal cell layer and the two enzymes showed a similar gene expression pattern at different developmental stages. These results suggest that ChtIII may be associated with chitin synthesis rather than chitin degradation (Liu et al., 2018). Moulting and wing development in adults were affected which resulted in multiple phenotypes including insects with elongated distal wing pads, thin thorax-abdominal junctions and adults with abnormal wings.

Group IV chitinases appear to have functions in the intestinal system because they are only expressed in different parts of the gut. It is also thought to be involved in insect immune defence, a gut-specific group IV chitinase from *O. furnacalis* (OfChtIV) was revealed to have antifungal activity toward phytopathogenic fungi isolated from maize, wheat, rice and soya bean (Arakane & Muthukrishnan, 2010; Liu et al., 2020). Group V chitinase like-proteins are predominantly expressed in embryonic yolk cells and fat bodies. They may act as circulating binding to cell surface receptors. Group V chitinase-like protein, IDGF2 appears to have immunity and

detoxification functions, it was induced upon injury in the larval stage in *Drosophila melanogaster* (Broz et al.,2017). The IDFG3 in *Drosophila melanogaster* played an immune-protective role during entomopathogenic nematode infections. Moreover, IDGF3 mutants displayed an extended development delay during wound healing, indicating that this chitinase is an essential component required for the formation of hemolymph clots that seal wounds (Kucerova et al., 2016). RNAi studies on cotton mealybug (*Phenacoccus solenopsis*), showed that some chitinase genes have an essential role during moulting and development, whereas others have a role during pupation and eclosion (Omar et al., 2019). However, it is well known that the same gene in the same chitinase group does not always have the same functional properties. In contrast to group I-V chitinases, the functions of group VI-VIII chitinases have not been addressed so far (Muthukrishnan et al., 2019; Noh et al., 2018).

The catalytic mechanism of Insect Chitinases

All insect GH18 chitinases have been shown to be endo-splitting enzymes that cleave chitin or chitooligosaccharides comprising three sugar moieties at least. The analysis of crystal structures of several family 18 chitinases with bound substrates or products have provided clues concerning their catalytic mechanism (Muthukrishnan et al., 2019). It indicated that in addition to the proton donor glutamate, the C2 N-acetyl group of the sugar bound in the -1 position of the substrate-binding site has a role in catalysis. The mechanism has been called substrate-assisted catalysis (anchimeric assistance) and involves an oxazolinium intermediate state.

The catalytic domains of family 18 chitinase assume the typical β_8 α_8 TIM barrel structure with eight β-strands in the centre of barrel end eight α-helices constituting the outer surface of the barrel. Four conserved motifs at characteristics positions in the structure have been identified. The conserved motif 1 is in the third β-strand and has the consensus sequence KXX (V/L/I) A (V/L) GGW. The second motif, FDG (L/F) DLDWE(Y/F) P in the β4 strand contains the catalytically critical glutamate, which is the proton donor in the hydrolytic reaction. The other two conserved motifs, MXYDL(R/HH)G and GAM(T/V)WA(I/L)DMDD are in the β6 and β8-strand respectively (Arakane & Muthukrishnan, 2010). The functions of motifs I, III and IV are uncertain. Based on their location and the presence of aromatic residues, they may provide sugar-binding sites and play a role in processivity and enzymatic activity toward crystalline chitin (Arakane & Muthukrishnan, 2010).

ROLES OF INSECT CHITINASES IN INTEGRATED PEST MANAGEMENT

Insect Chitinases in Biopesticides

Apart from synthetic pesticides, several other proper options are also available that work eco-friendly, biodegradable, and cost-effective too; these are microbes or their products used as biopesticides with an exclusive selectivity towards the targeted harmful pests (Berini et al., 2019; Yu et al., 2020). Insect chitinases are one of them, harmless for the plants, which do not possess chitin in their tissues. Resistance to adverse environments and predators is mainly composed of chitin and protein, therefore chitinase can be used to decompose chitin-containing tissues such as insect shells and peritrophic membrane to inhibit the normal physiological activities of pest to achieve pest control (Ye et al., 2019). The peritrophic membrane and insect shells work as a physiochemical barrier to protect against predators and environmental hazards. Microbial secondary metabolites such as hydrolytic enzymes as proteases, lipases and chitinases can be

used alone or better, in combination. These molecules can also be exploited in addition to chemical pesticides with the scope to favour their action thus reducing the introduction and impact of synthetic pesticides on the ecosystem (Berini et al., 2018).

One of the most successful biopesticides is Cry toxins produced by *Bacillus thuringiensis*. After their release from spore crystals in the midgut, they bind to their specific receptors at the apical membrane of midgut epithelial cells and damage the membrane by pore formation, and the cells eventually lyse (Sanahuja et al., 2011). Before Cry toxins can interact with the apical membranes, they have to pass the chitin-containing PM, which forms a physical barrier. It was suggested that chitinases increase the larvicidal effects by perforating the PM. It is tempting to speculate that the use of group IV insect chitinases that are expressed in the midgut might increase the synergistic effects of Cry toxins or the insect virulence of entomopathogenic fungi (Merzendorfer, 2013). Fusion proteins composed of chitinase and Cry1Ac expressed by *B. thuringiensis* strains have been shown to increase slightly toxicity in *Ephestia kuehniel* larvae in comparison to wild-type strains (Driss et al., 2011). The *Pseudomonas sp*. strain TXG6-1 isolated from soil chitinase enzyme (PsChiC) alone had limited insecticidal effect against *Spodoptera litura*, but significantly increased the impact of *Spodoptera litura* multicapsid nucleo-polyhedro virus (SpltNPV) against the larvae (Zhong et al., 2015). Insecticidal activity of three chitinases proteins (ChiA, ChiB, and ChiC) from *S. marcescens* showed insecticidal activity on *Malacosoma neustria* and *H. armigera* larvae (Danişmazoğlu et al., 2015). Also, purified chitinase produced from *Pseudomonas fluorescens* sprayed on fresh tea shoots and fed to *Helopeltis theivora* (tea mosquito bug) resulted in 100% mortality within 96h (Suganthi et al., 2017). Mortality was confirmed through the shrunken abdomen because the midgut of the insect covers with a layer of chitin.

Insect Chitinase dsRNA in Transgenic Plants

The first study that evaluated insect resistance of transgenic plants expressing an insect chitinase was reported in 1998. The bioassays using transgenic corn plants expressing a chitinase gene from *Spodoptera littoralis* against corn borer (*Sesamia cretica*) resulted in about 50% of the insects reared on transgenic corn plants, suggesting that transgenic maize plants have increased resistance against *S. cretica* (Osman et al., 2016).To knockdown chitinase genes in cotton mealybug (*Phenacoccus solenopsis)*, the third-instar nymphs were fed with cotton leaves whose petiole were immersed in a solution with dsRNA (Omar et al., 2019). Cotton mealybug's nymphs pupate as normal upon silencing of group I chitinase gene, but the colour of the pupa became dark and it failed to complete adult eclosion. Chitinase (HaCHI) gene from *H. armigera*, critically required for insect moulting and metamorphosis, was selected as a potential target of RNAi. Continuous feeding on leaves of RNAi lines abundantly reduced the target gene transcripts and hence affected the overall growth and survival of *H. armigera*. Various developmental deformities were also manifested in *H. armigera* larvae after feeding on the leaves of RNAi lines (Mamta et al., 2016). MicroRNA amiR-24, which targets the chitinase gene of *Helicoverpa armigera*, is expressed highly in transgenic tobacco plants, and larvae feeding on them ceased to moult further and eventually died (Agrawal et al., 2015).

Insect Chitinases RNAi

RNAi raises the possibility of directly spraying stabilized dsRNA formulation on host plants to silence the expression of vital genes in herbivorous insect pests. However, the stability of the dsRNA and the cost efficiency in synthesizing such dsRNA-based insecticides may be

problematic. Although many studies have shown promising results for RNAi-based approaches targeting the chitinase, all have been at the proof-of-concept stage (K. Y. Zhu et al., 2016). RNAi to silence the expression of group I chitinase in many insects including the red flour beetle *Tribolium castaneum*, the rice striped stem borer *Chilo suppressalis*, the fruit fly *Drosophila melanogaster*, and the migratory locust *Locusta migratoria*, resulted in common phenotypes were the larvae could not degrade the old cuticle and were trapped in the pupal cuticle, leading to pupation failure and death (Li et al., 2015; Pesch et al., 2017; Su et al., 2016). Silencing of target chitinase genes via dsRNAs mixed with artificial diet were fed to *Mythimna separata* larvae and mortality was observed (Cao et al., 2017). Oral delivery of bacterial dsRNA caused knockdown to target chitinase gene expression and mortality increase and bodyweight decline were observed in *Mythimna separata* (Ganbaatar et al., 2017). Feeding or injecting the dsRNA of group II chitinase in Lepidoptera such as the Asiatic corn borer *Ostrinia furnacalis*, the diamondback moth *Plutella xylostella* and *C. suppressalis* resulted in an ecdysis failure phenotype (Su et al., 2016; B. Zhu et al., 2019). Upon RNA interference to silence the expression of *Tribolium castaneum* ChtIII from the red flour beetle Tribolium castaneum, the newly synthesized cuticular layers were abnormal and contained amorphous fibres (Noh et al., 2018).

Chitinase Inhibitors in Pest Control

Inhibitors of GH18 chitinases demonstrate significant biological activities against insect pests, fungi, and protozoan/nematodal parasites, as they interfere with essential physiological functions. Most of them fall into two categories, carbohydrates-based inhibitors and cyclic peptide inhibitors (W. Chen et al., 2019). Natural products fall into two general categories: the carbohydrate based inhibitors, such as allosamidin, FPS-1, DP2S and GlcNAc(β1,4) Glc disaccharide, mimic the structure of the catalytic reaction intermediate, and the peptide-based inhibitors, such as argifin, argadin, and psammaplin A, mimic the carbohydrate-protein interactions (W. Chen & Yang, 2020). The advances in structural biology of insect chitinases have greatly promoted the development of small molecules targeting them, and a lot of novel inhibitors with diverse scaffolds have been reported. Argifin and argadin are an alternative class of GH18 chitinase inhibitors. Their chemistry is not based on sugars, and their synthesis is less challenging. These molecules are cyclopentapeptides. Argadin more strongly inhibits GH18 chitinases than allosamidin does, whereas argifin exhibits weaker inhibition due to structural differences between the two peptide backbones.

Phlegmacin B1, a microbial secondary metabolite derived from *Talaromyces* sp., displayed inhibitory activity against both OF Chi-h and Of ChtI, with Ki values of 5.5 and 79.3 μM, respectively (L. Chen et al., 2017). Berberine is a plant-originated compound with multiple medicinal and agricultural applications. Recently, berberine was reported to inhibit a broad spectrum of glycosyl hydrolases, such as Of ChtI, and exhibited insecticidal activities by inhibiting the growth and development of O. furnacalis larvae (Duan et al., 2018).

CONCLUSION

The knowledge about insect GH18 chitinases has increased in the past years. It has been proved that chitinase are involved in a variety of insect physiological processes including moulting, digestion and immune responses. Because of the inhibitory effects on the growth and development of insects, insect chitinases have been established as biopesticides and transgenes

in crop protection. Improved understanding of their structure and biochemistry will accelerate their usage in biotechnological processes. Not much research on insect chitinase has been done and lack of structural information on some insect chitinase has prevented the development of potential agrochemicals targeting insect chitinase. Further research on different groups of the GH18 family will improve the understanding of the roles of other insect chitinases.

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