Insect-Derived Chitinases

Hans Merzendorfer

Abstract Insect chitinases belong to family 18 of the glycoside hydrolase superfamily (GH18) and comprise endo-splitting enzymes that retain the anomeric β-(1,4) configuration of the cleavage products. However, some of them have lost their catalytic activity but retained the chitin binding activity and/or possess imaginal disc growth factor activity. In all sequenced insect genomes, multiple genes encode chitinases, which are differentially expressed during development and in various insect tissues. Some of them have nonredundant functions and are essential for growth and development. A characteristic property is their multidomain architecture, which comprises varying numbers of catalytic and chitin-binding domains that are connected by glycosylated serine/threonine linker regions. Based on sequence similarities and domain organization, they have been classified into eight different groups. Insect chitinases have gained increasing interest for use in the biological control of parasites, fungi, and insect pests, and some enzymes have properties that make them highly attractive for biotechnological applications.

Keywords Biopesticides · Carbohydrate binding module 14 · CBM14 · Chitin binding domain · Family 18 glycoside hydrolase · GH18 domain · Insect chitinase · Pest control

Abbreviations

Ac	Acetyl
CBM14	Carbohydrate binding module 14
GH18	Glycoside hydrolase family 18
20HE	20-hydroxyecdysone
IDGF	Imaginal disc growth factor
IPM	Integrated pest management
PM	Peritrophic matrix
STL	Serine/threonine-rich linker

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1 Introduction

Chitin is a β-(1,4)-linked polymer of N-aceytylglucosamine moieties, which is synthesized by a membrane-integral β-glycosyltransferase (chitin synthase; E.C. 2.4.1.16). It is secreted into the extracellular space, where it assembles into microfibrils [1]. Chitin is considered to be one of the most abundant macromolecules in the biosphere. The chitin microfibrils serve as structural scaffolds in cell walls, cuticles, shells, and intestinal peritrophic matrices (PMs). The capacity for chitin production is found in a vast variety of taxonomic groups including algae, fungi, protists, sponges, rotifers, nematodes, arthropods, cuttlefish, brachiopods, and mollusks (Table 1). Less known is the presence of chitin in tunicates and a few bony fishes. However, it seems that the ability to produce chitin has been lost at the root of the deuterostome lineage.

Chitin is particularly present in marine ecosystems because oceanic crustaceans produce most of its biomass (mainly pelagic zooplankton such as krill, which appears in gigantic swarms). In contrast to cellulose, whose biomass has been exploited since the early days of human history, chitin has remained an unused biomass resource for a long time. However, this has changed in recent years because it was recognized that chitin and its derivatives have unique physico-chemical properties that allow a broad spectrum of technical applications [14]. More than 10,000 tons of chitin are extracted every year from crab and shrimp shells, which end up as waste in the seafood industry. Most of the chitin produced

Table 1 Localization and function of chitinous structures in various organisms

Organism	Localization	Biological function	Ref.
α-Protobacteria (Rhizobiales)	Extracellular space	Signaling molecules involved in nodulation of leguminous plants	[2]
Protozoa	Cyst wall	Physical and chemical resistance	[3]
Porifera	Sponge skeleton	Mechanical stabilization	[4, 5]
Fungi	Cell wall	Turgor resistance	[6, 7]
	Yeast bud neck	Stabilization of cell division zone	
	Spore wall	Physical and chemical resistance	
	Septa	separation of mother and daughter cells	
Nematodes	Pharynx	Mechanical breakdown of food	[8]
	Egg shell	Physical and chemical resistance	
Arthropods	Epidermal cuticles	Exoskeletal functions	[1, 63]
	Shells	Protective functions	
	Tracheal cuticle	Tracheal structure and development	
	Peritrophic matrix	Mechanical protection, digestion, anti-infectious barrier	
	Egg shells	Physical and chemical resistance, oogenesis	
Bivalves	Shell	Shell and nacre formation	[9]
Gastropods	Snail-shell, radula	Shell formation, grazing	[10]
	Peritrophic matrix	Multiple protective functions	
Cephalopods	Squid pen	Endoskeleton	[11]
	Cuttlefish bone	Protective lining	
	Stomach cuticle		
Tunicates	Integument (test)	Reinforcement	[12]
	Peritrophic matrix	Multiple protective functions	
Teleost fish	Fin cuticle	Reinforcement	[13]

worldwide is used to obtain glucosamine and various oligosaccharides by acidic hydrolysis. Alkaline hydrolysis results in progressive deacetylation of chitin chains, eventually giving raise to chitosan, a polymer of β-(1,4)-linked glucosamines, which is a more soluble polymer. Chitosan is relatively nontoxic, has antimicrobial properties, and is a cationic polymer at acidic or neutral pH. Accordingly, it has manifold applications in industry. Chitosan-based materials are used as a preservative and dietary supplement in food industry, as a flocculation agent and for the adsorption of heavy metal ions in water treatment, and as antiseptic wound dressings and drug carriers in the pharmaceutical industry. Moreover, chitosan is subject of biopolymer research because the primary amino and the secondary hydroxyl groups are easy to modify. Many chitosan derivatives have been synthesized with the aim of developing intelligent biopolymers to fulfill specific functions. In contrast to cellulose, chitin is less stable in nature due to efficient decomposition. Accordingly, ancient chitin fossils are rare in contrast to cellulose fossils [15]. Biodegradation is mainly accomplished by hydrolytic enzymes called chitinases, which are found in a wide spectrum of organisms including viruses, archaea and eubacteria, protozoa, fungi, plants, and animals, independently of whether or not these organisms are capable of chitin synthesis.

In chitinolytic bacteria that do not synthesize chitin or chitooligomers themselves, chitinases are produced in the course of nutrition, enabling them to use chitinous material as a carbon and nitrogen source. Specifically, species of the genus *Streptomyces* transform insoluble chitin into soluble, metabolizable compounds by means of different chitinolytic enzymes and chitin-binding proteins that mediate adherence to chitinous substrates [16]. In plants, chitinases are thought to be involved in the defense against fungal pathogens, but some chitinase-like proteins lack antifungal activities and seem to play roles during development [17]. In carnivorous plants, they have been detected in the pitcher fluid where they appear to be involved in inducing the trap digesting machinery, in the defense against pathogens, and in chitin decomposition [18, 19]. In chitin-less mammals, chitinases and chitinase-like proteins have been shown to modulate immune responses (reviewed recently by [20]). In all chitin-producing organisms, however, chitinolytic enzymes are essential for maintaining normal lifecycle functions, such as cell division and sporulation in fungi or morphogenesis and metamorphosis in arthropods [21].

Chitinases from various sources have been attracting interest for biotechnological applications in the chemical and pharmaceutical industry because they can convert chitinous material from natural sources (such as crab shells) into usable components. Recombinant insect-derived chitinases may serve as powerful enzymes in such catalytic systems. In addition, chitinases and their inhibitors possess high potential as fungicides for the treatment of mycoses in animal and humans, therapeutic compounds against parasites, and biopesticides for the control of insect pests. This review focuses on insect-derived chitinases and discusses their potential in insect biotechnology (referred to as *yellow biotechnology* in this book).

2 Molecular Properties of Insect Chitinases

Biodegradation of chitin is accomplished by different types of enzymes that include chitinases (EC 3.2.1.14) and β-*N*-acetylhexosaminidases (EC 3.2.1.52), with the latter enzymes acting on chitooligosaccharides that were generated by chitinases cleaving longer chitin chains. Chitinases have been classified into two families of glycoside hydrolases, GH18 and GH19 (http://www.cazy.org; [22]). Although GH18 chitinases are widely distributed in all kingdoms, including insects, GH19 chitinases are restricted to plants, except for a few enzymes reported from viruses, bacteria, nematodes, and arachnids. GH18 and GH19 chitinases show only limited sequence similarities and differ in their catalytic mechanisms. GH18 chitinases and chitinase-like proteins are frequently endo-splitting enzymes but also contain catalytically inactive proteins such as imaginal disc growth factors (IDGFs), stabilin-1 interacting chitinase-like proteins (SI-CLPs), endo-β-*N*-acetylglucosaminidases (ENGases) and chitolectins [23]. The GH18 chitinases perform substrate-assisted catalysis involving a oxazolinium ion intermediate state [24]. This reaction results in the retention of the β-configuration at the anomeric

carbon of the cleavage product. In contrast, GH19 chitinases operate by an acidic catalytic mechanism through an oxocarbenium glycosyl-enzyme intermediate, resulting in the inversion of the anomeric carbon atom (i.e., α-anomeric configuration). The soluble products of the reaction catalyzed by chitinases are small chitooligosaccharides (predominantely chitobiose and chitotriose), which become substrates for exo-splitting β-N-acetylhexosaminidases (family GH20) and remove terminal nonreducing GlcNAc residues. GH18 and GH20 chitinioyltic enzymes frequently act in concert to facilitate chitin degradation.

2.1 Domain Architecture of Insect Chitinases

In insects, chitinases belong exclusively to family GH18 and exhibit mostly endo-splitting activity. They are presumably present in all insect orders. GH18 proteins have been reported in various dipteran, leipidoperan, coleopteran, hymenopteran, and hemipteran species, where they primarily function in remodeling chitinous structures, innate immunity, and development (see below). They exhibit a modular architecture composed of catalytic domains (GH18 domains), cysteine-rich chitin-binding domains (CBM14 or peritrophin A domains) and serine/threonine-rich linker domains (STL).

Insect chitinases have been particularly diversified during evolution, giving rise to eight different phylogenetic groups, which differ in their primary structures and domain architectures (Table 2, [25]). This classification was mainly based on genome-derived amino acid sequences and domain architectures for GH18 protein from the malaria mosquito *Anopheles gambiae*, the fruit fly *Drosophila melanogaster*, and the red flour beetle *Tribolium castaneum*. However, it has to be noted that a more recent study on GH18 proteins encoded in the genome of the pea aphid *Acyrthosiphon pisum* has revised this classification to some extent; this phylogenetic analysis, which included GH18 sequences from more insect species, did not well support groups IV, VI, and VII [26]. This finding is also reflected by the low bootstrap values at the corresponding branches in a phylogenetic tree of GH18 proteins from *A. gambiae*, the honey bee *Apis mellifera*, the silkmoth *Bombyx mori*, *D. melanogaster,* and *T. castaneum* (Fig. 1). Nevertheless, the old classification is used in this review article because most of the functional studies refer to this classification.

Group I chitinases are composed of a signal peptide, and one each of the GH18, STL and CBM14 domains. Interestingly, group I chitinase genes have expanded specifically in mosquitoes by gene duplication events [27]. Group II chitinases are significantly larger than group I chitinases because they are composed of a signal peptide followed by 4–5 GH18, multiple STL, and 4–7 CBM14 domains. Except for dipteran species, which have only 4 GH18 and 4 CBM14 domains, these domains are typically arranged as follows: GH18-CBM14-GH18-(CBM14)$_3$-(GH18)$_2$-CBM14-GH18. By contrast, dipteran group II chitinases are arranged like this: GH18-(CBM14)$_3$-(GH18)$_2$-CBM14-GH18. Some of the GH18 domains

Table 2 Domain architecture of group I-VIII insect chitinases

Group	SP[a]/TMH	GH18	CBM14	STL
I	SP	1	1	1
II	SP	4–5	4–7	Multiple
III	TMH	2	1	0
IV	SP	1	0 (1)	0
V	SP	1[b]	0	0
VI	SP	1	1	1[c]
VII	SP	1	0	1
VIII	TMH	1	0	0

[a] SP, N-terminal signal peptide; TMH, N-terminal transmembrane helix; GH18, catalytic domain similar to family 18 glycoside hydrolases; CBM14, chitin-binding domain belonging to family 14 of carbohydrate-binding modules; STL, serine/threonine-rich linker
[b] GH18 domain catalytically inactive
[c] Very large C-terminal serine/threonine-rich region

(in particular the second GH18 domain) appear to be catalytically inactive because a critical glutamate, which acts as a proton donor in the reaction cycle, is replaced by a nonacidic amino acid [28]. Group III chitinases are typically made up of a single *N*-terminal transmembrane helix (TMH), two adjacent GH18 domains, and one *C*-terminal CBM14 domain. From sequence alignments, it was concluded that the catalytic GH18 domains may have different functions and/or origins. The prediction of an *N*-terminal TMH region suggests that group III chitinases are membrane anchored. This assumption has been supported by analyzing Hi-5 cells expressing TcCHT7 from *T. castaneum*. The enzyme was apparently anchored by the TMH to the surface of the insect cells with the catalytic GH18 domains facing the extracellular space, as revealed by their ability to hydrolyze chitin without lysing the cells [29]. Group IV chitinases constitute a highly divergent group. They are usually encoded by multiple genes in a single insect species. Group IV chitinases are composed of a *N*-terminal signal peptide followed by a GH18 domain. Many of these chitinases (but not all) lack a CBM14 domain. Specifically, *T. castaneum* group IV chitinase genes have expanded in two separate mono-phyletic clades that consist of five and nine genes [26]. Group V chitinases always lack CBM14 domains, but possess an *N*-terminal signal peptide and a GH18 domain, which exhibits amino acid substitutions known to abrogate catalytic activity [30]. This group includes the imaginal disk growth factors (IDGFs), which are required for the proliferation, polarization, and motility of imaginal disc cells [31]. Group VI chitinases are similar to Group I chitinases. However, the *C*-terminal serine/threonine-rich region, which seems to be heavily glycosylated, largely extends the molecular mass of this protein. Group VII chitinases in turn resemble group IV chitinases in overall structure, but phylogenetic analysis revealed that this group is an outlier of group II chitinases. They have an N-terminal signal peptide and a GH18 domain, but they are devoid of a CBM14 domain. Group VIII chitinases have a GH18 domain but lack a signal peptide and a CBM14 domain. Like in group III chitinases, to which they are phylogenetically
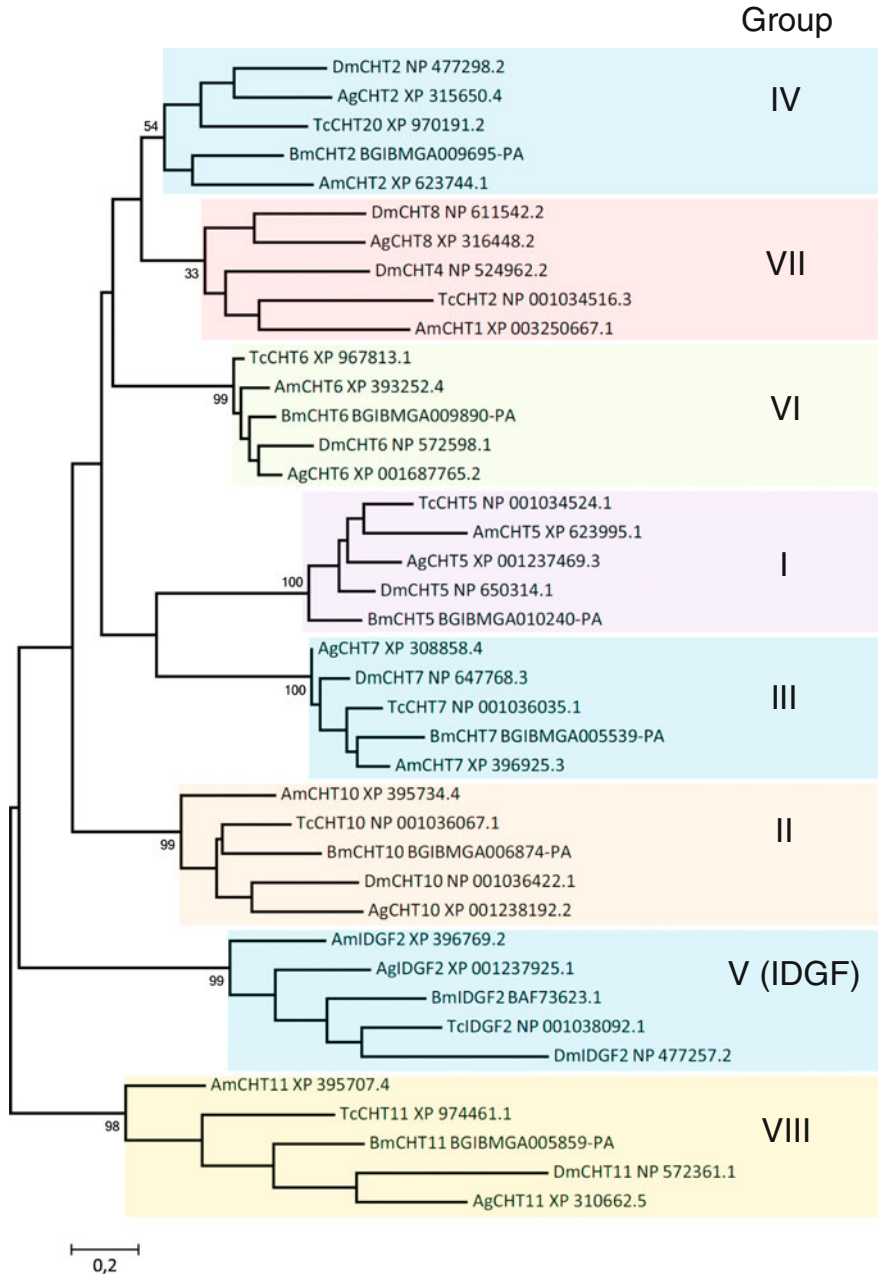
Fig. 1 Phylogenetic tree for various GH18 chitinases and chitinase-like proteins. The maximum likelihood tree was calculated on the basis of a ClustalW alignment (Blosum62) of amino acid sequences for chitinases and chitinase-like proteins. The scale bar indicates an evolutionary distance of 0.5 amino acid substitutions per site. Bootstrap values are given in percentages at the internodes. Different groups of GH18 chitinases are indicated by colored shadings. Am, *Apis mellifera*; Ag, *Anopheles gambiae*; Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Tc, *Tribolium castaneum*. Accession numbers are given after the species abbreviation
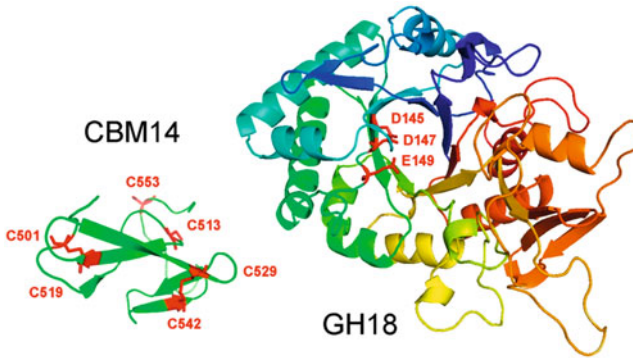
Fig. 2 Homology-based models of the GH18 and the CBM14 domains of a group I chitinase from *Anopheles gambiae* (XP_001237469.3). The positions of catalytic site residues of the GH18 domain and conserved cysteines of the CBM14 domain are indicated with red colors. The serine/threonine rich region linking the GH18 and CBM14 domains has not been modeled and is not included. The $(\alpha/\beta)8$ barrel (TIM barrel) is depicted in the center of the GH18 domain

most closely related, the GH18 domain is preceded by an *N*-terminal TMH. This suggests that these chitinases are also membrane-bound.

2.2 The Catalytic GH18 Domain

The crystal structures for several bacterial, fungal, plant, and mammalian GH18 chitinases have been determined [32–35]. The structural hallmark of the GH18 domain is a $(\beta/\alpha)8$ barrel (TIM barrel) fold, which is also illustrated in the center of a homology-based structural model for the group I chitinase from the malaria vector *A. gambiae* (Fig. 2). Some of the loops extending from the TIM barrel are thought to form a narrow but long substrate-binding cleft, which allows binding of at least five sugar units. In comparison, plant GH19 have a shorter and wider substrate binding site, which is predicted to allow binding of only three sugar units [36]. So far, only one crystal structure of an insect GH18 protein has been determined. Specifically, the structure of the imaginal disk growth factor (IDGF2) from *D. melanogaster* has been solved at a resolution of 1.3 Å [37]. IDGF2 has a classical TIM barrel of GH18 chitinases, however, with two prominent insertions. One insertion is highly conserved in group V chitinases (see below), and resides between the β-4 strand and the α-4 helix, a surface region that is exposed to the solvent. The other localizes between the β-7 strand and the α-7 helix and forms an additional $\alpha + \beta$ domain, which is present in all insect chitinases, although it is highly variable in sequence. Although the first insertion has been associated with proteolytic degradation, the second insertion may determine the cleavage modus of insect chitinases (whether they are endo- or exosplitting or processive enzymes). IDGF2 is catalytically inactive because amino acid substitutions in the conserved

GH18 domain appear to preclude chitin hydrolysis [38]. This is particularly due to the exchange of the catalytic glutamate by a glutamine, which abolishes hydrolytic activity in chitinases (see below).

The GH18 domain comprises four signature sequences, which are highly conserved in insect chitinases: motifs I-IV reside in the β-strand 3, 4, 6, and 8, respectively, and have the consensus sequences K(F/V)M(V/L/I)AVGGW, FDG(L/F)DLDWE(Y/F)P, M(S/T)YDL(R/H)G, and GAM(T/V)WA(I/L)D [25]. Site-directed mutagenesis performed with a recombinant group I chitinase from *Manduca sexta* has established the roles of three acidic amino acids in motif II (D142, D144, and E146, underlined above). The corresponding amino acids D145, D147, and E149 are also shown in the structural model for the group I chitinase of *A. gambiae* (Fig. 2). Although none of these amino acids were required for chitin binding in *M. sexta*, they were more or less critical for catalysis [28]. The glutamate E146 was most important for catalytic activity; its substitution by glutamine or even by the negatively charged aspartate led to a complete loss of enzymatic activity. This finding suggests that E146 acts as an acid/base catalyst in this reaction. The aspartates D142 and D144 are less critical; they seem to function in determining the pKa values of the other two residues and stabilizing the transition state, respectively. Similar to D142, tryptophan W145 within motif II appears to be necessary for optimal enzyme activity but is not required for chitin binding [39].

2.3 The Chitin-Binding CBM14 Domain

The CBM14 modules of chitinases are widely distributed chitin-binding domains, which are also found in insect PM proteins (PMPs) and cuticle proteins analogous to peritrophins (CPAPs) [40]. The chitin-binding function of the CBM14 has been shown in several cases [41]. The only structure available for an invertebrate CBM14 motif is that for tachycitin, a 73-residue polypeptide with antimicrobial activity from hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Nuclear magnetic resonance spectrometry revealed that the polypeptide consists of an *N*-terminal part made of a three-stranded β-sheet and *C*-terminal part made of a two-stranded β-sheet following a short helical turn [41]. Five disulfide bridges formed between 10 cysteines stabilize this β-sandwich structure. Some of the β-sheets form a hydrophobic binding pocket, which involves conserved polar and hydrophobic amino acid residues [42]. In contrast, insect CBM14 domains have only six conserved cysteines, presumably forming three disulfide bridges. The characteristic spacing between these cysteines is $CX_{13–20}CX_5CX_{9–19}CX_{10–14}CX_{4–14}C$ [40, 43]. Figure 2 shows a structural model of the CBM14 domain from a group I chitinase of *A. gambiae*, highlighting these conserved cysteines. The CBM14 domain is thought to enhance the affinity of the chitinase for its polymeric insoluble substrate, so that these enzymes are more effective on chitin than on chitooligosaccharides [44].

2.4 The Serine/Threonine-Rich Linker Region

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. This may affect protein stability, particularly in protease-rich environments, such as the molting fluid or the gut lumen, where insect chitinases frequently are operating [44]. The first step of the mucin-type O-glycosylation is catalyzed 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. Interestingly, 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 [45]. The STL region is predicted to be an unfolded polypeptide. Because it is therefore not possible to model the structure of this region, the GH18 and CBM14 of the *A. gambiae* chitinase domains were drawn separately in Fig. 2.

2.5 Enzymatic Properties of Insect Chitinases

In a comprehensive enzymatic study, Zhu et al. compared the properties of group I, IV, or V chitinases from *T. castaneum*, *D. melanogaster,* and *M. sexta*. They expressed the chitinases in Hi5 cells in insect cells using a baculoviral system and purified them by Ni–NTA or DEAE-Sepharose chromatography [38]. Subsequently, the enzymatic and chitin-binding properties were compared. Except for group V proteins, which comprise the group V IDGFs, all chitinases exhibited chitinolytic activities. However, the enzymes from different groups differed with respect to their ability to bind chitin, immunological cross-reactivity, kinetic properties, pH dependency of activity, and their preference for oligomeric or polymeric substrates. Group I chitinases cleaved both polymeric and oligomeric substrates, whereas group IV chitinases exhibited no and only little chitinolytic activity for oligomeric substrates. The tested group I chitinases showed two optima at approximately pH 6 and 9, whereas group IV chitinase either had only a single optimum at pH 6 or were highly active over a broad pH range from pH 4 to 9. All tested chitinases and chitinase-like proteins, including the group V IDGFs, bound tightly to colloidal chitin.

All catalytically active insect GH18 chitinases studied so far have been shown to be endo-splitting enzymes that cleave chitin or chitooligosaccharides comprising at least three sugar moieties. The recombinant 65-kDa chitinase from *Bombyx mori* preferentially cleaves the β-(1,4) glycosidic linkage of GlcNAc oligosaccharides after the second position from the nonreducing end, retaining the β-anomeric configuration of the product [46]. In contrast, the chitinase from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which may

have a bacterial origin but is expressed in virus-infected insect cells, exhibits both endo- and exo-splitting activities [47]. However, as in the case of insect chitinases, cleavage of olilgosaccharides occurs at the linkage between the second and third sugar moiety from the nonreducing end [48]. The catalytic mechanism of insect chitinases has not been explained in detail, but it is likely that—like other non-insect GH18 chitinases—they act through substrate-assisted catalysis involving an oxazolinium intermediate state, as described above.

Besides their chitinolytic activity, many bacterial, fungal, plant, and mammalian GH18 chitinase possess transglycosylation activity, which allows the formation of new glycosidic bonds between donor and acceptor saccharides. In retaining glycoside hydrolases, the transglycosylation reaction occurs through a double-displacement mechanism using another acceptor molecule (carbohydrate or an alcohol) instead of water [49]. This type of reaction leads to the interconversion of oligosaccharides of different compositions and lengths. Oligosaccharides of specific compositions and length, however, are attracting increasing interest in the food and pharmaceutical industries due to their potential use as therapeutics, prebiotics, or in plant protection [50–53]. Therefore, transglycosylation is considered to have a high potential in generating defined oligosaccharides because their chemical synthesis is highly challenging. The development of methods for enzymatic synthesis of oligosaccharides may include insect-derived GH 18 chitinases because it is likely that many of these enzymes exhibit transglycosylation activity.

2.6 Insect Chitinase Gene Expression During Development

In insects, chitinase encoding genes are differentially expressed during development, and the expression of some genes is restricted to certain tissues. The expression profiles have been determined in detail by reverse-transcription and qualitative polymerase chain reaction for *T. castaneum*, *A. gambiae,* and *A. pisum* [26, 54, 55].

In *T. castaneum*, the genome harbors in total 22 genes encoding chitinases and chitinase-like proteins [55]. The group I, II, III, and V genes (*TcCHT5*, *TcCHT10*, *TcCHT7,* and *TcIDGF2/4*, respectively) were found to be expressed throughout all stages of development. Transcripts for the group VI gene *TcCHT6* were detected in all stages except for adults, and transcripts for the group VII gene *TcCHT2* were found in all stages except for embryos and adults. The group VII gene *TcCHT11* was found to be expressed in all but penultimate instar larvae. Transcripts for all 14 group IV genes (*TcCHT4*, −8, −9, −12 to −22) were detectable in the feeding stages (larvae and adults). However, expression of two of these genes (*TcCHT4* and *TcCHT16*) was also found at lower levels in pharate pupal and pupal stages. Moreover, the group IV genes (except for *TcCHT4*) were found to be expressed exclusively in the larval midgut, and gene expression varied in different gut

regions. The latter finding suggests that group IV chitinases act on the chitin network of the PM and/or dietary components.

Overall, there are similarities and differences between the expression profiles of *T. castaneum* and *A. gambiae,* the latter of which has 20 chitinase genes [54]. Expression of group I, II, III, and V genes (*AgCHT5−1 to−5, AgCHT10, AgCHT7* and *AgIDGF2/4*, respectively) was detectable in all developmental stages at various levels, from embryos (except for *AgCHT10*) to adults. The two IDGF genes (*AgIDGF2* and *−4*) were constitutively expressed at higher levels. The expression of the eight group IV genes of *A. gambiae* (*AgCHT4, −8, −9, −12, −13, −16, −23, −24*) showed various levels of expression in the different developmental stages tested. Notably, the group IV chitinases *AgCHT12, AgCHT13* and *AgCHT23* were almost exclusively expressed in the larval stages, whereas *AgCHT8* was expressed in pupae and adults. All group IV genes were detected in the gut, either in the foregut (*AgCHT4, −12, −16, −23*), midgut (*AgCHT9, −13, −16, −23*), or hindgut (*AgCHT9, −16, −23*), except for *AgCHT24*, which was not tested for its expression in the gut. The group VII gene was also found to be specifically expressed in the *AgCHT4* in the foregut.

In *A. pisum,* the expression of *ApCHT2 (*group IV), *−3* (group VI), *−4* (group III), and *−8* (unclassified) was found to be highly upregulated in embryonic tissues [26], and that of two distinct chitinase-like genes, *ApCHT6* (group IV) and *Ap-ENGase* (ENGase), was significantly higher in the midgut than in other tissues (classification according to [25]).

Upregulation of the expression of chitinase-encoding genes involves transcriptional control mechanisms mediated by molting hormones. The injection of 20-hydroxyecdysone (20HE) into ligated larvae of *M. sexta* and *B. mori* stimulates the activity of chitinases in the integument [56, 57]. This increase in chitinolytic activity correlates with raised transcript levels that have been measured for group I chitinase genes from *M. sexta* following 20HE injection [58]. The 20HE-induced increase of transcript levels was suppressed when fenoxycarb, a juvenile hormone mimic, was topically applied. Similar results have been reported for chitinases from the silkworm *B. mori* [59], the spruce budworm *Choristoneura fumiferana* [60], the mealworm beetle *Tenebrio molitor* [61], and even from crustaceans such as the Chinese shrimp *Fenneropenaeus chinensis* [62]. Thus, 20HE-dependent control of chitinase gene expression and chitinolytic activity appears to be widely distributed among insects and possibly also other arthropods. Whether gene activities of chitinase-encoding genes are directly or indirectly controlled has not been elucidated yet.

2.7 Functions of Chitinases in Insects

The finding that gene expression of chitinases and chitinase-like proteins varies between different developmental stages and tissues indicates that chitinases have distinct functions. This conclusion was supported by an exciting study in *T.*

castaneum using systemic RNA interference to systematically knockdown transcripts for group I (*TcCHT5*), II (*TcCHT10*), III (*TcCHT7*), IV (*TcCHT8*, −*14*, −*16*), V (*TcIDGF2* and −*4*), VI (*TcCHT6*), and VII (*TcCHT2*) chitinase or chitinase-like protein encoding genes [55]. Injection of dsRNA specific for *TcCHT5* resulted in molting defects and death only during pupal-adult molting, whereas that for *TcCHT10* caused arrest at all larval–larval, larval–pupal, and pupal–adult moltings as well as defects in egg hatching. When penultimate instar larvae were injected with dsRNA specific for *TcCHT7*, they completed larval–larval and larval–pupal molts, but the pupae failed to contract their abdomens and to fully expand their elytra. Injection of dsRNA for *TcIDGF4* into penultimate or last instar larvae resulted in death of the insects during adult eclosion. The injection of dsRNAs for the genes *TcCHT2*, *TcCHT6*, *TcCHT8*, *TcCHT14*, *TcCHT16,* and *TcIDGF2* failed to result in observable phenotypes.

Chitinase and chitinase-like proteins are thought to have important functions during the growth and development of insects [21, 25]. During molting, they assist in the degradation of inner parts of the chitinous endocuticle, a process required to shed off the old cuticle (exuvia). In the intestinal tract, they have important roles in digestion of chitin-containing food (including exuvia, which is frequently eaten after molting) and in the turnover of the chitin-containing PM, which lines the midgut epithelium. It protects the midgut epithelium from abrasive particles, divides the gut lumen into distinct digestive compartments, and acts as an anti-infectious barrier [63]. In addition, chitinase-like proteins promote cell proliferation and have functions in the innate immune system. From the expression profiles and the RNAi studies, several important conclusions on the functions of chitinases in insects can be drawn.

Group I–II chitinases act in the degradation of the endocuticle during molting with activities that have different effects on larval–larval, larval–pupal, and pupal–adult molts. Partially, these chitinases may have redundant functions. They are secreted into the molting fluid, which is known to contain proetolytic and chitinolytic activities, and degrade the inner parts of the old cuticle to allow molting [64]. Interestingly, the chitinases appear not to be excluded from the newly synthesized cuticle by an impermeable layer (also called cuticulin or envelope) as previously assumed, because a recent study performed in *T. castaneum* provided evidence that the new cuticle is protected from chitinolytic activity by a cuticle-organizing protein named after the *Drosophila* mutant Knickkopf [65].

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. They appear not to be required for molting but for processes that occur immediately after pupation such as abdominal contraction and the extension of wings and elytra. How they accomplish these function is not known.

Group IV chitinases appear to have functions in the intestinal system because they are only expressed in different parts of the gut. This suggests that they are involved in the degradation of chitinous material either assimilated with food or as part of the PM. Some of these gut-specific chitinases may also have immune functions. For example, the group IV chitinase GmCHT1 from the tsetse fly

Glossina morsitans morsitans contains a GH18 domain and a CBM14 domain but lacks an STL region. The *GmChT1* gene is expressed in the fat body and in the milk glands of pregnant female flies. The tsetse flies undergo viviparous reproduction and the chitinase becomes transmitted to the larvae by feeding of a milk-like substance secreted in the mother's uterus. Once taken up by the larvae, it may have immunological function in the larval gut, protecting against fungal pathogens [66].

Groups V chitinase-like proteins (IDGFs) are predominantly expressed in embryonic yolk cells and fat body; they may act as chitolectins binding to cell surface receptors. They have been shown to promote cell lineages derived from *Drosophila* imaginal discs in cooperation with insulin-like growth factor [31]. In hemocyte and fat body cell lines derived from the cabbage armyworm, *Mamestra brassicae*, however, they stimulated cell proliferation independently from the presence of insulin-like peptides [67]. Next to their function in cell proliferation, some IDGFs also have immune functions. In *A. gambiae* two group V proteins, AgBr1 and AgBr2 are secreted into the hemolymph after challenging the mosquitoes by bacterial infections. AgBr1 and AgBr2 are proteolytically processed after exposure to bacteria or peptidoglycans, as reported for some other IDGFs [68]. Finally, honeybee salivary glands secrete IDGF4 into the royal jelly and honey. This finding implies that IDGF4 might affect growth, physiology, or even behavior of other bees in the hive [69]. In contrast to group I–V chitinases, the functions of group VI–VIII chitinases have not been addressed so far.

3 Chitinase Inhibitors in Human Health and Pest Control

3.1 Pseudosugars, Cyclic Peptides, and Purine Derivatives

Inhibitors of GH18 chitinases demonstrate significant biological activities against insect pests, fungi, and protozoan/nematodal parasites, as they interfere with essential physiological functions. Among the most potent natural inhibitors of chitinases are allosamidin, argifin, and argadin [70]. Allosamidin is one of the best characterized inhibitors. It was isolated from *Streptomyces* species and exhibits an inhibitory activity against GH18 chitinases, while being inactive on GH19 chitinases. It is a pseudosugar (pseudotrisaccharide) consisting of two units *N*-acetyl-D-allosamine and one unit of an aminocyclitol derivative (Fig. 3). Allosamidin is thought to specifically block the transition state of GH18 chitinases. The compound has been shown to inhibit chitinases from various insects, including the silk moth *B. mori*, the nonbiting midge *Chironomus tentans,* and the green peach aphid *Myzus persicae* [71–73]. When applied to larvae of *B. mori*, *Leucania separata* (common armyworm), *Tineola bisselliella* (webbing cloth moth), or *Lucilia cuprina* (Australian sheep blowfly), it inhibited larval to pupal molt [74, 75]. Feeding *Phlebotomus papatasi* sandflies, a vector for the *Leishmania* parasite, with allosamidin supplemented blood led to a thickening of the PM from the
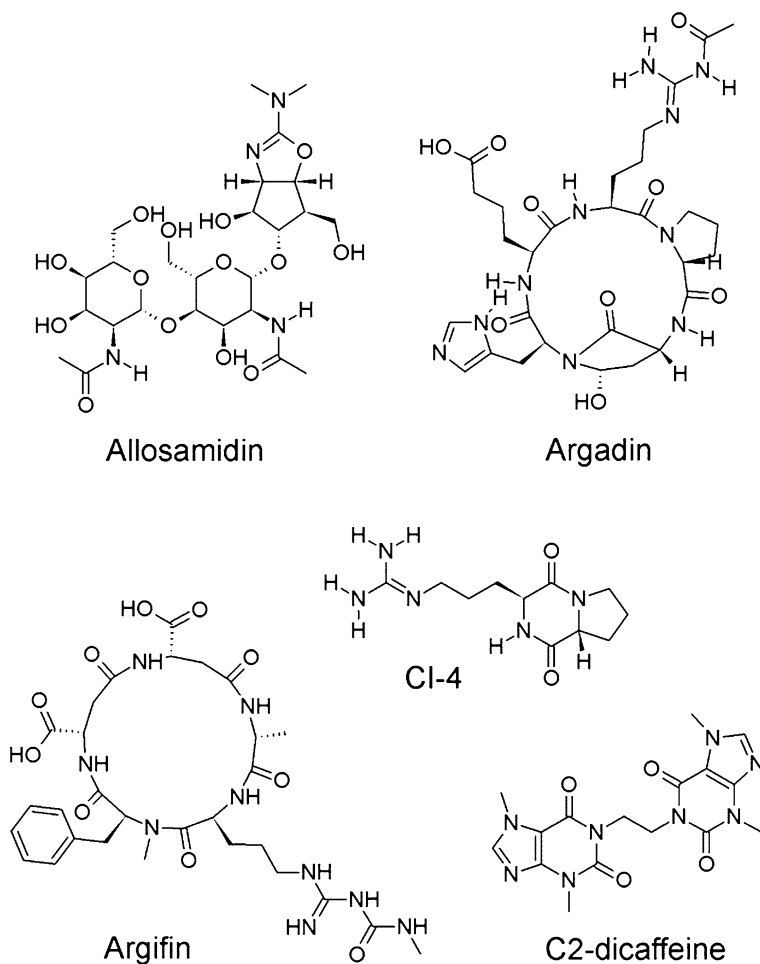
Fig. 3 Chemical structures of GH18 chitinase inhibitors. The depicted inhibitor structures include the pseudotrisaccharide allosamidin, the cyclopentapeptides argadin and argifin, the small cylic peptide CI-4, and the purine-derivate C2-dicaffeine. Chemical structures were obtained from the Binding database and visualized with ACD ChemSketch 8.0

midgut. It also prevented early parasite mortality seen in infected flies, suggesting an important role of the PM as an anti-infectious barrier [76].

Similar results were obtained when allosamidin-supplemented blood was fed to female yellow fever mosquitoes [77]. This treatment resulted in the formation of an atypical thick PM, whereas the addition of exogenous chitinase completely blocked PM formation. Most notably, allosamidin inhibits the transmission of the *Plasmodium* ookinetes by blocking the parasite's chitinase, which facilitates penetration of the mosquito's PM [78, 79]. Although allosamidin has evidently a high potential as an active ingredient of antiparasite drugs and insecticides, its total

synthesis is complex and expensive, thus currently preventing its use in parasite and pest control regimes [70]. The synthesis of allosamidin analogs provided insights into the structural requirements for chitinase inhibition. Blattner et al. evaluated different allosamidin analogues to provide insights into structure–activity relationships [75, 80]. For a chitinase from *Chironomus tentans*, the removal of a single *N*-acetylallosamine residue did not impair inhibitory activity and glucosamine can be exchanged against allosamine without any negative effect [80]. Similar results were obtained when testing the insecticidal activity for *Tineola bisselliella* and *Lucilia cuprina* larvae [75]. The authors came to the important conclusion that the *β*-(1,4) linkage between the sugar or pseudosugar moieties is necessary for chitinase inhibition and insecticidal activity. Thus, *β*-(1,4)-gluco disaccharides are attractive candidate molecules for chitinase inhibition. Based on these findings, Dusssouy et al. reported that GlcNAc-*β*-(1,4)Glc disaccharides containing 2-O-acetyl and/or 6-sulfate groups are highly potent inhibitors of insect chitinase activity and that they have strong aphidicidal activities when tested on hemipteran *Myzus persicae* [81]. However, the latter effects could not be explained exclusively on the basis of chitinase inhibition.

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 (Fig. 3), which were isolated from mycelia of *Gliocladium* and *Clonostachys* species, respectively [82, 83]. It has to be noted, however, that the taxonomic classification of these fungi is uncertain and needs to be reassessed using appropriate DNA markers [84]. Both inhibitors mimic the interactions of GH18 chitinases with chitooligosaccharides and interact with side chains D142, E144, and Y214 in the active site required for catalytic activity [85]. Argadin more strongly inhibits GH18 chitinases than allosamidin does, whereas argifin exhibits weaker inhibition due to structural differences between the two peptide backbones. The detailed structural information available for these types of inhibitors allowed identification of a tiny nine-atom active fragment of argifin, which is a micromolar inhibitor of GH18 chitinase from *Aspergillus fumigatus* [86]. Another peptide-based inhibitor is the small cyclic peptide CI-4 (cyclo (L-Arg-D-Pro)) (Fig. 3), which in contrast to the cyclopentapeptides blocks chitinase activity by structurally mimicking the reaction intermediate [87]. It was first isolated from the marine *Pseudomonas* species [88]. A screening of a compound library of 880 drug molecules identified recently three xanthine derivatives, theophylline, caffeine, and pentoxifylline as moderate inhibitors of bacterial, fungal, and human family 18 chitinases [89]. Moreover, a fragment-based, computer-aided approach to screen commercially available chemical structures allowed these investigators to identify a xanthine-derivative (C2-dicaffeine) as GH18 chitinase inhibitor that acts in the low micromolar range [90]. Some of the latter chitinase inhibitors may also be active on insect-derived enzymes.

3.2 Antibodies to Chitinases Block Parasite Transmission

Malaria is one of the most important parasitic diseases, affecting hundreds of millions of humans per year, with at least 1 million deaths. The parasite is transmitted by female *A. gambiae* mosquitos that inject infectious sporozoites while taking a blood meal. Before the mosquitoes can transmit the parasite, they have to get infected themselves by ingesting male and female gametocytes from a blood meal of a malaria-infected person. After mating, the resulting zygote elongates into the invasive motile form, which is called ookinete. The ookinete must penetrate the PM before it can invade the midgut epithelium to reach the hemolymph site, where it eventually develops into sporozoite-forming oocysts [91]. The ookinete produces a GH18 chitinase that facilitates traversal of the chitin-containing PM [92]. Both the targeted disruption of the *Plasmodium* chitinase-encoding genes and the inhibition of its chitinolytic activity by allosamidin led to a significant reduction of infectivity in *A. gambiae* mosquitos [78, 79, 93]. Because the *Plasmodium* chitinase is a potential target for blocking malaria transmission, the idea of developing antibodies to neutralize chitinase activity emanated. A monoclonal antibody (1C3-MAb) was generated against the recombinant *P. falciparum* chitinase PfCHT1. The 1C3-MAb indeed inhibited PfCHT1 activity and significantly reduced the infectivity of the *P. falciparum* parasite in mosquitoes [94, 95]. A recombinant single-chain antibody (scFv) derived from 1C3-MAb hybridoma cells also significantly reduced *P. gallinaceum* parasite transmission to mosquitoes by inhibiting the orthologous PgCHT2 enzyme [96]. The fact that the recombinant 1C3-MAb recognizes PfCHT1 as well as PgCHT2 allows using the avian parasite *P. gallinaceum* as a model system for elucidating the role of chitinases for invasion of the midgut epithelium. More importantly, however, transgenic strains of *Anopheles stephensi* expressing two scFvs directed to the parasite chitinase did not show the development of sporozoites after challenging them with *P. falciparum* [97]. The expression of a dual scFv transgene can completely block parasite development without affecting the mosquito's fitness. Interestingly, antibodies to microfilarial chitinases from *Brugia malayi,* a nematode which causes lymphatic filariasis in humans, also block parasite transmission [98].

4 Recombinant Chitinases in Pest Control

4.1 Chitinases Enhance Virulence of Baculoviral Biopesticides

Chemical pest management strategies may cause contaminations with toxic compounds that have problematic effects on the environment and eventually affect animal and human health. In addition, the extensive use of pesticides generates resistances that render many of the widely used insecticidal compounds ineffective. Therefore, alternate concepts of integrated pest management (IPM) have been

developed, including nonchemical strategies to control insects. A major objective of IPM is applying different methods to produce synergistic effects [99]. Pest populations are not controlled by attempting eradication but rather by reducing the number of pests to levels that do not cause economic damage. The methods that are combined in IPM strategies include environmentally safe and selective chemical pesticides, use of crop cultivars with pest resistance, intelligent cultivation practices, physical methods, natural biopesticides, and biocontrol with natural predators, parasites, and microbial organisms and agents.

Baculoviruses have been recognized as efficient biopesticides for the control of lepidopteran insect species in agriculture and forestry. The majority of baculoviruses used as biopesticides belong to the group of nucleopolyhedroviruses (NPV; α-baculoviruses); some are granuloviruses (GV; β-baculoviruses). These viruses exhibit high species-specificity and lack negative impact on plants, mammals, birds, fish, or even on nontarget insects. This is especially desirable when beneficial insects need to be protected or when an ecologically sensitive area is being treated. However, the narrow host range of baculoviruses and their slow working mode limit their use in pest control to some extent. Baculoviruses encode proteases and chitinases, which specifically degrade the protective PM in the midgut of infected insects; this presumably increases virulence and infectivity and mediates liquefaction of the integument. For instance, the *Mamestra configurata* (bertha armyworm) NPV (McNPV-A) encodes a metalloprotease enhancin, which evidently degrades PM proteins [100]. Chitinases are encoded by many baculoviral genomes, including those of the *Autographa californica* multicapsid NPV (Ac-MNPV) [101], *Spodoptera litura* multicapsid NPVs (SlMNPVs) [102], *Helicoverpa armigera* singlecapsid NPV (HaSNPV) [103], *Antheraea pernyi* NPV (ApNPV) [104], *Epiphyas postvittana* NPV (EpNPV) [105], *Orgyia pseudotsugata* multicapsid NPV (OpMNPV) [106], *Bombyx mori* NPV (BmNPV) [107] and the *Cryptophlebia leucotreta* granulovirus (ClCV) [108]. The function of the Ac-MNPV chitinase ChiA has been extensively studied, both in vitro and in vivo. The viral ChiA was expressed in *Sf*9 cells and purified using a pepstatin-aminohexyl Sepharose column [48]. Careful analyses of enzyme activity and cleavage products revealed that the mode of action was similar to that of chitinase A from the enterobacterium *Serratia marcescens* (SmChiA). It hydrolyzed the second β-(1,4) glycosidic bond from the non-reducing end of chitooligosaccharide substrates and acted also on solid β-chitin in a processive mechanism.

The viral chitinase seems to promote liquefaction of the larvae, a process that is usually observed in the late period of a baculoviral infection and involves partial degradation of the integument by proteolytic and chitinolytic activities. The processive working mode of ChiA may be especially advantageous for liquefaction. In AcMNPV-infected larvae of *Trichoplusia ni*, liquefaction depends, next to ChiA, on the baculoviral endoprotease cathepsin (v-Cath) [47], which physically interacts with the viral ChiA before being released from infected cells [109]. Site-directed mutagenesis of the ChiA active site residues D311 and E315 in the recombinant baculovirus caused a reduction in chitinolytic activity and an attenuated liquefaction of host larvae, indicating that ChiA is an important virulence factor of baculoviruses

[110]. Interestingly, ChiA contains a C-terminal KDEL motif retaining the protein in the endoplasmic reticulum [111]. When the nucleotide sequence encoding the KDEL motif was deleted in the viral chitinase gene, and subsequently *T. ni* cells were infected with the recombinant virus, the chitinase localized at the plasma membrane and was secreted into the supernatant of the culture medium. Consistently, the biological activity was enhanced in *T. ni* larvae infected with the recombinant virus encoding a KDEL-deficient chitinase version [112]. Why ChiA is retained in the endoplasmic reticulum to some extent is unknown, but it may attenuate liquefaction until enough polyhedra have been produced to embed the virions. The observed interaction of ChiA with v-Cath may be required for coordinating proteolytic and chitinolytic activities for liquefaction [109].

The potential of the ChiA chitinase as a biopesticide has been further explored by Rao et al. [113]. The *ChiA* gene was expressed in *Escherichia coli* cells and purified by affinity chromatography. When isolated peritrophic matrices from *B. mori* larvae were incubated with increasing amounts of the recombinant chitinase, a dose-dependent increase in PM permeability and number and size of PM perforations was observed. Feeding of the recombinant chitinase to *B. mori* larvae resulted in 100 % mortality at high doses, whereas lesser doses reduced larval growth. This finding implies that optimization of the baculoviral genome to increase chitinase expression by using alternate promoters may provide a powerful means for the development of environmentally safe biopesticides [114]. As insect chitinases have been optimized during evolution to degrade cuticle and peritrophic matrices, the insecticidal activities of baculoviruses may be improved by generating recombinant viruses encoding an insect chitinase with desired properties. One example supporting this hypothesis will be discussed in Sect. 4.3.

4.2 Chitinases Increase Insecticidal Activities of Biopesticides

Among the most successful biopesticides are the δ-endotoxins (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, so that the cells eventually lyse [115]. Before the 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, improving the accessibility of the Cry toxin to the epithelial membrane. An obvious idea was to coapply Cry toxins and chitinases, which indeed increased the insecticidal effect of *B. thuringiensis* on *Choristoneura fumiferana* larvae [116]. Also, the co-application of *B. thuringiensis* spore crystal suspension together with chitinolytic bacteria yielded significant synergistic insecticidal effects against *Spodoptera littoralis* larvae [117]. It was also shown that the addition of *Serratia marcescens* chitinase protein preparations to Cry toxin preparations caused synergistic toxic effects in *Spodoptera littoralis* larvae [118]. More recently,

recombinant plasmids containing the cry1Ac gene from *B. thuringiensis* and chitinase-encoding genes from tobacco *(Nicotiana tabacum)* were generated to transform acrystalliferous *B. thuringiensis*. The transformed bacteria exhibited significant chitinase activity, and when the insecticidal activity of the transformed bacteria was evaluated using *Helicoverpa armigera* larvae, it was more than tenfold higher compared to the bacteria that produced only the Cry toxin [119]. Even fusion proteins composed of a chitinase and Cry1Ac expressed by *B. thuringiensis* strains have been shown to increase slightly toxicity in *Ephestia kuehniella* larvae in comparison to wild-type strains [120].

Similar approaches have been undertaken to increase the insect virulence of entomopathogenic fungi that are used in biocontrol of insect pests. The genome of the entomopathogenic fungus *Metarhizium anisopliae* encodes several chitinases. One of these chitinases, chitinase 2 (Chi2), is involved in the pathogenicity of this fungus. Strains that overexpress Chi2 showed higher efficiency to kill its host, the cotton stainer bug *Dysdercus peruvianus*, whereas strains defective in the gene encoding Chi2 decreased infectivity of the fungus [121]. Likewise, a transgenic *Trichoderma konigii* strain expressing the chitinase 42 from *Metarhizium anisopliae* showed insecticidal activity against the Asian corn borer, *Ostrinia furnacalis* and the silkworm *B. mori* [122]. More recently, Araújo et al. showed that feeding of a bacterial chitinase disrupts the PM and reduces fecundity of female sandflies (*Lutzomyia longipalpis*), which are important vectors of visceral leishmaniasis [123]. Again, 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.

4.3 Insect Chitinases as Biopesticides

To date, only a few studies have been reported that directly used insect-derived chitinases as biopesticides for the control of pests. Gopalakrishnan et al. have constructed a recombinant AcMNPV baculovirus expressing a group I chitinase from *M. sexta* under the control of the polyhedrin promoter [124]. When the fourth instar larvae of *M. sexta* or *Spodoptera frugiperda* were injected with the recombinant virus, the chitinase was detectable in large amounts in the hemolymph. Liquefaction of infected *S. frugiperda* larvae occurred significantly earlier than when the insects were infected with a wild-type virus, indicating increased insecticidal activity. Similarly, a recombinant AcMNPV expressing a group III chitinase from the hard tick *Haemaphysalis longicornis* showed bioacaricidal effects against ticks when topically applied [125]. A mixture of recombinant virus and the purified recombinant protein was found to be more efficient in killing the ticks than the recombinant virus and pure chitinase alone. Mice that were immunized with the recombinant purified chitinase from *H. longicornis* developed a specific protective anti-tick immune response affecting tick molting [126]. These findings suggest that recombinant chitinases may be efficient antigens for

vaccination to biologically control ticks. Similar strategies may be helpful also to develop chitinase-based vaccines that block the transmission of leishmaniasis by sandflies [127, 128]. Interestingly, even the purified recombinant chitinase has insecticidal effects. Fitches et al. produced a recombinant chitinase from the tomato moth, *Lacanobia olerace* in the yeast, *Pichia pastoris*, and injected it into *L. oleracea* larvae [129]. They observed 100 % mortality already at a comparable low dose and a reduction in cuticle thickness when injected prior to molting. When fed orally, larval growth and food consumption were reduced.

Insect-derived chitinases may become also important for bioprocessing industries that, for instance, produce chitooligosaccharides with defined chemical properties. They have been expressed in different heterologous systems and purified in reasonable amounts. However, biotechnological processes will require cost-efficient large-scale production of highly active preparations. Most of the studies expressing insect chitinases so far used *E. coli* strains [102, 130–132] or insect cells [38, 39, 133]. Because insect chitinases may likely exhibit eukaryotic posttranslational modifications that improve folding and activity, cost-efficient eukaryotic cells such as the yeast *Pichia pastoris* could be the right expression system of choice to produce a high yield of stable and highly active preparations of genetically optimized enzymes for bioprocessing applications.

5 Chitinase Transgenes in Crop Protection

5.1 Transgenic Plants Expressing Chitinases

Because of the nematocidal, fungicidal and insecticidal properties of chitinases, transgenic plants were generated that heterologously express GH18 and GH19 chitinases from various baculoviral, bacterial, and insect sources to increase plant resistance [25, 134–137]. The high potential for transgenic plants expressing chitinases for use in plant protection against herbivorous insects has been established. The first study that evaluated insect resistance of transgenic plants expressing an insect chitinase was published in 1998. In this study, a transgenic tobacco was generated producing a group I chitinase from *M. sexta* [138, 139]. Although expression levels appeared to be generally low and the recombinant chitinase was truncated, larvae of the tobacco budworm *Heliothis virescens* were impaired in their growth when feeding on the leaves of the transgenic tobacco. Moreover, the larvae showed a higher mortality and caused less feeding damage compared to larvae fed on control plants. However, no effects on growth and mortality were observed for *M. sexta* larvae. This result may be explained by the greater thickness of the PM in the case of *M. sexta* larvae, indicating that susceptibilities to toxic chitinases may vary among different insect species. However, when the leaves of transgenic tobacco were sprayed with a formula containing a sublethal dose of Cry toxins from *B. thuringiensis*, a synergistic insecticidal effect

was observed in *H. virescence* larvae, as well as *M. sexta* larvae. The *M. sexta* chitinase was also expressed in transgenic cotton, which reportedly conferred insect resistance in the field. The chitinase was detectable in leaf tissues using antibodies developed to the recombinant enzyme [132].

To increase toxic effects on feeding insects, the gene encoding a scorpion insect toxin from *Buthus martensii* (BmkIT) was expressed in combination with the *M. sexta* chitinase in transgenic *Brassica napus* [140]. Some of the obtained transgenic plants showed high expression levels for both chitinase and scorpion toxin, and conferred significant resistance to the diamondback moth, *Plutella maculipenis*. Synergistic effects for the chitinase and the scorpion insect toxin have not been tested in this study; hence, it is not clear to what extent the chitinase contributes to the observed mortality. In an attempt to improve the resistance of papaya plants to the carmine spider mite (*Tetranychus cinnabarius*), McCarthy et al. generated a transgenic line of *Caprica papaya* expressing the *M. sexta* chitinase [141]. The transgenic papaya line showed increased tolerance to the spider mites under field conditions.

Likewise, chitinases from baculoviral sources have been used to generate transgenic plants. Although Shi et al. observed no insecticidal effects when *H. virescens* larvae were fed on transgenic tobacco leaf tissue expressing the baculoviral chitinase ChiA from AcMNPV, they reported significant tolerance against the fungal pathogen *Alternaria alternata* [142]. In contrast, Corrado et al. showed that transgenic tobacco plants expressing an active ChiA protein from AcMNPV are significantly protected against fungal pathogens (*Botrytis cinerea* and *Alternaria alternate*) and lepidopteran larvae (*B. mori*). However, they observed no insecticidal effects on aphid *M. persicae* populations [143]. As indicated by the latter two examples, the use of transgenic plants expressing chitinases has to be discussed carefully, particularly also because probiotic effects on insect pests have been reported in transgenic plants expressing chitinases. For instance, transgenic potatoes expressing a chitinase from the coleopteran pest *Phaedon cochleariae* revealed slightly positive effects on population growth of the aphid *M. persicae* [144]. Because aphids do not form a PM, they may be considered as nontarget insects for transgenic plants expressing baculoviral or insect chitinases.

Although some of the results obtained with transgenic plants expressing insect chitinases are inconsistent, it seems plausible that chitinase-mediated resistance can be improved by considering some of the recent insights on the different biochemical properties and physiological functions of chitinases. For example, so far only group I insect chitinases have been used for the construction of transgenic plants. However, these enzymes are located in the integument, where they have important function during molting. The primary target of plant-expressed chitinases, however, may be considered the PM in the midgut (in contrast to the chitinases acting systemically during a baculoviral infection). Therefore, it seems promising to use group IV chitinases as transgenes, which evidently are expressed

in the insect gut and hence may have higher chitinolytic activities due to evolutionary adaptations to this proteolytic environment. These adaptations may include resistance to proteolysis, distinctive pH optima, and different working modes that allow degradation of chitinous substrates which differ in the type of chitin and associated proteins.

Finally, it has to be noted that some of the observed insecticidal effects may be mediated by the stimulation of general defense mechanisms triggered by the exogenous chitinase rather than by direct chitinolytic action of the transgene. In addition, legal restrictions and problems with public acceptance of transgenic plants in some countries may raise concerns.

5.2 Insect Chitinases as Target Genes for RNAi

Using RNA interference as a powerful tool to investigate gene functions, a few studies revealed vital functions for chitinases for growth and development of insects. Zhu et al. reported lethal effects for the injection of the group I chitinase TcCHT5, the group II chitinase TcCHT10, and the group V IDGF protein TcIDGF4, whereas the injection of dsRNA for the group III chitinase TcCHT7 resulted in severe wing and elytral abnormalities [55]. Similar results were obtained when dsRNA specific for a group I and a group IV chitinase from the beet armyworm *Spodoptera exigua* was injected into pupae of this lepidopteran pest [145]. The injection of dsRNA specific for either chitinase led to a significant reduction in survival rates. Although the injection of dsRNA specific for the group I chitinase led to molting defects, that for the group IV chitinase prevented adult eclosion. Using a feeding-based RNAi approach, Khajuria et al. were successful in reducing transcript levels for a group IV chitinase in the European corn borer, *Ostrinia nubilalis*, which is predominantly detected in midgut of wild-type larvae [146]. In response to RNAi-mediated knock-down of transcript levels, the chitin content in the PM was slightly increased and larval growth was significantly impaired compared with control larvae. These findings indicate that the examined group IV chitinase is required for proper PM formation. If this function is disturbed, growth and development of the *O. nubilalis* larvae are negatively affected. As documented by the latter example, the knockdown of transcripts was accomplished by feeding dsRNA to larval pests. This approach 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 as well as the cost efficiency in synthesizing such dsRNA-based insecticides may be problematic. The expression of corresponding dsRNAs in transgenic plants, however, may be more promising, as this approach has been shown to provide protection against various pests [147].
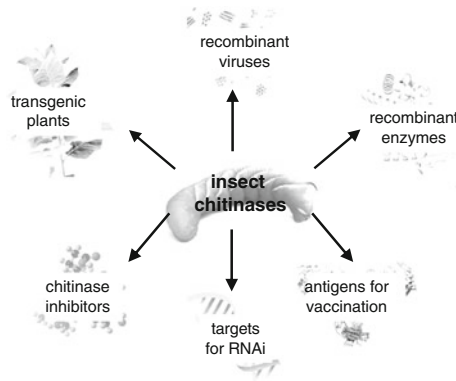
Fig. 4 Potential use of insect chitinases as biopesticides and targets for RNA interference, inhibitor- and antibody-based strategies of pest control

6 Conclusions

During the past decades, knowledge about the structure and function of insect GH18 chitinases has significantly increased. Next to the insights gained into their phylogenetic relationships, regulation, and working modes, significant progress has been made in understanding their distinctive physiological roles within different insect tissues. It became evident that chitinases are involved in a variety of physiological processes including molting, digestion, cell proliferation, and immune responses. Because of the inhibitory effects on the growth and development of fungi, parasites, and insects, chitinases from predominantly noninsect sources have been established as biopesticides and transgenes in crop protection, and as antigens for vaccination programs in animal and human health. Insect chitinases, however, may be considered as a largely unexploited resource for various applications in insect biotechnology due to their biochemical diversity resulting from evolutionary adaptations (Fig. 4). Improved understanding of their structure and biochemistry will accelerate their usage in biotechnological processes. Applications will include also recombinant insect chitinases that are expressed in heterologous systems and genetically optimized for bioprocessing industries.

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