Cell. Mol. Life Sci. (2010) 67:201–216
DOI 10.1007/s00018-009-0161-9

Cellular and Molecular Life Sciences

REVIEW

Insect chitinase and chitinase-like proteins

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Received: 26 July 2009 / Revised: 21 September 2009 / Accepted: 23 September 2009 / Published online: 9 October 2009

Abstract Insect chitinases belong to family 18 glycosylhydrolases that hydrolyze chitin by an endo-type of cleavage while retaining the anomeric β-(1→4) configuration of products. There are multiple genes encoding chitinases and chitinase-like proteins in all insect species studied using bioinformatics searches. These chitinases differ in size, domain organization, physical, chemical and enzymatic properties, and in patterns of their expression during development. There are also differences in tissue specificity of expression. Based on a phylogenetic analysis, insect chitinases and chitinase-like proteins have been classified into several different groups. Results of RNA interference experiments demonstrate that at least some of these chitinases belonging to different groups serve non-redundant functions and are essential for insect survival, molting or development. Chitinases have been utilized for biological control of insect pests on transgenic plants either alone or in combination with other insecticidal proteins. Specific chitinases may prove to be useful as biocontrol agents and/or as vaccines.

Keywords Chitinases · Insects · Classification · Expression · Function · RNA interference · Biocontrol · Vaccines

Introduction

Chitinases are widely distributed in nature as they are found in species from all kingdoms. These enzymes are found in archae- and eubacteria, protists, fungi, plants and animals, including arthropods and mammals. They have widely different functions and are involved in digestion, arthropod molting, defense/immunity and pathogenicity. Chitinases and chitooligosaccharides also serve as regulators of development or as morphogens in plants [1, 2]. Since our earlier review on insect chitinases was published more than a decade ago [3], there has been a substantial increase in our understanding of this group of enzymes, the genes encoding them and their biological functions. The reader is directed to other excellent reviews on chitin metabolism in insects [4, 5] as well as plant, fungal and microbial chitinases [6, 7] for some of the more current information now available. A comprehensive review of the evolution of all family 18 chitinases including mammals [8] provides a broader coverage of this field. This review will focus more narrowly on the more recent advances in the field of insect chitinases and chitinase-like proteins.

Family 18 chitinases

Chitinases (E.C. 3.2.1.14) are enzymes that hydrolyze the linear polymer of chitin consisting of β-1,4-linked N-acetylglucosamines. They are quite distinct from N-acetylhexosaminidases (E. C. 3.2.1.52) that act on the products of chitinase action, namely chitooligosaccharides, and convert them to the monosaccharide, N-acetylglucosamine. Some, but not all, chitinases exhibit lysozyme–like activity. These chitinases also hydrolyze cell wall polysaccharides consisting of alternating N-acetylglucosamine and N-acetylmuramic acid residues even though chitin may be the preferred substrate. Based on conservation of amino acid sequences, several conserved motifs and protein folding, chitinases have been classified into two families,

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namely family 18 and family 19 glycosylhydrolases [9, 10] (Carbohydrate-active enzymes family server, URL http://www.cazy.org/; http://afmb.cnrs.mrs.fr/~pedro/CAZY/db.html). Family 18 chitinases are widely distributed in representatives of all kingdoms, including bacteria, plants and animals. They share no sequence similarity with family 19 chitinases, which are found mostly in plants. However, there are quite a few reports of family 19 chitinases from an assortment of sources including bacteria [11, 12], a tick [32], viruses [13] and a nematode [14, 15]. Even though family 18 chitinases have undergone extensive evolutionary modification from protists to mammalia [8, 16], the most extensive expansion of the number of genes encoding chitinases with distinctive modular architecture appears to have occurred within the class of Insecta [17, 18, Kun-Yan Zhu, personal communication).

Family 18 insect chitinases are almost exclusively endochitinases and prefer to digest β-1,4-linkages in internal positions of the polymeric chitin yielding β-anomers at the reducing ends of the products. They have little or no exochitinase activity, i.e., the ability to remove terminal sugars, one residue at a time. This activity, termed N-acetylglucosaminidase activity is, instead, exhibited by group 20 glycosylhydrolases that often act in concert with endochitinases. The combined action of family 18 and family 20 chitinolytic enzymes is synergistic and leads to rapid depolymerization of the chitin polymer in insects [19]. In some cases, additional proteins with chitin-binding domains (CBD) but devoid of catalytic activity help in the degradation of chitin [20]. The main function of insect chitinases is in the turnover of chitin-containing extracellular matrices such as the insect cuticle and the peritrophic matrix (PM) during molting. In addition, chitinases may have a digestive function in insects, if their diet contains chitin. Chitinase-like proteins that lack enzymatic activity may have roles in immunity or as growth factors. We will discuss the functions of individual groups of chitinases later in this review.

Chitinases in different orders of insects

Chitinases or chitinase-like proteins have been found in all insect species studied belonging to different orders including dipterans, lepidopterans, coleopterans, hemipterans and hymenopterans. They are most easily purified from molting fluids in the ecdysial space between the old and new cuticles where they reach very high levels. Multiple chitinases differing in molecular weight have been identified and/or purified from lepidopteran molting fluid and the venom of a wasp [21–24]. Chitinases and the related proteins, which share extensive amino acid sequence similarities (imaginal disk growth factors, hemocyte aggregation inhibitory protein), have also been purified from insect cell lines and from gut and fat body tissues and hemolymph [25–28]. Several chitinases have been purified after introduction of the corresponding genes into insect cell lines using baculovirus vectors [29–35]. These purified enzymes have been very useful in characterization of the biochemical properties of chitinases. These aspects will be described later in this review.

Cloning of cDNAs and characterization of genes

Even though insect chitinases have been characterized at the protein level since the 1970s, the successful cloning of a cDNA encoding an insect chitinase was achieved only 2 decades later. Kramer et al. [36] first reported the isolation of a full-length cDNA clone from the tobacco hornworm, *Manduca sexta*. This finding was followed by the characterization of the corresponding gene in the *M. sexta* genome [37]. Since then, the cloning of more than 100 cDNAs encoding chitinases from numerous species of insects belonging to several different orders has been reported. The availability of whole insect genome sequences has enabled sequence similarity searches to be conducted using the available chitinase cDNA sequences from insects and other sources as queries [10, 17, 18]. These searches have revealed that insect genomes contain a rather large number of genes encoding proteins with high sequence similarity to well-characterized insect chitinases. The number of chitinase genes in insect species with fully characterized genomes is in the range of 17–24 in *Drosophila melanogaster*, *Tribolium castaneum* and *Anopheles gambiae*. The encoded chitinases in *T. castaneum* have been divided into eight subgroups based on sequence similarity and domain architecture (Fig. 1). This figure includes additional chitinase-like proteins and groups identified since our earlier publication [18].

A comparison of the number of genes in each subgroup of chitinases and chitinase-like proteins in different insects whose genomes have been completed has led to the following observations. The chitinases in all insects can be classified into multiple groups as in the case of *T. castaneum*. There is only one member in group I in all insects with the exception of *A. gambiae* and *Aedes aegypti* in which apparent gene duplications have occurred resulting in three or four additional members (Zhang and K. Zhu, personal communication). In group II, there is only one representative chitinase gene in the genomes of several species including *D. melanogaster*, *A. gambiae*, *A. aegypti*, *T. castaneum*, *Monochamus alternatus*, *Pediculus humanus corporis*, *Culex pipiens*, *Apis mellifera* and *Bombyx mori*. In groups III, VI, VII and VIII, there is only one

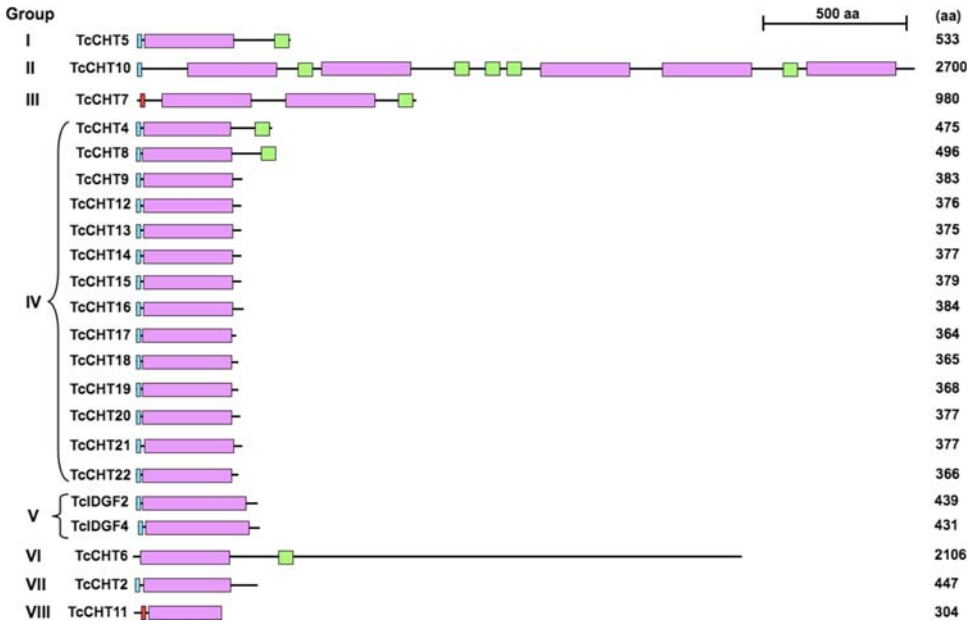

Fig. 1 Domain architecture of putative chitinases and chitinase-like proteins of *T. castaneum*. The program SMART was used to analyze the identified domains. TcCHT7 and TcCHT11 have a single transmembrane span at the N-terminal region. *Blue boxes*, signal peptide; *pink boxes*, catalytic domain; *green boxes*, chitin-binding domain; *red boxes*, transmembrane span; lines, linker regions

representative in a variety of species. In addition to the group III chitinase genes identified in fully sequenced genomes of *D. melanogaster*, *A. gambiae*, *A. aegypti*, *C. pipiens*, *T. castaneum*, *A. mellifera* and *N. vitripennis*, orthologs also have been detected in *Haemophysalis longicornis* (tick), *Pediculus corporis* (louse) and *Acyrthosiphon pisum* (pea aphid), indicating an ancient origin of this gene that predates the separation of the class Insecta. In all species studied in detail, group IV has the largest number of representatives with the most members in *D. melanogaster*, *A. gambiae*, *Aedes aegypti* and *T. castaneum* (5, 8, 10 and 14 genes, respectively). Many of these chitinase genes are found in large clusters within a small region of the genome with very small intergenic regions (e.g., *T. castaneum*; unpublished data), suggesting gene-duplication events. Group IV members also show the greatest variation in domain organization (see below). The number of genes in group V encoding chitinase-like proteins such as imaginal disk growth factors (IDGFs) or related proteins ranges from one in *A. mellifera* to as many as six in *D. melanogaster*.

Domain architecture of insect chitinases

The chitinases and chitinase-like proteins encoded by the various groups of genes discussed above all have a multi-domain structural organization that includes 1–5 catalytic domains (GH-18 domain), 0–7 cysteine-rich chitin-binding domains (peritrophin-A domain/CBM-14 domain) and serine/threonine-rich linker regions that can be heavily glycosylated (Fig. 1). In addition, most if not all chitinases are predicted to have a leader peptide or a transmembrane-spanning domain because they are targeted either to the extracellular space or sorted into plasma membrane, facing in both cases carbohydrates of the extracellular matrix [27, 34–36, 38].

Based on the predicted sequences of proteins encoded by these putative chitinase genes as well as the presence/absence of specific domains and conserved sequences, insect chitinases can been grouped into eight unique groups with different domain architectures as shown in Fig. 1 for *T. castaneum* chitinases. This classification represents an expansion of the previous classification proposed by Zhu et al. [18]. A brief description of these groups follows.

The group I chitinases (CHT5s) correspond to the enzymatically well-characterized chitinases isolated from the molting fluid and/or integument of *M. sexta* and *B. mori*, which contain a signal peptide, one catalytic domain, a Ser/Thr-rich linker region and one C-terminal chitin-binding domain (belonging to family CBM-14) with six cysteines.

The group II chitinases (CHT10s) are large molecular weight chitinases that have four or five catalytic domains and four to seven CBDs, whose distribution (number and location) between the catalytic domains shows similar arrangements. All species studied except the dipterans have five catalytic domains and five CBDs with the arrangement represented by ○-△-○-△-△-△-○-○-△-○ where ○ represents a catalytic domain and △ a CBD. Dipterans have only four catalytic domains and four CBDs with the arrangement ○-△-△-△-○-○-△-○. The domains corresponding to the most N-terminal catalytic domain and CBD found in the corresponding chitinases in coleopterans appear to be missing in the dipteran enzymes. Koga and associates have identified and cloned a full-length cDNA

encoding a group II chitinase from the pine sawyer beetle, *Monochamus alternatus* (personal communication). They also identified an alternatively spliced variant that is missing the third CBD from the N-terminus. The catalytic domain 2 (or in the case of dipterans, the catalytic domain 1) from this entire set of group II chitinases has an asparagine (N) instead of the proton donor glutamate (E) residue in a highly conserved sequence-motif (WNYP instead of WEYP found in catalytic domain 3) that is most critical for the chitinolytic activity of family 18 chitinases [39, 40]. In addition, an aspartic acid residue in this motif of catalytic domain 2 is substituted for by a histidine or serine [FXGL(H/S) instead of FXGLD], an observation that indicates that the catalytic domain 2 may be non-catalytic (Fig. 2). On the other hand, in catalytic domains 3 and 4 of all chitinases in Fig. 2, these critical amino acid residues are all present, suggesting that the catalytic domain 2 may have a function other than participating directly in the hydrolysis of chitin.

Group III chitinases (CHT7s) contain two catalytic domains and one CBD, typically in a ○-○-△-arrangement. The catalytic domain 1 of this group of chitinases exhibits greater sequence similarity to one another than to the catalytic domain 2 in the same protein(s), suggesting distinct functions and/or evolutionary origins for each of these two catalytic domains. This group of chitinases, unlike most insect chitinases, possesses a predicted transmembrane segment at the N-terminal region, whereas a hard tick (*Haemophysalis longicornis)* chitinase, whose amino acid sequence and domain architecture are highly similar to group III insect chitinases, has a signal peptide. Indeed, You et al. [32] found that recombinant *Haemophysalis* chitinase could be secreted into Sf9 culture medium. However, the recombinant *T. castaneum* chitinase 7 that was expressed in Hi-5 insect cells containing a recombinant baculovirus encoding this gene was bound to the cell membrane. Apparently, the catalytic domains of this chitinase face the extracellular space as revealed by its ability to hydrolyze an artificial chitin substrate added to the medium (Arakane et al., unpublished data).

Group IV chitinases constitute the largest and most divergent group. These chitinases have a signal peptide and a single catalytic domain. Most, but not all, members of this group of chitinases lack a CBD.

Group V chitinase-like proteins include the imaginal disk growth factors (IDGFs) with sequence similarity to the catalytic domain of chitinases. They have a leader peptide and a catalytic domain and conserved motifs but no CBDs. The glutamate residue in conserved motif II of all insect IDGFs, except for the two *T. castaneum* proteins, is replaced by a glutamine, and these chitinase-like proteins are devoid of chitinase activity. Even the two *T. castaneum* proteins, TcIDGF2 and TcIDGF4, which retain the glutamate residue in conserved motif II, which acts as the proton donor, lack chitinase activity, possibly because they have a D to A substitution in the conserved motif II [35]. Like the chitinases of other groups, homology modeling and amino acid sequence alignment revealed that all proteins belonging to this group have a $(\beta\alpha)_8$ triose-phosphate isomerase (TIM) barrel structure. However, group V chitinase-like proteins have an extra loop between the β-4 strand and α-4 helix immediately after conserved region II (see below).

Group VI chitinases are larger than the group I chitinases but similar in domain structure. Like group I chitinases, they have a signal peptide, an N-terminal

	catalytic domain				
	1	2	3	4	5
TcCHT10	FDGMDIHWEYP	FKGLHLDWNYP	FDGLDLDWEYP	FDGLDLDWEYP	FDGLDLDWEYP
TmCHT10	FDGMDIHWEYP	FRGLHFDWNYP	FDGLDLDWEYP	FDGLDLDWEYP	FDGLDLDWEYP
MaCHT10	FDGMDVHWQYP	FKGLHFDWNYP	FDGLDLDWEYP	FDGLDLDWEYP	FDGLDLDWEYP
BmCHT10	FDGLDLHWVYP	FKGLHLDWNYP	FDGLDLDWEYP	FDGLDLDWEYP	FDGVQIAWQYP
AmCHT10	FDGVEIDWEGS	FDGLSFEWNYP	FDGLDLDWEYP	FEGLDLDWEYP	FDGLDLLWEYP
CpCHT10	FDGIDLDWEFP	FSGLHLDWNYP	FEGLDLDWEYP	FDGLDLDWEYP	FDGLDLDWEYP
PhcCHT10	FDGIDLDWEFP	FQGLHFDWAYP	FDGLDLDWEYP	FDGLDLDWEYP	FDGLDLDWEYP
DmCHT10		FSGLHLDWNYP	FEGLDLDWEYP	FDGLDLDWEYP	FEGLDLAWEFP
AgCHT10		FAGLHMDWNYP	FEGLDLDWEYP	FDGLDLDWEYP	FDGLDLDWEYP
AaCHT10		FSGLHFDWNYP	FDGLDLDWEYP	FDGLDLDWEYP	FDGLDLAWEYP

Fig. 2 Comparison of the conserved motif II in each of the catalytic domains of group II chitinases from different insect species. Group II chitinases possess five catalytic domains except for chitinases from dipterans, which have four catalytic domains. In the second catalytic domain of coleopteran chitinase II or the first catalytic domain in the case of dipteran enzyme, the glutamate residue in conserved region II that is most critical for catalysis was substituted with Asn or Ala (shown in *grey color*). In the first and fifth CBDs of some chitinases other substitutions of acidic amino acids occur that are predicted to result in inactive chitinase domains (shown in *blue* or *grey*) [40]. Chitinases (Genbank accessions) compared are from: TcCHT10: *Tribolium castaneum* (ABG47448); TmCHT10: *Tenebrio molitor* (CAD31740); MaCHT10: *Monochamus alternatus* (BAG13448); BmCHT10: *Bombyx mori* (BGIBMGA006874-PA); AmCHT10: (XP_395734); CpCHT10: *Culex pipiens* (EDS34768); PhcCHT10: *Pediculus humanus corporis* (EEB13772); DmCHT10: *Drosophila melanogaster* (NP_001036422); AgCHT10: *Anopheles gambiae* (XP_001238192); AaCHT10: *Aedes aegypti* (XP_001655973)

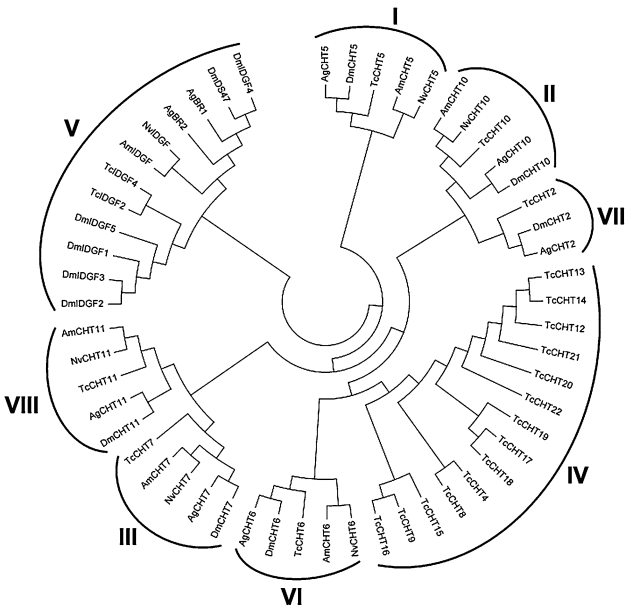

Fig. 3 Phylogenetic analysis of putative chitinases and chitinase-like proteins (IDGFs) from *T. castaneum*, *D. melanogaster*, *A. gambiae*, *N. vitripennis and A. mellifera*. ClustalW software was used to perform multiple sequence alignments prior to phylogenetic analysis. The phylogenetic tree was constructed by MEGA 3.0 software using UPGMA [91]. Group IV chitinases from *D. melanogaster*, *A. gambiae*, *N. vitripennis and A. mellifera* were not included due to incomplete gene annotations. *Dm D. melanogaster*, *Ag A. gambiae*, *Nv N. vitripennis*, *Am A. mellifera*

catalytic domain and one CBD. However, there is a long stretch at the C-terminus (1,000 to 2,500 amino acids) that contains 25–30% Ser/Thr residues, which accounts for their very large size. These proteins are presumed to be heavily glycosylated and are perhaps resistant to proteases.

Group VII chitinases (CHT2s) exhibit a domain architecture similar to that of group IV chitinases, which includes a signal peptide, single catalytic domain and no CBD. They are placed in a separate group because phylogenetic analysis indicates that group VII chitinases form an outlier group near group II chitinases (Fig. 3).

Group VIII chitinases (CHT11s) have a catalytic domain and no CBD. There is a predicted transmembrane span at the N-terminal region instead of a signal peptide, indicating they are membrane-associated proteins. Interestingly, they fall into the branch next to the one with group III chitinases (CHT7s; Fig. 3) that are also membrane bound.

Crystal structure of an insect GH family 18 protein

While crystal structures for several family 18 chitinases or chitinase family proteins, including *Serratia* chitinase A, hevamine and Ym1, are known [41–43], the crystal structure of only one insect protein belonging to this family is available [44]. The extracellular soluble protein, IDGF2 from *D. melanogaster*, is a member of group V insect chitinases and promotes the growth of imaginal cells in concert with an insulin-like protein. It is devoid of chitinase activity [35]. Its structure has the classical $(\beta\alpha)_8$ TIM barrel fold found in all family 18 glycosylhydrolases. The eight-stranded β-barrel at the center is surrounded by eight α-helices on the outside with an orientation anti-parallel to the β-strands. However, compared to the family 18 chitinase, hevamine, this protein has two prominent insertions, one between the β-4 strand and α-4 helix and another one between the β-7 strand and α-7 helix [35, 44]. The extra loop between the β-4 strand and α-4 helix is highly conserved among group V chitinases, indicating an important function (Fig. 4). This region with the consensus sequence KPRKVGXX(L/I)GSXWKFKKXF(T/S)GDXVVDE is not visible in the crystal structure, but is located on the surface and is fully exposed to the solvent. There is evidence of proteolysis in this loop between the F and T residues in *D. melanogaster* IDGF2 [44]. We have observed that IDGF4 from *T. castaneum* expressed in Hi-5 cells is similarly unstable and is broken down easily, presumably at a similar cleavage site [35]. The cleaved products have been shown to be just as active as the uncleaved proteins in imaginal disk cell proliferation assays, suggesting that this cleavage may be related to its function [44]. The putative substrate binding cleft of IDGF2 appears to be narrower than those in other chitinases, but is accessible, and several highly conserved aromatic amino acids implicated in chitin binding are located in the β-strands and in loops near the binding pockets of chitinases. However, IDGFs appear to have fewer of these conserved residues (see below).

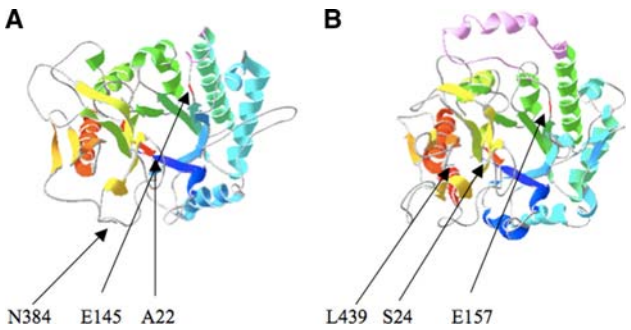

Fig. 4 Homology modeling of catalytic domains for *Tribolium* chitinase TcCHT5 and chitinase-like protein TcIDGF2. The SWISS-MODEL program was used to generate the models. The loop region between β-sheet 4 and α-helix 4 is labeled in purple. The *arrows* show the catalytic glutamate residue in conserved region II. A. TcCHT5 homology modeling was conducted using human chitotriosidase (PDB entry code 1lq0A) [92] as the template. B. TcIDGF2, *Drosophila* IDGF2 (PDB entry code 1jndA) [44] was used as the template. The glutamate residues in the conserved region II (E145 and E154) in the two proteins are indicated as are the beginnings (A22 and S24) and ends (N384 and L439) of the catalytic domains

Nonetheless, *D. melanogaster* IDGF2 and the orthologous IDGF4 do bind to colloidal chitin [35].

The other insertion in the classical TIM barrel motif is between the β-7 strand and α-7 helix. This insertion constitutes another domain with an α-plus-β-fold found in bacterial chitinases and all insect chitinases, but not in hevamine [44]. This additional domain is highly variable in length in different chitinases and IDGFs. A comparison of the activities and substrate preferences of three bacterial chitinases from *Serratia*, which differ in the presence or absence of this insertion, has led to the suggestion that this domain may be involved in determining whether the enzyme has an exo- or endo-activity and whether it is a processive enzyme [45]. Similar studies have not been done with insect chitinases, which are mostly endochitinases.

Conserved motifs in the catalytic domains

Insect chitinases of all groups possess signature motifs characteristic of family 18 glycosylhydrolases (Fig. 5). These include the four motifs K(F/V)M(V/L/I)AVGGW in strand β-3, FDG(L/F)DLDWE(Y/F)P in β-4, M(S/T)YDL (R/H)G in β-6 and GAM(T/V)WA(I/L)D in β-8 [46]. Only the residues in the second motif, FDG(L/F)DLDWE (Y/F)P, have been studied extensively by site-directed mutagenesis for their effects on the properties of the group I chitinase from *M. sexta*. The glutamate residue (E) is the most critical residue in this motif and is likely to be the proton donor required for the cleavage of the glycosidic bond. Replacement of this residue with a glutamine or by an aspartic residue resulted in total loss of activity [40]. Several group V chitinase-like proteins (IDGFs), which lack chitinase activity, have a substitution of this residue. The aspartic acid (D) residues also were critical for overall activity as their replacement by an asparagine resulted in either significant loss of activity or a shift to a more acidic pH optimum [40]. In all IDGFs, the aspartic acid closest to the W in conserved motif II is replaced by an alanine or glycine (e.g., FDGLDL**A**WEFP instead of FDGLDL**D**WEFP in *T. castaneum* IDGF2 and IDGF4). The TcIDGFs that also have this D to A substitution lack enzymatic activity even though they do retain the catalytically critical glutamic acid residue in this motif indicating that both of these acidic amino acids are crucial for retention of enzymatic activity. The presence of multiple acidic amino acid residues in this highly conserved motif probably contributes to the shift of the pH optimum to >8. Similar studies with the *Autographa californica* nuclear polyhedrosis virus-encoded chitinase have established the role of the glutamate residue as well as aspartate residues in affecting the exo- and endochitinase activities of the baculoviral enzyme [47]. Even the tryptophan (W) residue in the conserved second motif played a critical role in catalysis as shown by the loss of activity when this residue in *M. sexta* chitinase was replaced with a glycine. However, replacement of this residue with another aromatic residue phenylalanine (F) resulted in an enzyme that had only half the activity of the wild-type enzyme with significantly altered kinetic parameters as well as the pH vs. activity profile [48]. The functions of the other motifs are unknown, but it is interesting to note that all of these motifs are at the ends of the β-sheets that line the inner side of the $(\beta/\alpha)_8$ barrel structure or in loops that connect them to the adjacent α-helices that line the outer surface of the barrel. It is possible that these represent sugar-binding sites as they have aromatic residues that have been implicated in saccharide binding as well as in processivity and enzymatic activity on crystalline chitin of the chitinase from *S. marcescens* [49].

Linker regions contribute to immunogenicity and protein stability

The role of the linker regions containing multiple serine and threonine residues is likely to provide sites of O-glycosylation [34]. The sugar residues appear to be largely responsible for the immunogenicity of *M. sexta* chitinase, as antibodies directed against the wild-type enzyme do not recognize truncated enzymes lacking the linker region even

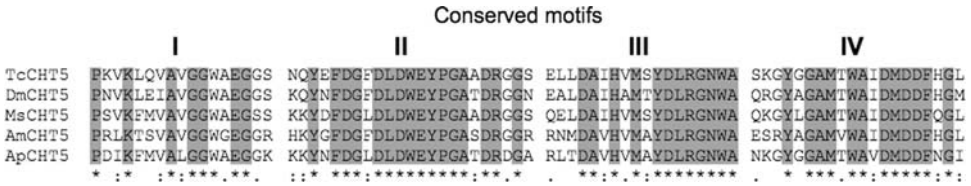

Fig. 5 Alignment of the four conserved motifs in the catalytic domains of group I insect chitinases from different orders. The amino acid sequences of the conserved motifs in the catalytic domains were aligned using ClastalW. *Symbols* below the aligned amino acid sequences indicate identical (*), highly conserved (:) and conserved residues (.). Chitinases compared are: TcCHT5, *Tribolium castaneum* (AAV74190); DmCHT5, *D. melanogaster* (NP_650314); MsCHT5, *Manduca sexta* (AAC04924); AmCHT5, *Apis mellifera* (XP_623995); and ApCHT5, *Acyrthosiphon pisum* (XP_001947416)

though they exhibit chitinase activity. The linker region is thought to be devoid of secondary structure and probably increases the stability of the proteins when present in a protease-rich environment such as the gut or the molting fluid.

C-terminal CBD with six conserved cysteines increases affinity of chitinase for the insoluble polymeric substrate

The CBD domains found in chitinases belong to the carbohydrate-binding module 14 (CBM14 family; http://www.cazy.org/fam/CBM14.html) found in peritrophins. They are characterized by the presence of six cysteines with a characteristic spacing between them ($CX_{11-24}CX_{5-6}CX_{9-19}CX_{10-17}CX_{4-14}C$) (Jasrapuria et al., manuscript in preparation). The CBD sequences within specific chitinase families show conservation of other residues in addition to the six conserved cysteines (Fig. 6).

The C-terminal CBD of group I *M. sexta* chitinase has been shown to increase the affinity of the enzyme for the polymeric substrate, but not for the oligosaccharide substrate [34]. It is likely that the CBD helps to anchor the enzyme on the chitin matrix and allows the enzyme to cleave bonds in a random endo type of cleavage mechanism. The CBD domain is predicted to have a β-sandwich structure based on homology modeling to tachycitin whose structure has been solved utilizing solution NMR experiments [50].

Chitinase isozymes exhibit differences in kinetic parameters

Zhu et al. [35] have expressed several insect chitinases and chitinase-like proteins belonging to three different groups, I, IV and V, using a baculovirus-insect cell expression system, and compared the properties of purified enzymes from three different insects, *T. castaneum, D. melanogaster* and *M. sexta*. These studies showed that there were differences among insect chitinases in immunological cross-reactivity, pH-activity profiles, ability to hydrolyze short versus long substrates, kinetic constants and ability to bind to colloidal chitin. Group I enzymes have lower K_m and V_{max}/K_m values for polymeric substrates than group IV enzymes and are active over a broader range of pH values with the highest activities in the alkaline range. Group IV enzymes had a more acidic pH optimum. Most striking was the absence of chitinase activity in members of group V. Representatives of the other groups of chitinases have not been fully characterized yet at the protein level. All chitinases with enzymatic activity exhibited product inhibition by the short oligosaccharides. The substantial differences among the physical and biochemical properties of the different chitinases provide a rationale for the occurrence of multiple chitinases in insects.

Substrate preference and anomer formation

The insect chitinases studied so far have been shown to be endochitinases. They act on chitin and chitooligosaccharides with three or more N-acetylglucosamine residues and exhibit little or no detectable exochitinase activity as shown by their inability to hydrolyze methylumbelliferyl-N-acetylglucosamine or p-nitrophenyl-N-acetylglucosamine or chitobiose. Only baculoviral chitinases, which may have a bacterial origin, have been reported to have both endo- and exo-chitinolytic activity [51]. With oligosaccharide substrates, the preferred site of cleavage is the linkage between the second and third GlcNAc moiety from the non-reducing end, but other sites were hydrolyzed [52]. The catalytic efficiency increases with increasing size of the substrate. The group I enzymes, which have been studied most extensively, act via a retaining mechanism and produce the β-anomer at the reducing end of the cleaved products, in contrast to family 19 chitinases, which act via an inverting mechanism and produce α-anomers [53]. The mechanism of action of insect chitinases has not been fully delineated, but based on extensive studies with other family 18 chitinases, it is likely that the hydrolysis of the glycosidic bond involves substrate-assisted catalysis (anchimeric assistance)

Fig. 6 Alignment of chitin binding domains in group I insect chitinases from different orders. The amino acid sequences of the CBDs of chitinases listed in Fig. 4 were aligned using ClastalW. *Symbols* below the aligned amino acid sequences indicate identical (*), highly conserved (:) and conserved residues (.). The C-terminal CBDs are probably involved in increasing the affinity of chitinases to the polymeric substrate

in which the carbonyl group of the N-acetyl group of the distorted sugar residue at the -1 subsite of the enzyme acts as the nucleophile, which leads to the formation of an oxazolinium ion intermediate that is stabilized by the substrate itself [54]. For a detailed discussion of the roles of various residues near the active site, the reader is referred to excellent articles by Brameld and Goddard [53] and Synstad et al. [55].

Inhibitors of insect chitinases

Insect chitinases are inhibited by substrate/products as well as transition-state analogs. There is substantial evidence to suggest that chitooligosaccharides, which are products of hydrolysis of chitin by insect chitinases, inhibit the enzyme at high concentrations likely to be encountered during the molting process [34, 56]. Presumably, the inhibition occurs as a result of the non-productive binding of chitooligosaccharides (trimers or smaller) at sub-sites that are farther removed from the -1 subsite [57]. The appearance of N-acetylglucosaminidase activity ahead of the induction of chitinases during molting may be to minimize the accumulation of inhibitory chitooligosaccharides during cuticle digestion [24, 58].

Allosamidin, a compound extracted from a *Streptomyces* species, inhibits several family 18 chitinases, but not family 19 chitinases, with the insect chitinases being most affected by sub-micromolar concentrations. Allosamidin is a β-1-4-linked dimer of β-N-acetyl-allosamine (a C-3 epimer of N-acetylglucosamine) attached to an aminocyclitol derivative at its reducing end. This compound inhibits the larval-pupal molt of *Leuconia separata* [59]. Its oxazolinium moiety is similar to the reaction intermediate and binds to family 18 chitinases at the active site [60]. The much tighter binding of this inhibitor to insect chitinases compared to microbial family 18 chitinases suggests differences in the active sites within family 18 chitinases and may provide a rationale for the use of allosamidin-type inhibitors for selective insect control.

Some species of fungi produce cyclic pentapeptides named argifin and argadin, which inhibit insect chitinases at nM concentrations. These peptides are peptide mimetopes that mimic the substrate and bind to the -1, $+1$ and $+2$ subsites in family 18 chitinases [61]. These peptides affect molting of cockroach larvae, indicating their potential as insecticides. They also inhibit fungal and human chitinases, but not a hevamine-like family 18 chitinase from *S. marcescens* chitinase C, suggesting that it may be possible to selectively inhibit specific family 18 chitinases using peptide derivatives.

Regulation of expression of chitinases

Chitinase activities show periodic changes during development

During development, the insect cuticle is constantly being deposited and remodeled to allow for growth, while providing protection against mechanical injury and predators. The old cuticle is periodically shed at each molt, and a new cuticle is synthesized under the old cuticle. During apolysis, the period of initial separation of new cuticle from old cuticle, molting fluid begins to accumulate between the old and the new cuticular layers both during larval–larval and larval–pupal molts. Molting fluid contains high concentrations of chitinolytic and proteolytic enzymes (for a review, see Reynolds and Samuels, [24]. After ecdysis, molting fluid is either resorbed or ingested, minimizing inappropriate exposure of the developing cuticular layers to these degradative enzymes. The expression patterns of chitinolytic enzymes are cyclical, reaching high levels just about the time of apolysis and disappearing at ecdysis in *B. mori* [62]. Similar results have been reported for other insect species [63].

There have been some suggestions that proteolysis may be involved in the activation of chitinases [38, 64, 65]. However, in vitro studies with purified preparations of group 1 chitinases have not indicated a requirement of proteolytic activation at least for group I and III chitinases ([35], Arakane et al. unpublished data). On the other hand, the group II chitinases with multiple catalytic domains are potential targets for activation by proteolysis. But no experimental evidence is currently available to prove or disprove this possibility.

Hormones control the rise and fall of chitinolytic activity

The rise and fall of chitinolytic activity at each molt correlate well with increasing and falling ecdysteroid titers prior to ecdysis as observed initially by Kimura [21]. The need for ecdysteroid to induce chitinase activity was directly demonstrated by isolating the sources of developmental hormones (ecdysteroids and juvenile hormone) from their target tissues by ligating the larval abdomens below the second thoracic segment of *M. sexta*. In these isolated larval abdomens, injection of 20-hydroxyecdysone (20HE) resulted in a sharp increase in activity of chitinolytic enzymes, reaching a ten-fold or higher peak levels 4 days after injection [66]. Similar results were observed utilizing isolated *Bombyx* abdomens by Koga et al. [67], indicating that hormonal regulation of chitinase induction occurs in a broad range of insects.

Insect hormones control at least two chitinase genes

Definitive proof that the 20HE-mediated increase in chitinolytic activity is due to an increase in transcript levels for a chitinase gene came from the work of Kramer et al. [36] who measured transcript levels for an *M. sexta* chitinase gene (group I) using the isolated abdomen system described above. In 20HE-injected abdomens, the levels of chitinase transcripts increased and reached a plateau about 2 days after injection in both epidermal and gut tissues. This increase was abolished by simultaneous injection of 20HE and the juvenile hormone (JH) mimic, phenoxycarb, indicating that JH negated the effects of 20HE. These results suggest that this chitinase gene is under both positive and negative control by developmental hormones. Transcripts for this gene peaked just prior to ecdysis/pupation in the fourth and fifth larval instars, coinciding with the peaks of ecdysteroid titers. Expression of a gene encoding a group I chitinase that accumulated in the molting fluid, integument, tracheae, spiracles and salivary glands was observed by Zheng et al. [68] in *Choristoneura fumiferana* during larval-larval and larval pupal molts. This gene was induced by an ecdysone agonist in the integument during early stages of the sixth instar, which led to an incomplete molt and resulted in the premature separation of the cuticle from the epidermis.

Hormonal control of another chitinase gene with five catalytic domains belonging to group II has been studied in the beetle, *Tenebrio molitor* [38]. The abundance of transcripts for this gene correlated well with 20HE titers during metamorphosis. When pupae were injected with 20HE, the transcript levels increased within 2–4 h after hormonal treatment. Interestingly, even topical application of the JH analog, methoprene, resulted in an induction of transcripts for this chitinase gene 8 h after treatment. These results are different from those reported for *M. sexta*, which indicated no effect of the application of JH alone on a group I chitinase transcript [36]. In *B. mori*, the induction of another chitinase gene, BmChiR1, paralleled the induction kinetics of a group I chitinase and required 20HE for induction. This gene was also suppressed by the simultaneous application of a JH analog [69]. Even though this chitinase was identified as an inactive chitinase with two inactive catalytic domains and one CBD, based on sequence data we have concluded that this gene from *B. mori* actually encodes a protein with five catalytic domains and seven CBDs, and have classified it as a group II chitinase. Interestingly, one recent study in shrimp suggests regulation of chitinase gene expression by ecdysteroids. Hence, transcriptional regulation by ecdysteroids may occur in arthropods as well [70]. While it is clear that the expression of more than one chitinase gene is controlled by ecdysteroid and possibly by JH, it is likely that these effects are indirect and mediated through one or more transcription factors induced by ecdysteroids [71].

Chitinase genes exhibit differences in tissue specificity and developmental patterns of expression

Earlier studies that monitored the levels of chitinolytic activity in molting fluid or in extracts of whole insects measured total activity and did not address the issue of how individual genes responded to developmental cues. The availability of whole genome sequences and annotation of individual genes/cDNAs for all members of the chitinase gene family in *T. castaneum* has allowed such questions to be answered. Zhu et al. [72] used gene-specific primer pairs in reverse transcriptase-polymerase chain reactions (RT-PCR) to follow the expression of individual *T. castaneum* chitinase genes during embryonic, larval, prepupal, pupal and adult stages. These results demonstrated substantial differences in expression patterns of individual groups of chitinases (Fig. 7). Chitinases belonging to groups I, II, III, V, VI, VII and VIII were expressed at almost all developmental stages including embryonic, larval, pupal and adult stages, whereas those belonging to group IV are expressed only during larval stages and in adults, but not in prepupal, pupal or embryonic stages. More importantly, all genes of group IV were expressed in the larval gut tissue, but not in the carcass (whole body minus gut and head), suggesting that the target of these enzymes may be gut-derived PM-associated chitin (Fig. 8). The lone exception is *TcCHT4*, which is expressed at high levels in the gut, but also at a lower level in the carcass. Likewise, the low level expression of *TcCHT5*, *7*, *10* and *11* in midgut tissue may be due to the presence of small amounts of tracheal tissue in the midgut preparations. At any rate, it is clear that there are distinct differences in the developmental patterns of expression of individual chitinase genes. Similarly, it appears that there are differences in the tissue specificity of expression of individual chitinase genes even though this has not been studied in sufficient detail to assign their expression to specific tissues comprising the carcass (fat body, muscle, CNS, etc.). From limited studies in *T. castaneum*, it appears that chitinases belonging to groups I, II and III are expressed in epidermal (and possibly tracheal) tissues and act on cuticular chitin, whereas the group IV and VIII chitinases are expressed in the gut and are presumed to act on PM-associated chitin.

There are interesting differences in the locations of expression within the gut of members of group IV chitinase genes. Some are expressed predominantly in the anterior and middle midgut (TcCHT9, TcCHT13, TcCHT14, TcCHT15, TcCHT18, TcCHT19 and TcCHT20), whereas others are expressed predominantly in the posterior midgut

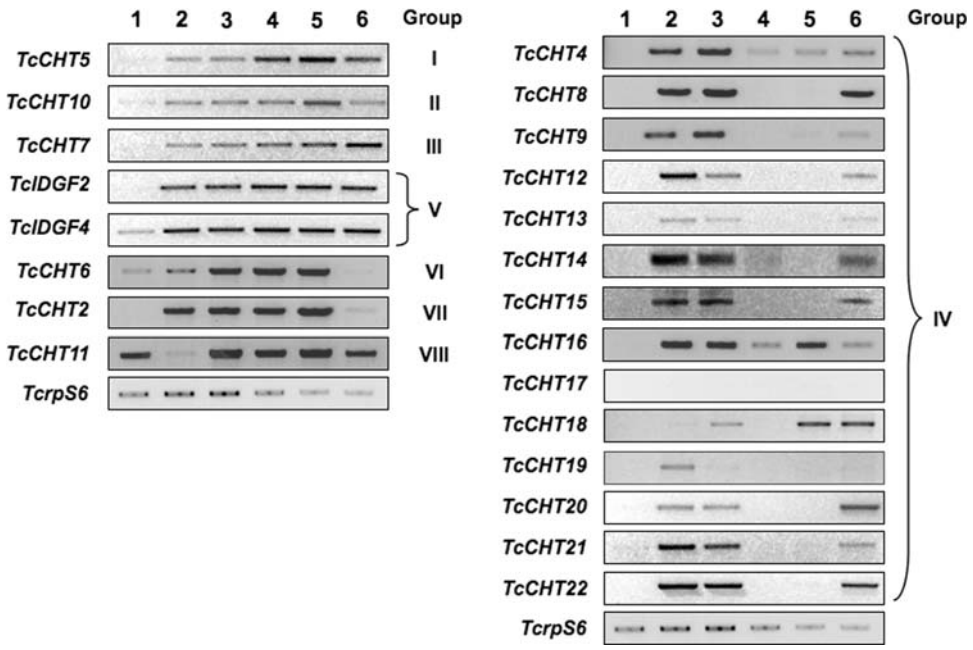

Fig. 7 Developmental patterns of expression of chitinase gene family in *T. castaneum*. RT-PCR was done with first strand cDNA synthesized from total RNA isolated from different developmental stages. *Lanes: 1* embryos, *2* penultimate instar larvae, *3* last instar larvae, *4* pharate pupae, *5* pupae, *6* adults. Transcript for *T. castaneum* ribosomal protein 6 (*rpS6*) was used as an internal loading control. RT-PCR was carried out for 30 cycles for all genes except *rpS6* (24 cycles) (modified from Zhu et al. [72])

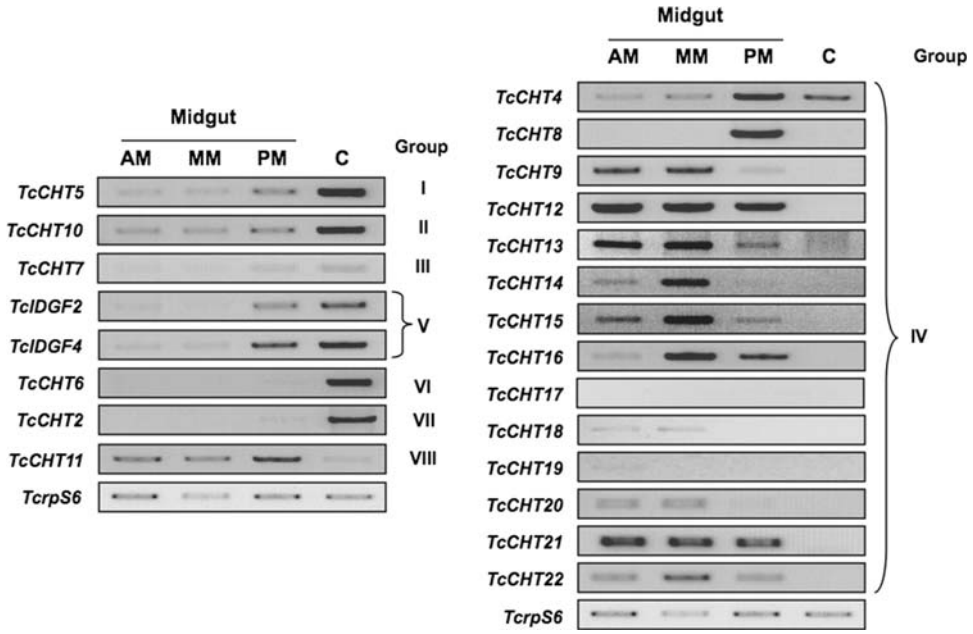

Fig. 8 Expression of chitinase genes in different parts of the *T. castaneum* midgut and in carcass. Ten midguts were dissected from last instar larvae and then divided roughly equally into three parts, which were labeled anterior (AM), middle (MM) and posterior (PM) midguts. The carcass (whole body minus gut, head and posterior tip) was also collected to analyze transcript levels of individual chitinase genes. Transcript for *T. castaneum* ribosomal protein 6 (*rpS6*) was used as an internal loading control. RT-PCR was carried out for 30 cycles for all genes except *rpS6* (24 cycles) (modified from Zhu et al. [72])

(TcCHT4 and TcCHT8). Some genes are expressed throughout the midgut (TcCHT12, TcCHT16, TcCHT21 and TcCHT22) (Fig. 8).

Individual chitinases may have specialized functions

The differences in the developmental patterns of expression and tissue specificity of expression of different chitinases in insects strongly suggest that they have distinct functions. This hypothesis is further supported by the retention of the distinctive groups of chitinases with different domain organization in insect species from several (perhaps all) orders of insects (coleopterans, lepidopterans, dipterans, hemipterans and hymenopterans). Strong experimental evidence for this idea has come from RNA interference (RNAi) studies in *T. castaneum* [72]. In this species, RNAi can be accomplished by injection of small amounts (typically 2–200 ng/insect) of dsRNAs specific for the target gene. Furthermore, the role of each chitinase gene could be studied by carefully choosing the stage at which the injections were carried out to allow the determination of the role of each gene during larval-larval, larval-pupal and pupal-adult molts, as well as in the

developing or mature adults. These studies have led to the following observations.

The most dramatic effects were seen following injections of dsRNA for the group II chitinase gene, *TcCHT10*, which led to molting arrest at all stages (Fig. 9). Embryo hatch, larval molting, pupation and adult morphogenesis were affected, indicating a role for this chitinase at every life stage of the insect when old cuticle is shed and a new cuticle is synthesized. Since this chitinase, which is expressed at all stages of insect development, has a distinct arrangement of catalytically competent and inactive subunits interspersed with CBDs (see Fig. 1 and Fig. 2), it has been proposed that this group of chitinases may have a role in disrupting the crystalline arrangement of chitin fibrils in the cuticle followed by random endo-type cleavage of chitin chains leading to their rapid depolymerization [72]. Since this gene is expressed only in the integument and not in the gut tissue, it is likely to be involved in the turnover of cuticular chitin. The role of the catalytically incompetent domains and/or the CBDs may be similar to that proposed for the chitin-binding protein CBP21 from *S. marcescens*, which is devoid of chitinase activity, but strongly promotes the hydrolysis of β-chitin by other chitinases when it is added to the reaction [20]. Homologues of this protein, which are specific for α-chitin, are found in *Streptomyces olivaceoviridis* [73].

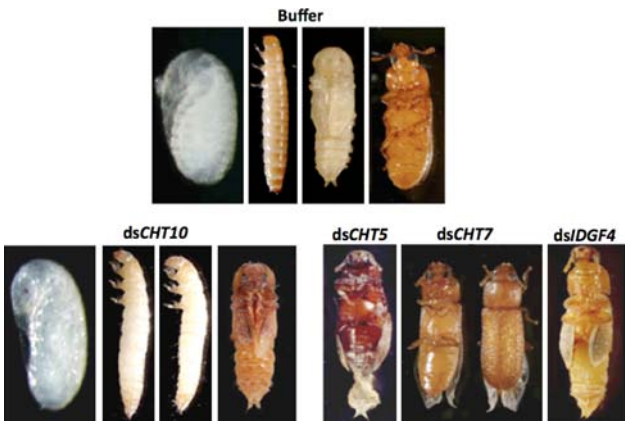

Fig. 9 Terminal phenotypes produced by injection of dsRNA for *TcCHT5, TcCHT7, TcCHT10* and *TcIDGF4*. dsRNA of *TcCHT10* (200 ng per insect, $n = 20$) was injected into penultimate instar larvae, last instar larvae, pharate pupae or adult females as indicated above each panel. All *TcCHT10* dsRNA-injected animals died at the ensuing molt. The embryo from adult females injected with dsRNA for *TcCHT10* developed fully but failed to hatch. Unlike RNAi of *TcCHT10*, injection of dsRNA (200 ng per insect) for *TcCHT5* and *TcIDGF4* into penultimate instar and last instar larvae as well as pharate pupae prevented only adult molt. When dsRNA for *TcCHT7* (200 ng per insect) was injected into pharate pupae, normal phenotypes were observed in the pupal stage. However, unlike buffer-injected controls, *TcCHT7* dsRNA-treated insects failed to expand their adult elytra, and their wings did not fold properly (modified from Zhu et al. [72]). These data suggest that individual chitinase may have specialized functions

The group I chitinase, *TcCHT5*, which is also expressed at all developmental stages, appears to be essential only for adult metamorphosis because injections of dsRNA for this gene do not interfere with larval-larval and larval-pupal molting, but does affect pupal–adult metamorphosis [72]. The pharate adults had nearly completed their metamorphosis, but just failed to close and were trapped in their pupal cuticle, indicating that this chitinase has a critical role in digesting the old pupal cuticle (Fig. 9). Even though this gene is expressed during all developmental stages, the failure to see developmental arrest at larval or pupal stages suggests that perhaps other chitinases (including CHT10) compensate for CHT5 during earlier molts, but not in the pupal-adult metamorphosis.

The group III chitinases, which have two catalytic domains and one CBD, are the chitinases having a membrane-spanning domain. The catalytic domains are predicted to be exposed to the extra-cellular space. In *T. castaneum*, this gene is required for abdominal contraction and wing/elytra extension immediately after pupation, but not for molting. The insects injected with dsRNA for this gene developed into adults, but exhibited wing/elytra abnormalities (Fig. 9; 72). Even though this enzyme has chitinase activity, it is not clear whether the activity is critical for its function or whether the enzyme just complexes substrates with N-acetylglucosamines such as glycoproteins, which have β-1-4 linked N-acetylglucosamine residues.

No evidence for any developmental defects was observed when expression of individual members of the family IV chitinases was down regulated by RNAi. It is unclear whether these chitinases perform redundant functions. This is plausible because all of these genes are expressed only in the gut with only minor variations in expression profiles. Perhaps they have a role in digestion of chitin-containing material (in food or exuvia) or in immunity against pathogens containing chitin. Indeed, a role for chitinases in defense against plasmodium has been suggested as chitinase activity in the mosquito gut goes up after blood-feeding [64].

Chitinase-like proteins of group V have been shown to regulate cell proliferation and remodeling in insect and mammalian cells. Since they do not possess chitinase activity they probably act as chitolectins and bind to cell surface receptors. Kawamura et al. [27] demonstrated that four imaginal disk growth factors, IDGF1-4, isolated from the conditioned medium from *D. melanogaster* imaginal disk cells as well as recombinant versions of these proteins, promoted cell proliferation in cooperation with an insulin-like growth factor. Cell motility as well as shape was altered in the presence of IDGFs. The genes encoding

IDGFs are expressed in the yolk cells and fat body (and some other tissues) and are transported in the hemolymph and cause proliferation of imaginal disc cells.

The chitinase like protein, TcIDGF4, belonging to group V, which has no chitinase activity, is nevertheless needed for adult eclosion. This is surprising because *D. melanogaster* IDGFs have been shown to promote proliferation of imaginal disk cells. Perhaps the multiple IDGFs perform redundant functions at all developmental stages except during adult metamorphosis. One possibility is that the rapid proliferation of trachea known to occur at this stage requires TcIDGF4 (Fig. 9; 72). Two *A. gambiae* proteins, AgBR1 and AgBR2, belonging to this family have been shown to be cleaved rapidly upon exposure of the mosquitoes to bacteria. This is reminiscent of the cleavage of *D. melanogaster* IDGF2, though the precise cleavage site in AgBR1 and AgBR2 has not been determined [74]. Clearly some members of the group V proteins have immune functions.

A fat body-specific chitinase may play a role in immune defense

Yan et al. [31] have described an unusual chitinase gene that is expressed in the fat body of the tsetse fly (*Glossina morsitans morsitans*). This 50-kDa chitinase has the chitinase catalytic domain and a CBD, but is missing the ser/thr-rich linker region. This chitinase is detected in adult fat body extracts, milk glands of pregnant females and in intrauterine larvae and pupae. Since no transcripts for this gene could be detected in the larval and pupal stages, it was proposed that the chitinase protein was passed on to the larvae by the mother via milk. There was increased expression of this chitinase gene following a blood meal. It is possible that this fat body-specific chitinase gene may have a role in immune defense against chitin-containing pathogens. Similar defense roles for other chitinases from other insects are likely, but this possibility has not been investigated in detail.

Chitinases increase potency of venom and baculoviruses

Chitinases are also components of some insect venoms. Krishnan et al. [23] isolated and characterized a group IV chitinase without a CBD from the venom of the wasp, *Chelonus* (sp), and proposed that the chitinase allowed rapid penetration of prey tissue by venom components. Baculoviruses also encode chitinases (and proteases) presumably to allow spread of the virus from cell to cell by digesting chitinous barriers of the host. Hawtin et al. [75] reported the identification of a gene encoding a chitinase from *Autographa californica* nuclear polyhedrosis virus (AcMNPV), which did have a eukaryotic signal sequence, but was not secreted from the infected insect cells. This protein has no CBD domain, but possesses a C-terminal KDEL motif, which leads to ER retention [76]. This enzyme, which has both endo- and exo-chitinase activity, is closely related to a chitinase from *Serratia* and only marginally to insect chitinases, and it has been proposed that it was acquired by the baculovirus from a bacterial source. Mutations of the nucleotides corresponding to the active site aspartate and glutamate residues of this chitinase gene from the baculoviral genome resulted in a virus that was defective in liquefaction of the host and reduced virus spread within the host, indicating that chitinase is a virulence factor in baculoviruses [47].

Insect chitinases as pesticides in transgenic plants

There have been a few reports of the use of insect or baculoviral chitinases for control of insect or fungal infestations of transgenic plants with mixed results. The expression level appeared to be low and in the case of the transgenic tobacco lines expressing an *M. sexta* group I chitinase in which the recombinant protein was truncated [77, 78]. Nonetheless, the transgenic plants did not allow growth of tobacco budworms (*Heliothis virescens*), but growth of *M. sexta* larvae (which have a much thicker PM) was not affected. However, when the transgenic plants were sprayed with a sub-lethal dose of *Bacillus thuringiensis (Bt)* toxin, there was greater mortality of *M. sexta* larvae, suggesting that damage to PM integrity increased the access of midgut epithelial cells to Bt toxin. Similarly, transgenic tobacco plants expressing a baculoviral chitinase, which accumulated the recombinant protein in vascular tissues, did not impair the development of *H. virescens* that were allowed to feed on transgenic plants expressing the baculoviral chitinase. On the other hand, there was significant protection of transgenic plants against a fungal pathogen, *Alternaria alternata* [79]. More recently, McCafferty et al. [80] reported that papaya plants expressing a group I *M. sexta* chitinase had greater tolerance to spider mites. Field trials also confirmed increased resistance to spider mite infestations. Synergistic action of chitinase and other insecticidal proteins such as a scorpion insect toxin has also been reported [81].

It may be useful to revisit the mixed results of these attempts to improve resistance of plants to insects by genetic engineering of plants in view of the recent improvements in our understanding of the roles of different groups of chitinases in different tissues of the insects. Group IV chitinases are expressed only in the gut and are likely to be involved in turnover of the PM and or in

digestion of chitinous food constituents. It is possible that the PM-associated chitin and cuticular chitin may be differentially susceptible to different chitinases, perhaps as a result of their different crystalline forms (α versus β) or as a result of their association with different proteins and/or due to the extent of cross-linking. Strategies involving a combination of several chitinases, proteases and different routes of administration of inhibitors may be more effective in insect control than those employing individual chitinases.

Chitinases in disease control

Plasmodia, including the parasitic nematode, *Brugia malayi*, the malarial pathogen *Plasmodium falciparum* and the trypanasomatid protozoan pathogen *Leichmania mexicana* secrete chitinases for exsheathment of microfilaria and/or for penetrating the PM of the vector host mosquitoes by ookinetes or for damaging chitin-containing structures, such as the stomadeal valve at the junction of the sand fly midgut and foregut, respectively [82–84]. Antibodies against some of these chitinases of the parasites are often found in infected mammalian hosts. Elevated levels of chitinolytic enzymes are also found in human blood as a result of macrophage activation in patients infected with the malarial pathogen. *A. gambiae* mosquitoes allowed to feed on human blood with different levels of chitinase (either as a result of disease conditions or infection or artificial supplementation) exhibited progressively greater damage to their PM. Differences in chitinase levels in blood of healthy individuals in different geographic regions have been correlated with susceptibility to malaria [85]. A single-chain antibody capable of neutralizing *Plasmodium falciparum* and *P. gallinaceum* chitinases was shown to be effective in reducing parasite transmission to mosquitoes, leading to the suggestion that malaria transmission could be controlled using such strategies in transgenic mosquitoes [86].

Chitinases for control of insect pests

Recombinant baculoviruses expressing insect chitinases have been shown to increase the rate of death of insects compared to insects infected with the virus alone [29]. Rao et al. [87] reported 100% mortality of fifth instar larvae of *B. mori* fed a diet containing AcMNPV ChiA at a dose of 1 μg/g of larval body weight. Fitches et al. [88] showed that injections of pure chitinase from tomato moth larvae (*Lacanobia oleracea*) decreased cuticle thickness and that oral administration of chitinase led to a high mortality at low doses (2.5 μg chitinase/g of insect). Insect chitinases have also been used as bioacaricides for insect control. Topical application of a recombinant baculovirus expressing a chitinase from the hard tick *Haemophysalis longicornis* (which has two catalytic domains) was effective in controlling tick infections, and this bioacaricidal activity was potentiated by the addition of pure chitinase [89]. Mice immunized with this tick chitinase antigen exhibited protection against tick infection [90].

Concluding remarks

In recent years a great deal of new information concerning the presence in insects of a large assortment of chitinases and chitinase-like proteins falling into multiple groups with distinct domain organizations has been obtained from studies that have utilized the tools of bioinformatics and RNA interference. Biochemical studies have lagged behind, although some progress has been made in comparing the properties of purified chitinases belonging to different groups. These advances have replaced our earlier notions about a single chitinase responsible for turnover of chitin in all tissues with an improved understanding of the large variety of insect chitinases in many insect orders. The evolutionary conservation of these groups of chitinases in all of the insects with completely sequenced genomes suggests that these chitinase isozymes have distinctive biological roles in various tissues and at different developmental stages. Functions as diverse as molting, digestion, cell proliferation and tissue remodeling appear to be influenced by chitinases and related proteins. Differences in their physical and biochemical properties reinforce the notion of distinctive biological functions for specific chitinases. We can clearly anticipate substantial new insights in the area of cuticle and PM assembly and turnover as well as insect development from future studies focused on the roles of individual isozymes of the chitinase family of proteins in insects. In addition, chitinases of pathogens such as plasmodia or nematodes could be targeted for disease control by using them as vaccines to generate blocking antibodies.

Acknowledgments We thank Drs. Karl J. Kramer, Hans Merzendorfer and Richard W. Beeman for their critical reading and editing of this manuscript. We are grateful to Professors Daizo Koga and Kun-Yan Zhu for sharing and allowing us to cite some of their unpublished data in this review. This work was supported by NSF grants IBN-0316963 and IOS-615818. This is contribution 10-021-J from the Kansas Agricultural Experiment Station.

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