

Chapter 2

Chitin Metabolic Pathways in Insects and Their Regulation

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Abstract Chitin, the matrix polymer of arthropod exoskeleton, occurs in three forms that have different structural and mechanical properties. In the insect cuticle and the peritrophic matrix, individual chitin chains are further organized into higher order structures that give chitinous matrices their unique and widely differing properties such as rigidity, elasticity and waterproofing. In this chapter, we review the biochemical pathways of chitin biosynthesis, degradation and modification. In many cases, there are multiple isozymes for carrying out each step of chitin metabolism with specialization among members of families of isozymes. The gene families encoding the enzymes of chitin metabolism and their regulation are presented. The roles of chitin metabolism isoenzymes within families as revealed by gene deletion studies or RNA interference are discussed. How the association of chitin with different assortments of proteins modifies the properties of chitin matrices is briefly outlined.

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2.1 Introduction

Chitin, a linear, unbranched aminopolysaccharide, made up of *N*-acetylglucosamine monomers, is a structural constituent of extracellular matrices such as the cell walls of fungi and the exoskeletons of arthropods including insects (Muzzarelli 1973). In arthropods, the choice of chitin rather than cellulose as the matrix polymer seems to have been designed to increase the versatility of the cuticle in response to the need to adapt to widely different environmental stresses to which they have been subjected during hundreds of millions of years of evolution in both aquatic and terrestrial environments. The presence of the amide function in chitin polymer increases additional hydrogen-bonding opportunities and possibly confers the ability to cross-link with other functional groups in the components of the cuticle. In particular, the large and anisotropic variations in the physicochemical and mechanical properties of the insect cuticle derive from the ability of chitin to be arranged in unique ways and its potential to interact with a large assortment of proteins and possibly tanning agents during the process of cuticle deposition. In this review, we will focus mostly on the metabolic pathways leading to chitin synthesis, modification and degradation, but we will also address how chitin might interact with different proteins to alter the physicochemical properties of chitin-containing matrices. The properties of the mineralized exoskeletons of crustaceans and the hydrated peritrophic matrix will be dealt with in other chapters in this book.

2.2 Structure of Chitin

In natural materials chitin can occur in different forms including the α , β and γ forms (Lotmer and Picken 1950; Ruddal 1963). The α -form is the predominant form characterized by its strong mechanical properties and low solubility and is the form found in the exoskeletons of arthropods including insects. In crustaceans, it is heavily mineralized with calcium or magnesium salts, further increasing its strength. In the crystal structure of α -chitin, which forms orthorhombic crystals, the unit cell has two chains in an antiparallel orientation stabilized by intra-chain H-bonds (Carlstrom 1957; Minke and Blackwell 1978). More recent analysis and refinements of high resolution synchrotron X-ray diffraction data of highly crystalline α -chitin fibers have indicated that in this form, there is a three dimensional network of H-bonded sheets in which two distinct conformations of C6-O6 hydroxymethyl groups exist. These two distinctive O6 atom conformations allow the formation of a three-dimensional network of chitin sheets. This maximizes the formation of intra-chain as well as inter-chain H-bonds involving this H-atom, the carbonyl group (C7) and amino group of the side chain (Sikorski et al. 2009; Beckham and Crowley 2011) that accounts for the extraordinary stability of α -chitin compared to cellulose II (Fig. 2.1a).

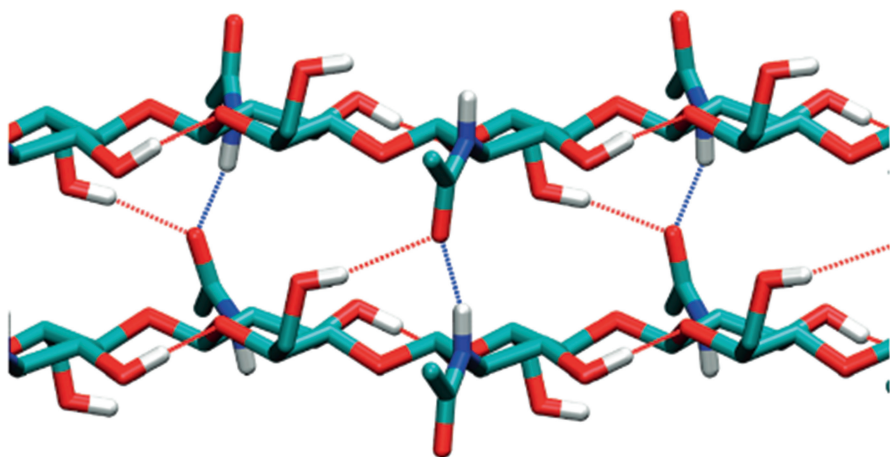


Fig. 2.1a Hydrogen bonding in α -chitin: Bifurcated hydrogen bonds (*broken red and blue lines*) are formed between the GlcNAc side chain and the O6 side chain of every two residues in a layer to the GlcNAc side chain in the layer above. The hydrogen bonds internal to a chain between O5 and O3/HO3 (*solid red lines*) are self-hydrogen bonds (From Sarkar and Perez 2015)

In β -chitin, which forms monoclinical crystals, the chains are organized in a parallel orientation as in cellulose I and forms mostly intra-chain H-bonds involving O3H and O5 except for two H-bonds in the a-axis (NH...O7 and O6H...O7). There are no H-bonds in the b-c plane (Sarkar and Perez 2015; (Fig. 2.1b)). Strong interactions between pyranose rings in adjacent chains do allow formation of stacked sheets, but due to the absence of H-bonds between chains in different planes, β -chitin can be easily hydrated unlike α -chitin. β -chitin is found in squid pens, spines of diatoms, tubes of giant tubeworms and possibly in the peritrophic matrix lining the midgut epithelial cells of insects. These linings lack the structural rigidity of cuticles and, in fact, are highly flexible and hydrated. The chitin in these structures is associated with a large number of glycoproteins with mucin domains, which increase their hydration and help forming hydrogels. γ -chitin is found in the cocoons of some beetles and consists of both β -chitin and α -chitin but its precise structure is not resolved to the same extent as the other two forms of chitin.

2.3 Higher Order Structures Involving Chitin Fibers in the Cuticle

Arthropod cuticles have, in addition to chitin, a wide assortment of proteins, quinones and minerals that contribute to and alter the physicochemical properties of the exoskeleton. The estimates of the size of the chitin molecules vary widely (from 5 to 700 kDa; ~20–3000 monomeric sugars) depending on the technique used for the preparation of chitin for these analyses (Kaya et al. 2014). The low molecular

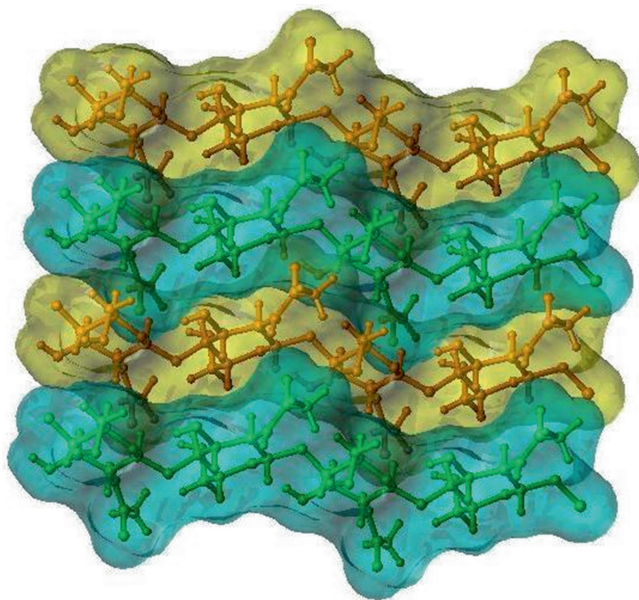


Fig. 2.1b Stacking of adjacent chains in β -chitin without involvement of hydrogen bonds (From Sarkar and Perez 2015)

weight chitins may represent degradation products occurring naturally due to the action of endogenous chitinolytic enzymes or artifacts of the isolation procedures, which require harsh conditions. It is often assumed that the average chitin nanofibers have 18–25 chitin chains that form a chitin core, which associate with various proteins to form chitin-protein composite fibers with an effective diameter of 50–250 nm and lengths of 300 nm (Fabritius et al. 2009). Since this size is significantly smaller than the width of an epidermal cell ($\sim 10\ \mu\text{m}$), several chitin bundles must be attached end to end to cover the epidermal cell. These larger fibrils are arranged in parallel to form a sheet (or lamina) of chitin parallel to the apical surface of the underlying epidermal cells. Additional laminae consisting of chitin-protein complexes are added continuously to the growing cuticle from the assembly zone between the epidermis and the cuticle during intermolt periods. These laminae can be stacked on top of one another in two ways. In the first pattern, each successive layer of fibers is twisted with respect to the layers above and below by a constant angle of rotation of the lamina. This helicoidal pattern of arrangement of successive laminae is known to result in isotropic stress resistance. This helicoidal arrangement also known as Bouligand structure results in an optical illusion in oblique sections in which the chitin fibrils assume a parabolic shape corresponding to each 180° stack (Bouligand 1972). A second type of arrangement involves a “pseudo-orthogonal stack” with two stacks of multiple laminae arranged orthogonally with respect to each other with a transitional zone of helicoidal laminae between them (Fig. 2.2; Fabritius et al. 2009). This is similar to a “cross-ply” laminate used in

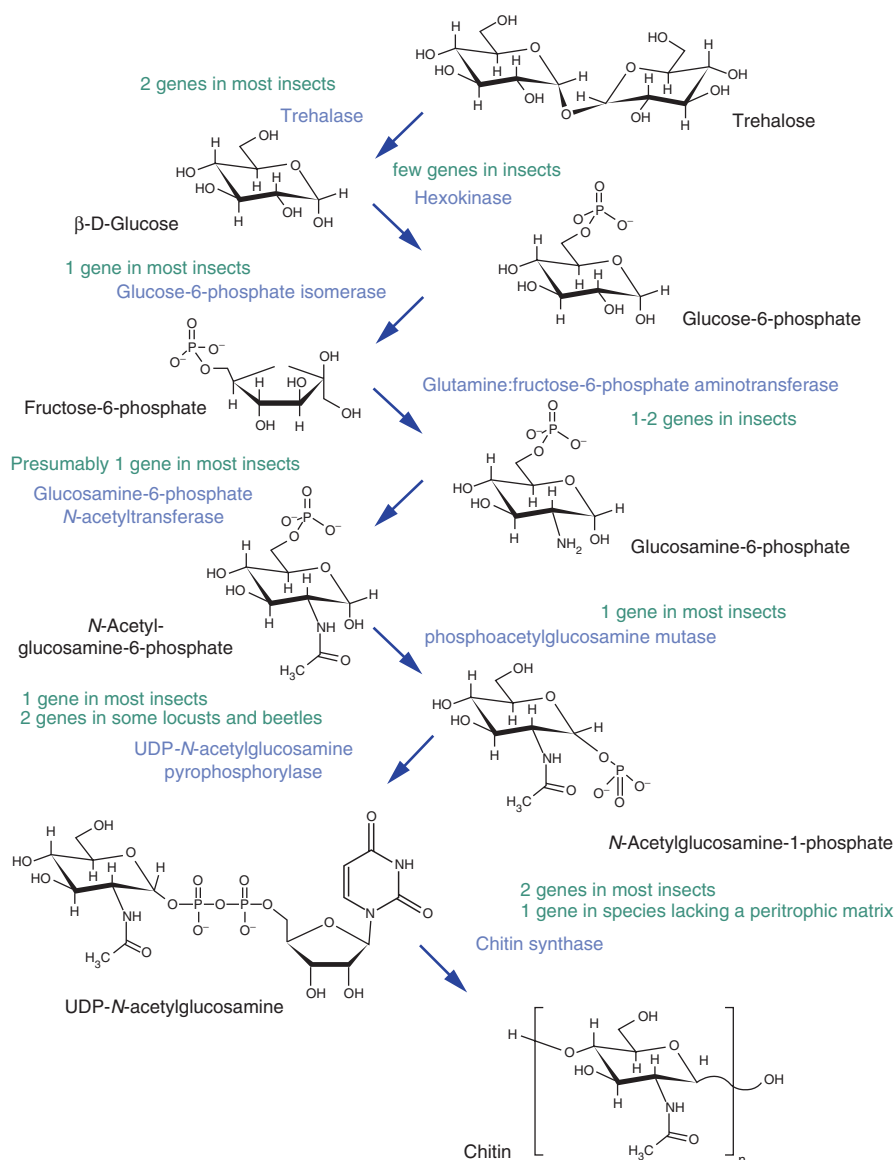


Fig. 2.2 Pathway of chitin biosynthesis in insects. Enzymes of the chitin biosynthesis pathway are depicted in *blue*, and gene numbers are given in *green* (Modified from Merzendorfer and Zimoch 2003)

constructions. In addition, the vertical stacks of laminae are further stabilized by pore canals that traverse the procuticle in a cork-screw-like arrangement. In the middle of these canals are pore canal fibers made of chitin (Fabritius et al. 2009). The composition of the outer boundary of the pore canals is unknown, but presumably is made up of proteins. Immunostaining using gold labeled secondary

antibodies indicate that the presence of cuticular proteins in these pore canals is distinct and often in unique locations (Mun et al. 2015).

2.4 Higher Order Structure in the Peritrophic Matrix

In contrast to the cuticle, the peritrophic matrix (PM)-associated chitin is well hydrated, flexible and can vary widely in thickness and number of layers (Hegedus et al. 2009; Merzendorfer et al. Chap. 8). The PM appears to emerge from the microvilli of the epithelial cells that line the midgut of several lepidopteran and coleopteran insects both in the larval and adult stages. Successive layers of PM (consisting of chitin and proteins associated with them) delaminate from the microvilli and form mesh-like tubules covering the luminal surface of the entire midgut epithelium. They do not form laminae as in the cuticle, but form loosely associated grid-like structures where the chitin-protein complexes criss-cross each other nearly orthogonally (Harper and Hopkins 1997; Harper et al. 1998). Additional proteinaceous material is added to the forming meshwork, which becomes thicker and has a reduced pore size. The structure of the PM and the nature of the proteins associated with it are discussed in more detail in the Chap. 8 by Merzendorfer and colleagues in this book.

2.5 Precursors of Chitin and Generation of Activated Substrates

While it is logical to assume that the supply of carbon for the chitin chains found in insect cuticle and PM would come ultimately from stored form of sugars such as glycogen and from the highly abundant disaccharide, trehalose, in circulating hemolymph (10–50 mM), direct proof for this is lacking for insects. However, several studies are consistent with this assumption. The enzyme trehalase that is needed to convert trehalose into glucose is widespread in species that contain chitin (Becker et al. 1996; Thompson 2002). Two isoforms have been identified in most insects, one of them a soluble form and the other a membrane-bound form that acts on the substrate found in the hemolymph. The glucose liberated by the action of trehalase is presumed to be taken up by cells for utilization in chitin synthesis. In a detailed study, the two genes encoding the beet armyworm, *Spodoptera exigua* trehalase proteins showed differences in tissue specificity of expression and were found to be highly expressed in cuticle-forming tissues and tracheae. Their transcript levels also showed developmental control with the highest levels reached during pupal stages when the rate of chitin synthesis is high (Tang et al. 2008; Chen et al. 2010). RNA interference studies using dsRNA for the trehalase resulted in molting defects and loss of chitin in the cuticle suggesting the importance of trehalose as a precursor for chitin (Chen et al. 2010).

The activated substrate for chitin biosynthesis by the membrane-bound enzyme, chitin synthase, is UDP-*N*-acetylglucosamine. Its formation requires the activity of several enzymes including trehalase, hexokinase, glucose-6-phosphate isomerase, glutamine-fructose-6-phosphate aminotransferase, glucosamine-6-phosphate *N*-acetyltransferase, phosphoglucosamine mutase and UDP-*N*-acetylglucosamine pyrophosphorylase (UAP) (Merzendorfer and Zimoch 2003; Cohen 2010; Muthukrishnan et al. 2012). These enzymes are shared by the glycolytic pathway and the pathway leading to the formation of aminosugars (Fig. 2.2). In particular, the enzyme, glutamine-fructose-6-phosphate aminotransferase (GFAT) is the first committed step in the formation of a common pathway for the biosynthesis of chitin and complex carbohydrates containing *N*-acetylglucosamine. RNAi of one of the two genes encoding this enzymes in the yellow fever mosquito, *Aedes aegypti* led to loss of chitin in the midgut and this was reversed by administration of glucosamine into the hemocoel (Kato et al. 2006). RNAi of this gene in the blood sucking cattle tick, *Haemaphysalis longicornis* resulted in less blood feeding, lower body weight gain and ultimately in death (Huang et al. 2007). GFAT is also sensitive to feedback inhibition by UDP-GlcNAc indicating the importance of this enzyme in regulating the flow of carbon into this pathway. The functional significance of the presence of two genes encoding GFAT has not been explored so far.

Another enzyme of the pathway leading to the formation of UDP-*N*-acetylglucosamine (UDPGlcNAc) has also been studied in some detail. This enzyme, named UDP-*N*-acetylglucosamine pyrophosphorylase (UAP) catalyzes the transfer of UMP moiety from UTP to *N*-acetylglucosamine to form UDPGlcNAc, the substrate for chitin synthase, and pyrophosphate, which is hydrolyzed to inorganic phosphate by the ubiquitous pyrophosphatase. The fruit fly, *Drosophila* UAP enzyme is encoded by a gene named *mummy/cystic/cabrio* and homozygous mutants of this gene show severe cuticular defects and tracheal deformities (Araujo et al. 2005; Schimmelpfeng et al. 2006; Tonning et al. 2006). RNAi of the *Uap* gene in the olive fruit fly, *Bactrocera dorsalis*, results in mortality and abnormal cuticular defects (Yang et al. 2015). There is only one copy of this gene encoding this enzyme in most insects with the exception of the red flour beetle, *Tribolium castaneum*, the migratory locust, *Locusta migratoria* and the Colorado potato beetle, *Leptinotarsa decemlineata* (Arakane et al. 2010; Liu et al. 2013; Li et al. 2015). Down-regulation of transcripts for either gene from *T. castaneum* results in lethality. However, the phenotypes were quite different. Only *TcUap1* appeared to be essential for chitin formation in both the epidermal cuticle and gut. *TcUap2* RNAi resulted in developmental arrest and shrinkage and pupal paralysis without affecting chitin content of either the cuticle or the PM (Arakane et al. 2010). It is likely that *Tribolium* UAP2 may be affecting glycosylation (addition of *N*-acetylglucosamine) of some glycoproteins or some other metabolites. RNAi of *L. migratoria* *Uap* resulted in molting defects but no phenotype was seen after RNAi for *Uap2* from this insect (Liu et al. 2013). RNAi of *L. decemlineata* *Uap1* gene reduced chitin content of integument and tracheal taenidia and affected molting. RNAi of *Uap2* gene from this insect adversely affected peritrophic matrix integrity and larval growth and led to fat body depletion (Shi et al. 2016). Thus, the role of the *Uap2* gene varies in different

insects. The presence of a single *Uap* gene in many other insects indicates that a single UAP protein contributes UDPGlcNAc needed for both chitin synthesis and glycosylation involving addition of *N*-acetylglucosamine, with *T. castaneum*, *L. migratoria* and *L. decemlineata* being exceptions in which the duplicated *Uap* genes have assumed specialized functions. It is noteworthy that the two human UAP enzymes can accept glucosamine-1-phosphate and galactosamine-1-phosphate as co-substrates, though with different kinetic constants (Peneff et al. 2001).

The pathway leading to chitin from trehalose is shown in Fig. 2.2. In the final step, the membrane-bound chitin synthase (CHS) utilizes cytosolic UDP-GlcNAc to synthesize and extrude the product chitin chains across the lipid bilayer to the extracellular matrix side. The number of genes encoding CHS in most insect species genomes is two (Tellam et al. 2000; Ibrahim et al. 2000; Gagou et al. 2002; Zhu et al. 2002; Arakane et al. 2004; Hogenkamp et al. 2005). However, several hemipteran insects that do not elaborate a PM appear to harbor only one *Chs* gene. Examples are the brown planthopper, *Nilaparvata lugens*, the soybean aphid, *Aphis glycines*, the pea aphid, *Acyrtosiphon pisum* and the kissing bug, *Rhodnius prolixus* (Wang et al. 2012; Bansal et al. 2012; Mansur et al. 2014). This is similar to the situation in nematodes, with some nematode genomes having only one *Chs* gene, whereas others have two *Chs* genes (Harris et al. 2000; Harris and Fuhrman 2002). Once again, when there is more than one *Chs* gene, there is specialization in the functions and/or tissue specificity of expression. For example, one *Chs* gene in the nematode, *Caenorhabditis elegans* is responsible for producing chitin in the pharynx, whereas the other one is involved in production of chitin in the eggshell (Veronico et al. 2001; Zhang et al. 2005).

CHS-A protein (encoded by *Chs-1* gene) is expressed in epidermal cells of insects, tracheal lining cells and embryonic serosal cells that specialize in the synthesis of cuticular chitin, whereas CHS-B protein (encoded by *Chs-2* gene) specializes in the synthesis of PM-associated chitin (Arakane et al. 2004, 2005, 2008; Bolognesi et al. 2005; Hogenkamp et al. 2005; Zimoch et al. 2005; Chen et al. 2007; Kumar et al. 2008; Rezende et al. 2008; Jacobs et al. 2015; Chaudhari et al. 2015). Membrane preparations from larval integuments or guts do exhibit chitin synthase activity that is sensitive to inhibitors of chitin synthases (Cohen and Casida 1980a, b). Maue et al. (2009) showed that this enzyme forms an oligomeric complex and there is indication that the enzyme may require proteolytic activation for gaining full activity (Broehan et al. 2007).

While the two enzymes share extensive amino acid sequence and structural similarities and have all the conserved motifs of the glycosyltransferase II family enzymes, they differ in some significant ways. The most interesting difference is the presence of alternate exons in *Chs-1* gene while they are absent in *Chs-2* genes. Depending upon the insect species, there are two sets of alternate exons in *Chs-1* gene, one near the 5' end and one close to the 3'-end. The set of two alternate exons that occurs near the 3'-end of the *Chs-1* gene encodes a 59 amino acids-long region that includes the penultimate transmembrane helix in CHS-A protein. Each of these alternate exons is of the exact same length (177 nucleotides long). The two 59 amino acids-long segments encoded by the two alternate exons of the same species have

significant sequence similarity/identity to each other. But phylogenetic analysis of this region alone from multiple insects belonging to different orders indicates that alternate exon “a”-encoded regions from all insect species are more closely related to one another than to the “b” form encoded by the second alternate exon-encoded region from the same species (Arakane et al. 2004; Ashfaq et al. 2007; Wang et al. 2012; Yang et al. 2013). Thus, the utilization of alternate exons must have occurred long before the branching of the insect orders. Furthermore, there must be an evolutionary pressure to conserve this variation in different orders of insects. Of particular interest is the finding that the relative abundances of the two alternative transcripts are developmentally controlled at least in *T. castaneum*, *Manduca sexta*, and the oriental fruit fly, *Bactrocera dorsalis* (Arakane et al. 2004; Hogenkamp et al. 2005; Zimoch et al. 2005; Zhang et al. 2010; Yang et al. 2013). The relative abundance of the transcript with the exon “a” or “b” changes during developmental stages, with the “b” exon becoming prominent in the pupal stage. In addition, the transcripts with the “b” exon seem to be enriched in tracheae of *M. sexta* and *B. dorsalis* during late pupal stages. There are two notable exceptions to this rule. The water flea, *Daphnia pulex* and the aphid, *A. pisum*, seem to have only the “b” form of this alternate exon (Wang et al. 2012). It has been suggested that the “a” form was lost in species that had no need to make a PM (Wang et al. 2012).

The fruit fly, *Drosophila melanogaster*, embryos with homozygous mutations of the *Chs-1* gene (also known as *kkv*), have a blimp phenotype and the embryos are unable to break open the egg shell (Ostrowski et al. 2002; Moussian et al. 2005a). RNA interference studies in several holometabolous insects have established that the two *Chs* genes have specialized functions. Down-regulation of transcripts for *T. castaneum Chs-1* gene results in reduction in cuticular chitin, failure to molt, mortality and failure of eggs to hatch without affecting PM-associated chitin. On the other hand, RNAi of *Chs-2* gene resulted in loss of PM-associated chitin, growth retardation, and loss of PM integrity (Arakane et al. 2005; Agrawal et al. 2014; Kelkenberg et al. 2015). RNAi at the adult stage resulted in fat body depletion and mortality and loss of chitin in the PM. Similarly, RNAi of *Chs-2* gene from *A. aegypti* led to failure to form a PM after having had a blood meal (Kato et al. 2006). Feeding of *Anopheles gambiae* with dsRNA for *Chs-1* or *Chs-2* genes along with chitosan to promote uptake of dsRNA also led to greater sensitivity to chitin inhibitors and calcofluor (Zhang et al. 2010). Tian et al. (2009) also reported that *S. exigua* larvae fed a diet containing *Escherichia coli* expressing dsRNA for *SeChs-1* gene had reduced survival rate. Mansur et al. (2014) demonstrated that injection of dsRNA for the single *Chs* gene in *R. prolixus* led to severe deformations of the cuticle. In female adults, RNAi of this *Chs* gene affected oogenesis, reduction in ovary size and a significant increase in degenerated oocytes indicating a novel role for CHS in ovary and egg development. Using the same system, Souza-Ferreira et al. (2014) also reported eclosion defects and alterations in the cuticle morphology.

DsRNAs specific for splice variants of *Chs-1* gene have also been performed. In several insects, dsRNA's for either of the two alternatively spliced transcripts resulted in molting failure and death (Arakane et al. 2005; Wang et al. 2012; Yang

et al. 2013). However, the phenotypes and timing of developmental arrest were not identical suggesting that the protein products of this *Chs* gene derived from alternatively spliced mRNAs may have distinctly different functions and/or different tissue specificity. One attractive possibility is that one of these isozymes is uniquely required for tracheal cuticle development and that it cannot be substituted by the other isozyme (Hogenkamp et al. 2005; Yang et al. 2013).

Alternative splicing of an upstream exon of *Chs-1* has been described in some lepidopteran species, including the Asian corn borer, *Ostrinia furnacalis*, the silk worm, *Bombyx mori* and *M. sexta*. In well-studied *Chs-1* gene of *O. furnacalis*, there are two promoters that give rise to two proteins with different *N*-terminal sequences (Qu and Yang 2011, 2012). The shorter of the two transcripts uses a promoter located in the 2nd intron of the larger transcript and codes for a protein that is nine amino acids shorter. From the third exon, the sequences of the transcripts and the encoded proteins are identical. The promoter regions of the two transcripts differ in the presence of binding sites for the ecdysone receptor or the down-stream transcription factors elements, BR-C or FTZ, of the ecdysone-signaling pathway, suggesting differences in the control of their expression by molting hormone. Consistent with this possibility, the relative abundances of the two transcripts also differed at different developmental times, most notably during the pupal period and in different tissues. 20-hydroxyecdysone (20E)-treatment of early fifth instar larvae resulted in an earlier increase in transcripts with exon 2b and 19b indicating that the two promoters respond differently to this hormone (Qu and Yang 2012). Down-regulation of each of these two transcripts using splice isoform-specific dsRNAs resulted in molting abnormalities in a significant number of individuals, with the RNAi of the longer transcript being more likely to produce severe molting defects (Qu and Yang 2011).

More recently, alternative splicing of transcripts for a gene encoding the CHS-B protein from the corn earworm, *Helicoverpa zea* has been reported (Shirk et al. 2015). One of the two alternative spliced transcripts is predicted to result in a truncated protein and to be devoid of chitin synthase activity. The functional significance of this observation and whether similar splicing of *Chs-2* transcripts occurs in other insects remains to be explored.

2.6 Towards the Mode of Action of Insect Chitin Synthases

Chitin synthase is a member of the glycosyltransferase-2 (GT-2) class of enzymes that includes cellulose synthase and hyaluronan synthase. Insect CHS enzymes have 15–16 membrane-spanning helices in a characteristic arrangement of three sets of transmembrane helices (TMH) with 8–9 helices in the *N*-terminal domain followed by the catalytic domain in the cytosolic side, immediately followed by a second 5-TMH-domain presumed to translocate the nascent polysaccharide, and a C-terminal domain with two TMHs in the central catalytic domain. There are several conserved motifs characteristic of all chitin synthases as well as cellulose

synthases and hyaluronan synthases. These motifs include the CHS signature sequence motifs, EDR, QRRRW, and WGTRE (Zhu et al. 2002; Moussian et al. 2005a, b; Merzendorfer 2006) (Fig. 2.3a). Of these, the EDR and QRRRW motifs are close to the active site and are implicated in catalysis and/or binding of substrates based on mutagenic studies with yeast chitin synthases.

Even though there are no crystal structures of GT-2 enzymes from insects, crystal structure of a bacterial cellulose synthase is available. Based on this structure, a model for a chitooligosaccharide synthase encoded by the bacterial *NodC* gene has been proposed (Dorfmüller et al. 2014). These studies have led to the conclusion that the NodC protein has three membrane spanning regions that traverse the lipid bilayer in the following order: outside → inside, inside → outside and the last one outside → inside with the catalytic domain inside the cytosol where it will have access to UDP-GlcNAc (Dorfmüller et al. 2014). This proposed model is consistent with the role of the conserved aspartate in the [EDR] motif acting as the base in the nucleophilic attack of the 4-OH group of the acceptor sugar. It also explains the role of the [Q(Q/R)XRW] motif as part of an α -helix that lines the active site where the W residue can stack on the GlcNAc residue at subsite +2 and the R residue interacts with the negatively charged diphosphate of UDP-GlcNAc (Dorfmüller et al. 2014). We have modeled an insect chitin synthase based on the crystal structures of eight GT-2 enzymes including these two bacterial structures for cellulose synthase and NodC (Fig. 2.3b). The notable difference between the NodC structure and that of the cellulose synthase and insect chitin synthase is the presence of a long tunnel at the active site, which can accommodate several sugars of the elongating chains of cellulose and chitin, whereas the NodC enzyme has a closed pocket that can accommodate only a chain with 5 sugars. The homology model further predicts a central, narrow channel formed by 6 TMH. Interestingly, one helix of the 5-TMS cluster, which was originally predicted to traverse the membrane, is not membrane-integral but is attached to the cytosolic side of the membrane (Fig. 2.3b). This helix is bent and seems to control the entry of the chitin-conducting channel. Mutations in the *Chs-1* gene of the spider mite associated with resistance for the chitin synthesis inhibitor, etoxazole, was found to locate in this helix in a bulk-segregant analysis (van Leeuwen et al. 2012; Demaeght et al. 2012).

Dorfmüller et al. (2014) proposed a reaction mechanism in which during the first synthetic step both the +1 and −1 sugars rotate, but during elongation, the sugar at +1 position rotates only once during two sugar additions as shown in Fig. 2.3. This proposal solves a vexing problem of generating β -1-4 linked sugar polymers where alternating sugars are turned 180° with respect to one another, while utilizing a α -UDP-*N*-acetylglucosamine sugar as the substrate (Fig. 2.4).

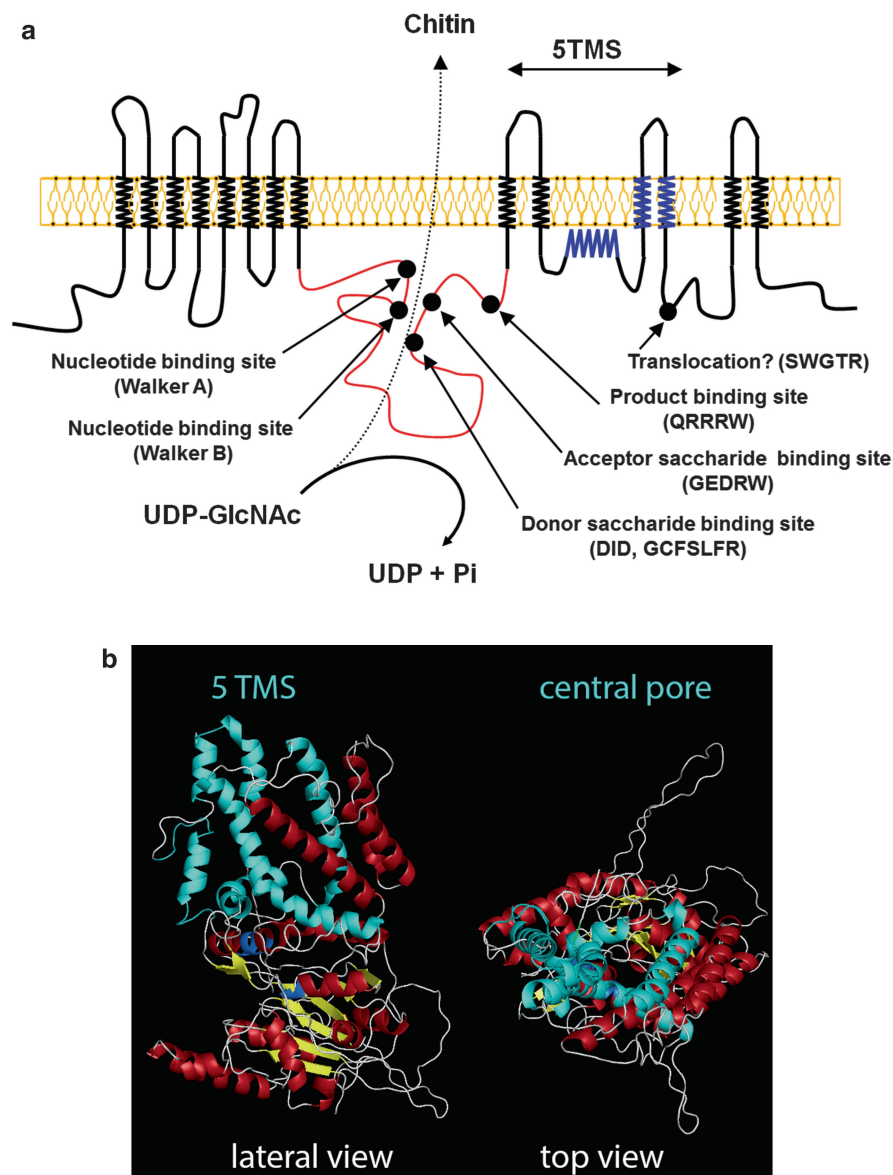


Fig. 2.3 Structural models of the tripartite domain organization of the insect CHS. **(a)** Topology of transmembrane helices (TMHs) and soluble domains. The *N*-terminal *domain A* of *Drosophila* CHS-1 contains 8 TMHs, but this number may vary, from 7 to 10 in other insects. The central *domain B*, forms the catalytic site facing the cytoplasm. *Domain C* was predicted to contain 5 + 2 conserved TMHs. However, homology modeling revealed that one helix does not span the membrane but is attached to the cytosolic side of the plasma membrane, resulting in an intracellular orientation of the *C*-terminus. Putative motifs involved in nucleotide, donor, acceptor, and product binding are indicated. **(b)** Partial 3D structure predictions for CHS-2 from *M. sexta* generated by PHYRE2–Homology modeling was performed using the crystal structures of eight GT2 enzymes as templates. (Left) lateral view, (right) top view. Yellow, β -strands; red, α -helices; cyan, highly conserved α -helices of the 5 TMS cluster; blue, catalytic site motifs (Structure predictions and models were made by S. Gohlke and H. Merzendorfer)

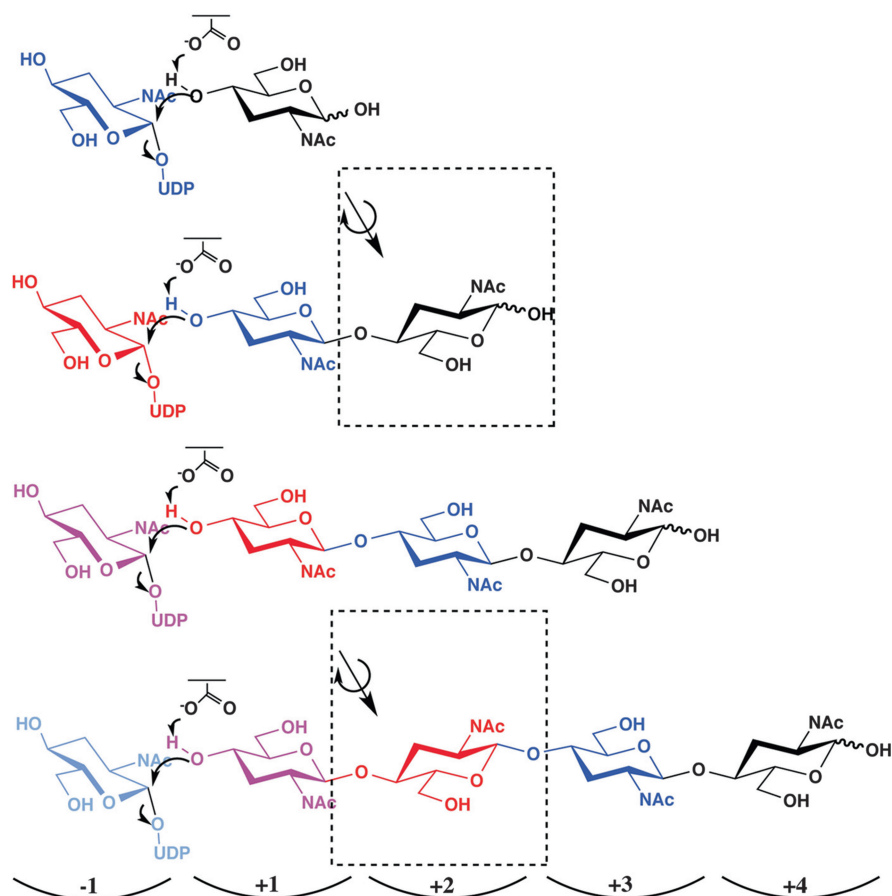


Fig. 2.4 Proposed reaction mechanism of chitin synthesis. Chemical drawing of the proposed reaction mechanism for NodC and chitin synthesis. The 1-hydroxyl group of the donor substrate UDP-GlcNAc is transferred onto the non-reducing end of the growing acceptor oligosaccharide. When the transfer reaction is completed, UDP leaves the active site. In the first synthesis step, the two terminal sugars (-1 and +1) of the growing chain would both rotate while moving into the next binding site (+1 and +2). During further elongation, the +1 sugar would only rotate every second synthesis step (*red sugar* compared with *blue sugar*). All sugars moving into the +3/+4 subsites would remain of a fixed orientation. This rotation and translocation enables the newly added non-reducing sugar to be in the same acceptor position as the previous one (From Dorfmueller et al. 2014)

2.7 Regulation of Chitin Synthesis

During every intermolt period, insects develop a multi-laminate cuticle which seems to arise from the tips of microvilli that are enriched for electron-dense “plasma membrane plaques” (Locke and Huie; 1979; Locke 2001). Presumably, these are the sources of cuticular material including cuticular proteins and chitin synthase, which synthesize and secrete chitin into the procuticle. The procuticle of

several lepidopteran and coleopteran insects is very thin in the early stage of the last instar and keeps growing to a thickness of several hundred laminae, almost until the first appearance of ecdysial droplets. However, this is the period of low ecdysone titers making it unlikely that ecdysone response elements in promoter regions of *Chs* genes regulate chitin synthesis directly (Gagou et al. 2002).

In *O. furnacalis*, the core promoter of *OfChsB* contains the binding sites of only early ecdysone-inducible elements (BR-C and E74A), but not ecdysone-response elements (EcR and USP) (Qu and Yang 2011). The consensus sequences for BR-C and E74A were also predicted to be within the promoter region of *DmeChsB* (Gagou et al. 2002). The presence of ecdysone-inducible instead of ecdysone-response elements indicates that *Chs-B* might be in an ecdysone-dependent regulatory pathway and not directly stimulated by ecdysone. On the contrary, both of the alternative promoter regions of *OfChs-A*, contain the ecdysone-response element EcR, suggesting that *OfChs-A* could be regulated by ecdysone directly (Qu and Yang 2011; Qu and Yang 2012).

2.8 Chitin Deacetylation and Possible Role in Cuticle Assembly

The role of chitin deacetylation is still shrouded in mystery. Naturally occurring chitin is partially deacetylated as revealed by Fourier transform infrared spectroscopy, chemical analysis and analysis of enzymatic digestion products by thin layer chromatography. Depending on the source of chitin, the degree of deacetylation can vary ranging from 5 to 25 %. These estimates are further complicated by the effects of the harsh extraction methods utilized for obtaining chitin free of protein and other cuticular components including lipids and quinone derivatives. But it is clear that deacetylation is vital to proper function of cuticles, which diverge widely in their physicochemical properties. Interfering with deacetylation by RNAi results in abnormal tracheal tubes, cuticle and joint defects, and molting failure and mortality. Since no enzyme is known to synthesize chitosan directly from precursors, chitosan can only arise by chemical or enzymatic deacetylation of preformed chitin. Chitin deacetylation may be coupled with chitin synthesis in the assembly zone of the procuticle because chitin deacetylase (CDA) in the epidermal cuticle is confined to the assembly zone (Arakane, unpublished data). Even the distribution of chitosan in different layers of procuticle has not been studied yet.

2.9 Chitin Deacetylases in Insects

CDAs are metalloproteins belonging to the class of carbohydrate esterase family 4 (CE4) (EC 3.5.1.41) that remove the acetyl group from chitin. They are present in all species that have chitin including fungi, nematodes, and arthropods including

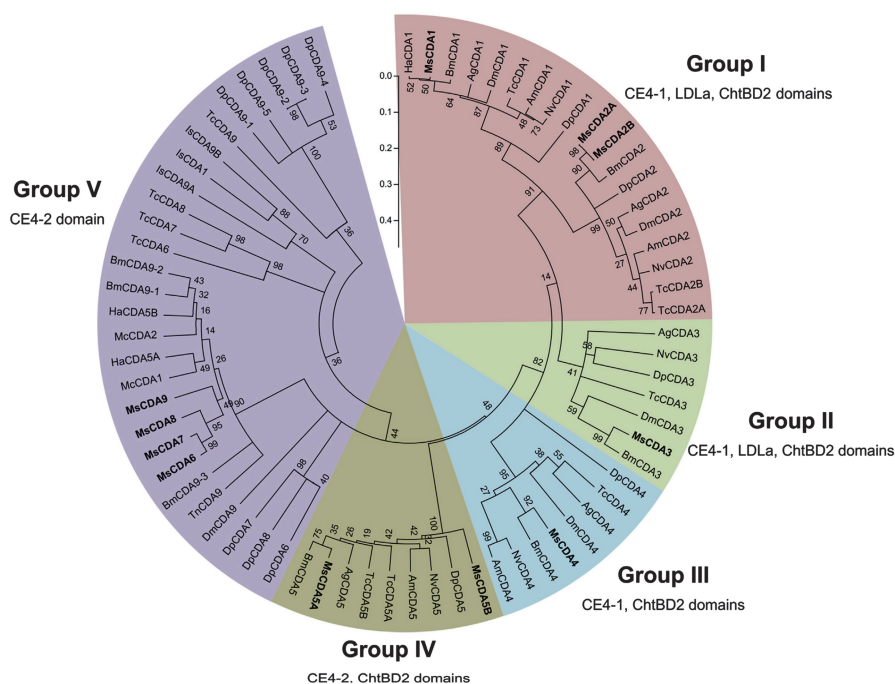


Fig. 2.5 Phylogenetic analysis of chitin deacetylases (CDA) from 12 different species: *Anopheles gambiae* (Ag), *Apis mellifera* (Am), *Bombyx mori* (Bm), *Daphnia pulex* (Dp), *Drosophila melanogaster* (Dm), *Helicoverpa armigera* (Ha), *Ixodes scapularis* (Is), *Mamestra configurata* (Mc), *Manduca sexta* (Ms), *Nasonia vitripennis* (Nv), *Tribolium castaneum* (Tc) and *Trichoplusia ni* (Tn). A bootstrap analysis of 2000 replications was carried out on the trees inferred from the neighbor joining method and bootstrap values are shown at each branch of the tree. CDAs are divided into five different groups: group I (pink), group II (green), group III (light blue), group IV (brown) or group V (dark purple) as described in Dixit et al. (2008) (Reproduced with permission from Tetreau et al. (2015b))

insects. In insects, these proteins are encoded by a family of genes that number from 4 to 9 (Dixit et al. 2008; Campbell et al. 2008; Xi et al. 2014; Tetreau et al. 2015b). Phylogenetically, insect CDAs fall into five groups with distinct domain organizations (Fig. 2.5). While all groups have the CE-4 domain at the C-terminal part, groups I and II have two additional domains, a low density lipoprotein receptor-a (LDLa) domain and a chitin binding domain (CBD) at the N-terminal part. The group III and IV CDAs lack the LDLa domain but retain the CBD domain (see Muthukrishnan et al. 2012 for a more complete description of the five phylogenetic groups). The purpose of the additional domains besides the catalytic domain (CE4) is not established, but the conservation of representatives of all five groups in most insects (some hemipterans and anapleurans lack group II and group V CDAs) suggests that these CDAs are essential for insect survival.

Evidence for the requirement of distinct CDAs belonging to different groups comes from studies of *D. melanogaster* Cda mutants, RNAi studies in *T. castaneum*

and *N. lugens*, and studies on the suppression of specific *Cda* transcripts following baculovirus infection of *H. armigera* (Jakubowska et al. 2010). Homozygous null alleles of two group I *Cda* genes from *D. melanogaster* (*serpentine* and *vermiform*) exhibit convoluted, elongated, and wider dorsal tracheal trunks during embryonic development indicating a role for these proteins in regulating tracheal tube dimensions and rigidity. The embryos had a bloated phenotype reminiscent of *Chs* mutants indicating loss of rigidity of the cuticle as well (Luschnig et al. 2006; Wang et al. 2006). A detailed RNAi study of all 9 *Cda* genes from *T. castaneum* was carried out by Arakane et al. (2009). These studies have revealed distinct and specific functions of several CDAs and, in some cases, specific splice isoforms. Group I CDAs (TcCDA1 and TcCDA2) were both critical for molting and survival during development (but not in adult life) indicating that they performed non-redundant, but essential functions, even though both were expressed in the same tissues. The two splice isoforms of CDA2 appeared to regulate different cuticles. RNAi of *Cda2a* isoform affected movement of femoral-tibial joints and led to failure of egg hatch. On the other hand, RNAi of CDA2b did not affect these functions but led to wrinkled elytral surface. It is likely that there are differences in expression (and requirement) for specific CDAs and their isoforms in different regions of the insect anatomy. RNAi of group I CDAs does lead to reduction in chitin content and loss of laminar organization (our unpublished data).

RNAi of the CDAs belonging to the other four groups in *T. castaneum* failed to produce any visible effects and, therefore, no conclusions regarding their function could be drawn, except some speculations based on the tissue specificity and developmental patterns of expression. However, RNAi studies using a dsRNA for a group IV CDA in *N. lugens* did result in molting failure (Xi et al. 2014). The finding that several hemimetabolous insects and even mosquitoes do not have group V CDAs and the failure to see RNAi effects for this group of CDAs in insects that are expressed predominantly in the gut tissue is intriguing. This finding suggests that the group V CDA may be involved in digestion of chitinous material in the diet or some immune function rather than modification of endogenous chitin. It is interesting to note that there is wide variation in the number of proteins belonging to this group.

CDAs are proteins with leader peptides and are without membrane-spanning or obvious membrane-anchoring segments. Therefore, they are expected to be secreted from the cells in which they are made. This is consistent with their location in the lumen of tracheal tubes and in the procuticle (Luschnig et al. 2006; Wang et al. 2006; Arakane et al. 2009). However, a second tissue, namely the fat body has been shown to be a source of this protein as well (Dong et al. 2014). All CDAs except group V members have CBD or LDLa domains that have several cysteines presumably involved in the formation of disulfide bonds that stabilize the three dimensional structures of these proteins. They are thought to partially deacetylate chitin consistent with a degree of deacetylation of ~20% observed in naturally occurring chitin. Toprak et al. (2008) reported that an *M. configurata* CDA expressed in *E. coli* could deacetylate colloidal chitin using an *in gel* assay that detects formation of chitosan. Zhong et al. (2014) expressed a *B. mori* CDA in yeast and claimed

it has deacetylase activity using an assay involving conversion of p-nitroacetanilide to p-nitroaniline. Whether or not this enzyme can deacetylate native chitin remains to be proven. Three CDAs belonging to family 1, 2 and 4 were found in the molting fluid of *B. mori* (Qu et al. 2014). The recombinant products of these CDAs were catalytically inactive when assayed with the formaldehyde-fluorescamine method (Blair et al. 2005). Other attempts to demonstrate deacetylation of chitin using ^3H -chitin, colloidal chitin in gel assays, or with chitooligosaccharides as substrates using purified CDAs, expressed in a baculovirus expression system, have failed as well (Guo et al. 2005; Jakubowska et al. 2010; Arakane, Dittmer and Muthukrishnan unpublished). However, Jakubowska et al. (2010) reported increased permeability of PM incubated *in vitro* with the CDA preparation from *H. armigera*. They also reported suppression of transcripts coding for a specific CDA after baculovirus infection, which suggests a biological function for this protein. Since chitosan is present in cuticle and there is no pathway for direct synthesis of this polymer except via deacetylation of chitin, we suspect that CDA may be active only on nascent chitin and that it may require assistance from other proteins in this process.

2.10 Chitin Degradation

The pathway of chitin degradation is almost as complex as the biosynthetic pathway, because the native substrate is crystalline chitin in association with a large assortment of proteins in the cuticle, which is often sclerotized. In the gut, chitin is in association with an assortment of chitin-binding peritrophic matrix proteins, of which many have mucin-like linker domains (Tellam 1996; Wang et al. 2004; Campbell et al. 2008; Dinglasan et al. 2009; Hegedus et al. 2009; Merzendorfer et al. Chap. 8). Very little is known about the reactions that strip away the matrix-associated proteins to expose the chitin nanofibrils to chitinases. In the gut and in the molting fluid, there is a large assortment of proteases, which assists in this process. The process of matrix-associated chitin degradation must be regulated, because transcripts for the major chitinase are not detectable in the integument tissue until after cessation of the feeding period in each larval instar, and appears only just prior to pupation (Kramer et al. 1993). In addition, the transcripts encoding chitinases essentially disappear after a very short period of a day or two in the molt cycle. Likewise, transcripts for some chymotrypsin-like enzymes implicated in molting also exhibit developmental control (Broehan et al. 2008, 2010).

Just prior to molting and apolysis, dramatic changes occur at the interface between the old cuticle and the underlying epidermal cell layer. The inner unsclerotized part of the old procuticle begins to get degraded as a result of the accumulation of hydrolytic enzymes in the molting fluid, which appears in the space between the epidermal cell and the overlying cuticle. The apical plasma membrane-associated microvilli enriched in electron-dense “plasma membrane plaques” begin to degrade and disappear presumably due to endocytosis (Locke and Huie; 1979). This is followed by the appearance of new microvilli associated with epidermal

cells, which are initially devoid of these plaques but become electron-dense soon after. These authors also noted that the peak of 20E coincided precisely with the timing of disappearance of the plaques and the onset of apolysis. It is likely that ecdysteroids control the expression of genes encoding several proteins in the molting fluid either directly or indirectly. Injection of 20E into last instar *B. mori* and *M. sexta* larval abdomens isolated from the source of prothoracic hormones by a ligation results in premature appearance of chitinase activity in several insect systems (Kimura 1976; Fukamizo and Kramer 1987; Koga et al. 1992). This induction is at the level of transcription, as shown by the increase in transcripts for chitinase in the same ligated abdominal system described above (Kramer et al. 1993; Zheng et al. 2003). Similarly, Royer et al. (2002) reported that transcript levels for a larger chitinase (a class II chitinase) increased within 2–4 h after injection of 20E. This induction was unaffected in the presence of the protein synthesis inhibitor cycloheximide indicating that the effect of 20E was directly at the level of transcription and did not require continued protein synthesis. However, analysis of promoter regions of individual chitinase genes has not identified the presence of ecdysteroid responsive elements. No studies on the binding of 20E-inducible transcription factors to promoter regions of chitinase genes have been reported.

2.10.1 Chitinases Are Encoded by a Very Large Family of Genes

The initial report of the isolation and characterization of a molting fluid-associated chitinase, cloning of a full length cDNA encoding this protein and characterization of the corresponding gene by sequencing along with Southern blot analysis with probes from the chitinase cDNA, gave rise to the false impression that a single chitinase gene was responsible for the appearance of chitinolytic activity in the molting fluid and gut (Kramer et al. 1993; Choi et al. 1997; Koga et al. 1997). As whole insect genome sequences became available and functional analyses of individual chitinases were increasingly conducted, it became clear that insect chitinases belong to a large family of enzymes with different domain organizations, expression profiles, tissue specificity and function. The number of genes in insect genomes encoding chitinases or related proteins ranges from a low of seven to as many as 22 (or more) chitinases (Zhu et al. 2004, 2008a; Nakabachi et al. 2010; Zhang et al. 2011; Pan et al. 2012; Merzendorfer 2013). They have been grouped into ever increasing number of subgroups based on a combination of domain organization and/or tissue specificity of expression and phylogenetic analyses as well as functional analyses (Zhu et al. 2008c; Nakabachi et al. 2010; Zhang et al. 2011; Tetreau et al. 2015a) (Fig. 2.6).

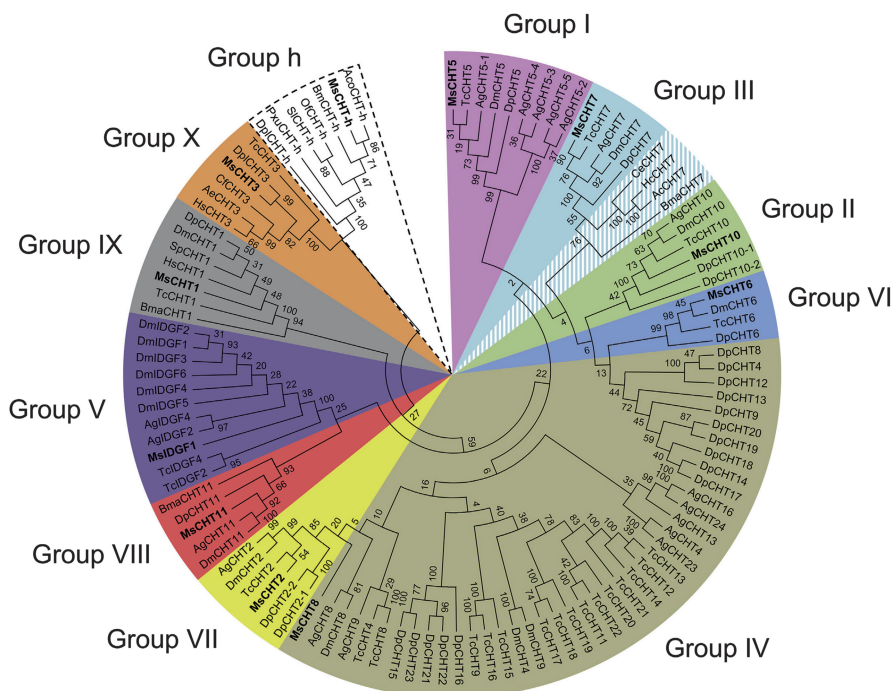


Fig. 2.6 Phylogenetic analyses of insect chitinase-like proteins: *Ancylostoma ceylanicum* (Ac), *Agrius convolvuli* (Aco), *Anopheles gambiae* (Ag), *Bombyx mori* (Bm), *Brugia malayi* (Bma), *Caenorhabditis elegans* (Ce), *Camponotus floridanus* (Cf), *Danaus plexippus* (Dpl), *Daphnia pulex* (Dp), *Drosophila melanogaster* (Dm), *Haemonchus contortus* (Hc), *Harpegnathos saltator* (Hs), *Manduca sexta* (Ms), *Ostrinia furnacalis* (Of), *Papilio xuthus* (Px), *Plutella xylostella* (Px), *Spodoptera litura* (Sl), *Strongylocentrotus purpuratus* (Sp) and *Tribolium castaneum* (Tc). A bootstrap analysis of 2000 replications was carried out on the trees inferred from the maximum likelihood method and bootstrap values are shown at each branch of the tree. CHTs are grouped into 10 different groups: group I (light purple), group II (green), group III (light blue), group IV (brown), group V (dark purple), group VI (dark blue), group VII (yellow), group VIII (red), group IX (gray), group X (orange) or group h (white and dotted lines). As CHT7 chitinases from Nematoda (*A. ceylanicum*, *B. malayi*, *C. elegans* and *H. contortus*) have a divergent domain organization from other group III chitinases they are highlighted by a hatched light blue area. CHT from *M. sexta* are indicated in **bold** (Figure reprinted with permission from Tetreau et al. (2015a))

2.10.2 Domain Organization and Structure of Chitinases

The individual chitinases differ in the number and assortment of catalytic domains, chitin-binding domains, serine-threonine-rich linker or mucin domains and polycystic kidney disease domains (PKD). It is clear that while there are at least seven groups of chitinases with one member in each group in all insects (the body louse, *Pediculus humanis corporis* with seven chitinases represents the minimal set), expansion of groups and duplications within groups has occurred to raise this number to ten at present (Fig. 2.7).

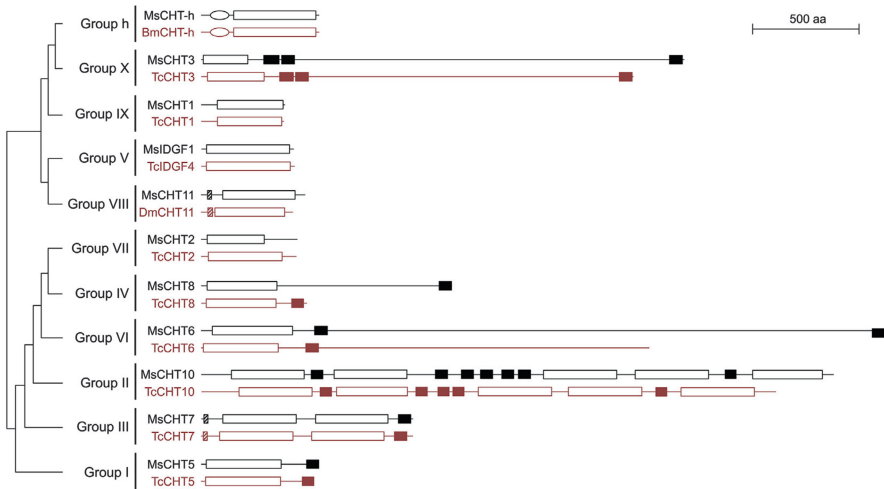


Fig. 2.7 Domain organization of chitinases (CHT) and chitinase-like (IDGF) proteins in insects. For each *M. sexta* CHT from each group, one ortholog from *T. castaneum* (groups I, II, III, IV, V, VI, VII, IX, and X), *B. mori* (group h) or *D. melanogaster* (group VIII) is represented. These orthologs are representative of the typical domain organization for each group. The domain organization was generated with the SMART tool (<http://smart.embl-heidelberg.de/>) by using the protein sequences. *M. sexta* proteins are represented in black, while proteins from other species are in red. Catalytic GH18 domains (white boxes), chitin-binding CBM14 domains (dark and red boxes), transmembrane spans (hatched boxes), polycystic kidney disease 1 (PKD1) domain (ovals), and linker regions (lines) are indicated (Figure reprinted with permission from Tetreau et al. (2015a))

The domain organization and conserved motifs of insect chitinases have been reviewed recently (Arakane and Muthukrishnan 2010; Merzendorfer 2013). The simplest and smallest chitinases have just the glycosylhydrolase 18 (GH18) domain (with a signal peptide coding region), while the largest chitinases have 5 GH18 domains, and 5–7 CBDs. Full-length cDNA clones for representative chitinases from several of these groups have been obtained (Kramer et al. 1993; Abdel-Banat et al. (2001); Royer et al. 2002; Zhu et al. 2008a; Wu et al. 2013). In some cases, they were expressed in baculovirus-insect cells or yeast expression systems and shown to have chitinolytic activities (Gopalakrishnan et al. 1995; Arakane et al. 2003; Wu et al. 2013; Zhu et al. 2008b). They differ in their pH optima, kinetic constants for chitin versus chitoooligosaccharide substrates and affinity for chitin beads. Vast majority of them have the signature motif 2 (FDGLDLDWEYP or variations thereof) that has been implicated in catalysis (Watanabe et al. 1993; Lu et al. 2002; Arakane and Muthukrishnan 2010). Notable exceptions are chitinases belonging to group V (also known as imaginal disc growth factors, or IDGF's that lack catalytic activity; Kawamura et al. 1999; Kanost et al. 1994) and have additional insertion loops (Zhu et al. 2008b). In particular, the proton donor, glutamate (E residue) that is critical for catalysis in the conserved region 2 found in all family 18 chitinases (Watanabe et al. 1993; Lu et al. 2002) is replaced with an asparagine or a glutamine residue. Unlike the plant derived family 19 chitinases with an inverting

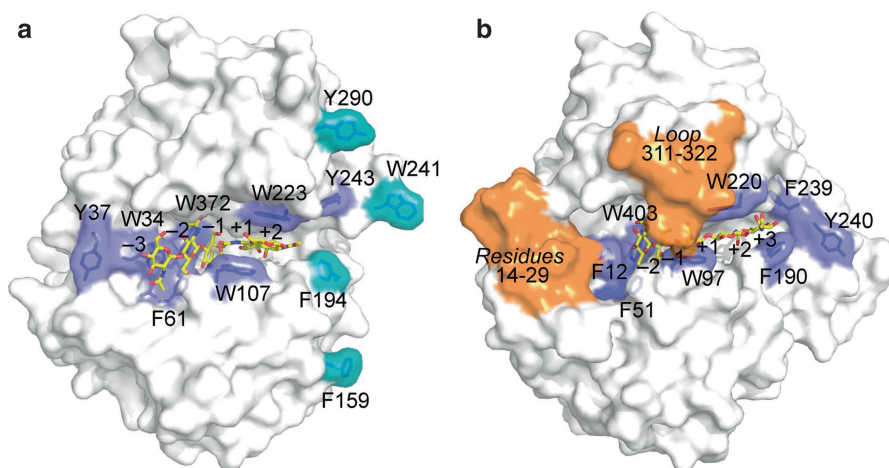


Fig. 2.8 The substrate-binding clefts of the OfChtI-CAD (**a**) and SmChiB (**b**) complexes. The carbohydrate-binding module and the linker of SmChiB are not shown. The (GlcNAc) 2/3 bound to OfChtI-CAD and the (GlcNAc) 5 bound to SmChiB (PDB entry 1e6n) are shown as sticks with yellow *C* atoms. The numbers indicate the subsites to which the sugar is bound. The aromatic residues in the substrate-binding clefts of OfChtI-CAD and SmChiB are labeled and are shown as blue sticks. In OfChtI-CAD, the four residues forming the hydrophobic plane are shown in cyan. In SmChiB, loop 311–322 forming the roof of the tunnel and residues 14–29 forming the blunted non-reducing end are labeled (With permission granted on behalf of IUCr)

mechanism that yields α -anomer products, insect chitinases that belong to family 18 have a retraining mechanism of action and produce β -anomers. It is also likely that the action of these enzymes involves substrate-assisted catalysis utilizing the carbonyl group of the *N*-acetylglucosamine and the formation of an oxazolium ion intermediate (Brameld et al. 1998).

The crystal structures of a family 1 chitinase from *O. furnacali* in the unbound form and in complex with substrates have been determined recently by Chen et al. (2014). This study has revealed that the substrate-binding site takes the shape of an open groove-like cleft and that the reducing sugar at the -1 site is in the energetically unfavorable boat conformation (Fig. 2.8). This is in contrast to the bacterial enzyme SmChiB from *Serratia marcescens*, which has a tunnel-like substrate-binding pocket. Further, the presence of four aromatic amino acids forming a hydrophobic plane near the catalytic site were shown to be involved in substrate anchoring, but not in catalysis using mutant forms of these enzymes in which these aromatic amino acids were substituted by other residues.

2.10.3 Tissue Specificity and Regulation of Expression of Chitinase Families

The timing of appearance and locations of the chitinases belonging to different groups provide some clues regarding their putative functions. Chitinases belonging to groups I and II are found in molting fluid (for examples see Koga et al. 1992; Qu et al. 2014) and are expressed only in cuticle forming tissue (notably they are absent in the gut). Group IV chitinases show the opposite specificity with strong expression in the gut during larval stages and low (or no) expression in integument (Zhu et al. 2008b; Zhang et al. 2011). Some of them were expressed in insect cells using baculoviral expression systems and were also shown to be enzymatically active (Gopalakrishnan et al. 1995; Zhu et al. 2008b). The members of group III (and the more recently discovered group VIII) chitinases have a membrane-spanning domain. Group III chitinases have also been shown to be enzymatically active (Arakane, unpublished data; Qing Yang, unpublished data) and their catalytic domains are pointing to the cuticle side and therefore are presumed to digest chitin. Group III chitinases are expressed predominantly in pupal stages in *An. gambiae* (Zhang et al. 2011; Noh and Arakane unpublished results; Qing Yang unpublished data). This group of chitinases is not required for molting *per se*, but seems to affect the ultrastructure of the cuticle.

2.10.4 Specialization in the Functions of Families of Chitinases

The diversification in the domain architecture of the many families of chitinases may be related to their distinctive biological functions. There are substantial variations in the tissue specificity, timing of expression and regulation of each family of chitinases. RNAi experiments have revealed that group I chitinases are critical for molting especially at the pharate adult stage. Down-regulation of transcripts for these genes resulted in molting failure and trapping of the fully developed insects inside their pupal cuticles (or cuticle from the previous instar) (Zhu et al. 2008c; Zhang et al. 2012a, b; Xi et al. 2015; Li et al. 2015). However, at earlier stages, this enzyme appears dispensable, presumably because other enzymes can fill in for this enzyme. RNAi of group II chitinases that have four or more catalytic domains affected hatching of embryos and molting at every developmental stage indicating their indispensable nature. This chitinase is also likely to be present in the molting fluid as it has a cleavable signal peptide, though this point has not been studied carefully. RNAi of group III chitinases that have two catalytic domains in tandem did not prevent adult eclosion but the insects had reduced chitin content in their procuticle, loss of laminar organization and abnormal adult cuticles (Arakane, unpublished data). RNAi of group IV chitinases from *T. castaneum* using dsRNAs for a single group IV chitinase gene or combinations of dsRNAs for three genes of this

group did not result in any visible phenotypes (Zhu et al. 2008c). However administration of dsRNA for a group IV chitinase from *O. nubilalis* resulted in an increase in chitin content of PM and reduced weight gain of larvae (Khajuria et al. 2010). RNAi of a group V chitinase-like protein from *T. castaneum*, TcIDGF4, prevented adult eclosion but had no effect at other developmental stages (Zhu et al. 2008c). Similarly in *N. lugens*, RNAi of chitinases belonging to group I, II, III and V resulted in failure of nymph-nymph molt (Xi et al. 2015) confirming that at least these four groups of chitinases are indispensable in this insect too.

The presence of chitinases with distinct domain organization and non-redundant, but essential functions suggests that they have functions besides degradation of chitin in the old cuticle. These could include providing primers for elongation of chitin and processing of mature chitin chains for higher level of organization.

2.11 Chitinolytic *N*-Acetylglucosaminidases (EC 3.2.1.52) and Their Genes

N-acetylglucosaminidases (NAGases) have been identified or purified from a variety of sources from insects including molting fluid, hemolymph, integument and gut and shown to be enzymatically active (Koga et al. 1982; Nagamatsu et al. 1995; Zen et al. 1996; Filho et al. 2002; Tomiya et al. 2006; Leonard et al. 2006; Yang et al. 2008; Kokuho et al. 2007). They belong to family 20 glycosylhydrolases and produce hexosamines (*N*-acetylglucosamine and *N*-acetylgalactosamine) by cleaving aminosugars from the non-reducing ends of chitoooligosaccharides or the terminal residues of *N*-glycans, glycoproteins and glycolipids (Intra et al. 2008; Liu et al. 2012). While some of them may be specific for degrading chitoooligosaccharides, others may be involved exclusively in the removal of hexosamines from *N*-glycans (Léonard et al. 2006; Yang et al. 2008), glycoconjugate degradation and egg-sperm recognition (Cattaneo et al. 2006; Okada et al. 2007; Liu et al. 2012). The enzymes of the former group act synergistically with endochitinases, which cleave crystalline chitin into chitoooligosaccharides. They may also alleviate the inhibition of chitinases by accumulating chitotriose and chitotetraose in the molting fluid (Fukamizo and Kramer 1985a, b).

NAGases in insects have been divided into three major groups based on phylogenetic analyses of proteins predicted from several insect genomes (Hogenkamp et al. 2008). In addition, insects have additional proteins with lower sequence similarity to these three groups and constitute another branch that includes several well-characterized mammalian hexosaminidases involved in glycan processing (Fig. 2.9).

NAGases belonging to group I are the major chitinolytic enzymes and are found in abundance in insects. They have the highest catalytic efficiency with chitoooligosaccharide substrates (Liu et al. 2012). The crystal structure of NAG-1 (Hex-1) from *O. furnacalis* was determined by Liu et al. (2012). In the crystal structure, this enzyme exists as a homodimer and has a deeper and larger substrate-binding pocket

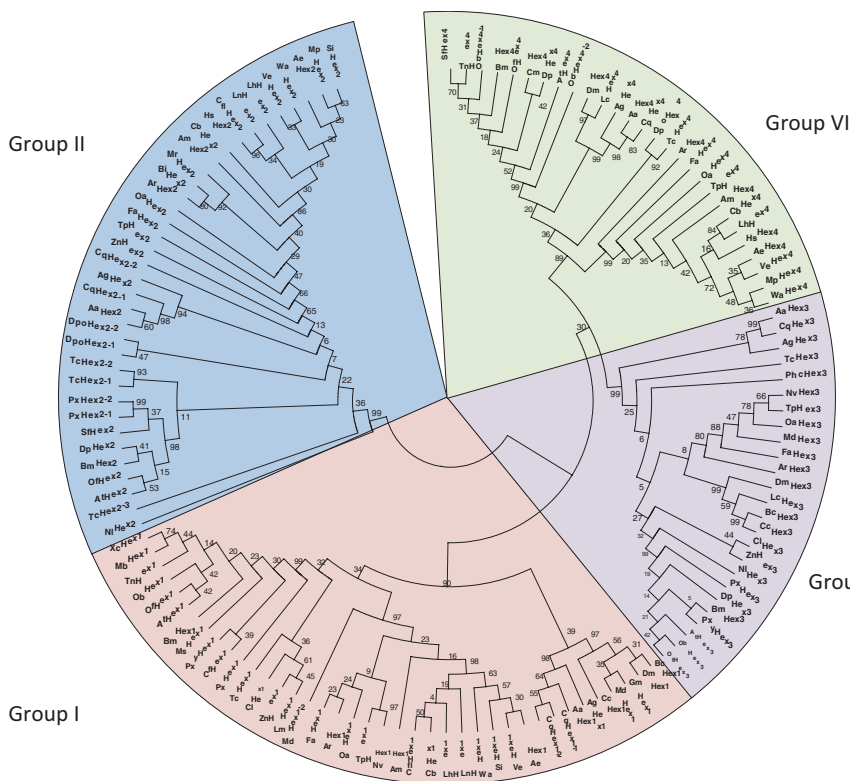


Fig. 2.9 Phylogenetic analysis of insects GH20 *N*-acetylhexosaminidases (Hex) from 48 different species; *Aedes aegypti* (Aa), *Acromyrmex echinator* (Ae), *Anopheles gambiae* (Ag), *Apis mellifera* (Am), *Athalia rosae* (Ar), *Amyelois transitella* (At), *Bactrocera cucurbitae* (Bc), *Bombus impatiens* (Bi), *Bombyx mori* (Bm), *Cerapachys biroï* (Cb), *Ceratitis capitata* (Cc), *Choristoneura fumiferana* (Cf), *Camponotus floridanus* (Cfl), *Cimex lectularius* (Cl), *Cnaphalocrocis medinalis* (Cm), *Culex quinquefasciatus* (Cq), *Drosophila melanogaster* (Dm), *Danaus plexippus* (Dp), *Dendroctonus ponderosae* (Dpo), *Fopius arisanus* (Fa), *Glossina morsitans morsitans* (Gm), *Harpegnathos saltator* (Hs), *Lucilia cuprina* (Lc), *Linepithema humile* (Lh), *Locusta migratoria* (Lm), *Lasius niger* (Ln), *Mamestra brassicae* (Mb), *Musca domestica* (Md), *Monomorium pharaonis* (Mp), *Megachile rotundata* (Mr), *Manduca sexta* (Ms), *Nilaparvata lugens* (Nl), *Nasonia vitripennis* (Nv), *Orussus abietinus* (Oa), *Operophtera brumata* (Ob), *Ostrinia furnacalis* (Of), *Pediculus humanus corporis* (Phc), *Papilio xuthus* (Px), *Plutella xylostella* (Pxy), *Spodoptera frugiperda* (Sf), *Solenopsis invicta* (Si), *Tribolium castaneum* (Tc), *Trichoplusia ni* (Tn), *Trichogramma pretiosum* (Tp), *Vollenhovia emeryi* (Ve), *Wasmannia auropunctata* (Wa), *Xestia cnigrum* (Xc), *Zootermopsis nevadensis* (Zn). A bootstrap analysis of 2000 replications was carried out on the trees inferred from the neighbor joining method and bootstrap values are shown at each branch of the tree. Hexs are divided into four different groups: group I (light red), group II (light blue), group III (light purple), group IV (light green) (Figure reprinted with permission from Tetreau et al. (2015a))

capable of binding oligosaccharides compared to the human and bacterial enzymes, which can accommodate only one sugar in their active site pockets. They are expressed at high levels in the larval carcass, and down-regulation of the transcripts for these genes has been shown to result in severe molting defects and death at the next molt in *T. castaneum* and *O. furnacalis* (Hogenkamp et al. 2008; Liu et al. 2012).

TcNag2 is expressed predominantly in the midgut in the larval stage. However, RNAi of this gene resulted in only lower mortality during larval-larval and larval-pupal molts but substantial mortality at the pharate adult stage indicating its role in molting. The tissue specificity of expression of this gene at the pupal stage has not been reported so far. The enzymatic properties of Hex2 show that it is an enzyme with broad substrate-spectrum capable of hydrolyzing *N*-acetylhexosamine from chitin oligosaccharides, *N*-glycan and glycolipids (Liu et al. 2012). But unlike human Hex A, OfHex2 could not degrade charged substrates such as ganglioside GM2 and peptidoglycan. Real-time PCR analysis demonstrated that the expression of the *O. furnacalis* Hex2 was up-regulated in larva and pupa, and mainly occurred in the carcass rather than in the midgut during the feeding stage of fifth (final) instar larva. RNAi of Hex2 caused non-lethal but severe abnormalities of larval abdomen, pupal and adult appendages (Liu et al. 2013). Thus its properties are consistent with a more limited role in degradation of chitooligosaccharides compared to NAG1.

The third group of NAGases is called FDLs based on prior nomenclature of an orthologous gene from *D. melanogaster*, which has “fused lobes” phenotype (Leonard et al. 2006). The DmFDL was shown to release α -1,3-linked mannose of the core pentasaccharide of *N*-glycans, but does not act on chitotriose or on the GlcNAc-GlcNAc bonds in *N*-glycans. DmHex3 may be involved in sperm-egg recognition and in fertilization (Cattaneo et al. 2006). The tissue-specific expression pattern analysis indicated that OfHex3 was mostly localized in the fat body and testis. Thus the enzymes of this group are like to be involved in deglycosylation of hexosamine residues from terminal positions of *N*-glycans or glycolipids. In the molting fluid of *O. furnacalis*, an interaction between OfHex3 with OfHex1 was detected by co-immunoprecipitation (Qu et al. 2014). Enzymatic activity analysis indicates that OfHex3 is able to degrade chitooligosaccharides, but at a lower rate than that of OfHex1, and had no ability to hydrolyze other glycans from glycoproteins and glycolipids. A recent proteomic analysis proved the presence of Hex-3 (FDL) in the molting fluid of *B. mori* (Qu et al. 2014). Thus the precise function of enzymes of the FDL group remains to be resolved.

The fourth group of *N*-acetylglucosaminidases in insects is grouped along with hexosaminidases from mammalian and other sources. The enzymes of this group have a broad specificity for sugars containing either *N*-acetylglucosamine or *N*-acetylgalactosamine and there is only limited evidence for their role in chitin metabolism and will therefore be not discussed in this review.

2.12 Additional Proteins Involved in Chitin Protection and Degradation

One of the reasons that chitin in the cuticle is remarkably stable is its firm association with other proteins. In fact, the formation of chitin laminae requires the participation of CDA and Knickkopf (KNK) (our unpublished results; Moussian et al. 2005a, b; Chaudhari et al. 2011, 2015) as well as Obstructor-A (CPAP3-A) (Pesch et al. 2015). KNK, CDA as well as cuticular proteins analogous to peritrophins (CPAPs) that have one or three CBDs have been predicted or shown to bind to chitin (Jasrapuria et al. 2010, 2012; Petkau et al. 2012). Removal of these proteins from chitin-protein complexes may be an essential prerequisite for chitin degradation. The specific protease(s) involved in this turnover have not been characterized. But the finding that RNAi of some chymotrypsin-like proteins in *T. castaneum* results in molting defects similar to RNAi of chitin synthase indicates that these proteins may be important for digestion of the old cuticle (Broehan et al. 2010). The roles of PMPs that associate with chitin in the midgut will be discussed by Merzendorfer in Chap. 8.

2.13 Cuticular Proteins Analogous to Peritrophins (CPAPs)

Several cuticular proteins contain peritrophin-A type chitin binding motifs, and they have been described in all insects. These are all expressed only in cuticle forming epithelial cells and predicted to be secreted proteins. Some of them have been localized either within the cuticle or the molting fluid. They have been grouped into two families based on the number of these chitin-binding domains they contain. The family with one CBD has been named CPAP1 family and that with three CBDs was named CPAP3 family (Jasrapuria et al. 2010). The CPAP3 family members were originally named GASPs or “Obstructors” in *D. melanogaster* (Barry et al. 1999; Behr and Hoch 2005). The CPAP1 family has been subdivided into 15 subfamilies (Tetreau et al. 2015a, b) and the CPAP3 family was divided into 9 subfamilies (Behr and Hoch 2005; Jasrapuria et al. 2010). RNAi for some of the genes encoding these proteins indicated that they affect either molting, integrity, morphology or the ultrastructure of the cuticle. Some of them are essential for survival while others produce visible morphological defects in cuticles of different parts of the insect’s anatomy or joint defects (Petkau et al. 2012; Jasrapuria et al. 2012; Pesch et al. 2015). These proteins may be involved in organization of chitin into laminae or higher order structures, or the formation of pore canals or tracheal tubules or other chitin-containing structures such as denticles.

2.14 Cuticular Proteins Belonging to R & R and Other Groups

The chitin in the procuticle is also associated with several other proteins that have chitin binding domains other than the peritrophin-A domains. A large assortment of cuticular proteins with one of the three types of Rebers & Riddiford (R & R) consensus motifs have been identified and classified into multiple groups. The reader is referred to recent excellent reviews on this topic (Willis 2010; Willis et al. 2012) and to the chapter on cuticular proteins by Heideki Kawasaki (Chap. 1). Other proteins were shown to be associated with some specialized exoskeletal structures. Some insects such as locusts, fleas and click beetles use deformations of exoskeleton as a way to store energy for sudden release. The exoskeletons of these insects have, in addition to chitin, an elastic protein known as resilin, which contains di- or tri-tyrosine linkages thought to be involved in energy storage. These insects use the combination of the stored energy in the chitinous cuticle and resilin to power their sudden movements. For example, froghoppers have bow-shaped pleural arches linking the coxa to the hinge of the hindwing that contains resilin to store energy by deformation of these bow-shaped chitin-resilin composites (Burrows et al. 2008). A more extensive description of resilin and its role is described by Gorb et al. in Chap. 4 of this book.

2.15 Concluding Remarks

In the past, studies of biosynthesis, turnover, and assembly of matrices containing chitin had been hampered by the difficulties of developing soluble systems to study them biochemically and by the fact that many of these reactions occur in extracellular spaces. Advances in genomics and proteomics and applications of RNA interference and ultra-structural analyses have provided new insights into how chitin-composites are assembled and turned over. In the near future, we can expect to see several new studies that will provide more details of the dynamics of assembly and turnover of cuticle and PM. They will also reveal the roles of the numerous proteins and small molecules that participate in the overall process of how these complex extracellular structures are shaped and how they function in supporting several physiological processes that are vital for insect survival and extraordinary resilience.

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