Chapter 5
Chitin Organizing and Modifying Enzymes and Proteins Involved In Remodeling of the Insect Cuticle

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Abstract Chitin, the extracellular matrix polysaccharide of insects and arthropods is widely distributed in nature in all kingdoms of life and serves a variety of functions. After synthesis by membrane-bound chitin synthases, it is extensively remodeled before incorporation into divergent matrices with wide-ranging physical and biological properties. This chapter discusses the properties of a variety of insect enzymes and proteins involved in this process. Chitin remodeling involves chitin synthases, which make the nascent chitin chains, and chitin deacetylases that partially deacetylate some of the *N*-acetylglucosamine residues either randomly or sequentially to yield local chitosan-like regions. Other proteins secreted into the procuticle or the midgut help in the assembly of single chitin chains into larger crystalline aggregates that measure in a few 100 nanometers. They are further embedded in a complex matrix of cuticular proteins or become associated with proteins containing chitin-binding domains to constitute the laminar procuticle or the lattice-like peritrophic matrix. During molting, previously formed laminar cuticle or PM are decrystallized/depolymerized to unmask the chitin chains, which then are degraded by a mixture of chitinolytic enzymes consisting of chitinases and *N*-acetylglucosaminidases present in molting fluid or in gut secretions. Some of the degradation products may be recycled for the synthesis of new matrices. We present a model of chitin synthesis, assembly, and degradation and the roles of these chitin-remodeling enzymes in this overall process.

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5.1 Introduction

Chitin (along with cellulose and hyaluronan) is one of the three major matrix polysaccharides found in nature that provides structural support to cell walls or extracellular matrices of multicellular organisms (Muzzarelli 1973; Cohen 2010; Muthukrishnan et al. 2016). All three are β-1-4 linked linear sugar polymers that provide shape, rigidity and mechanical strength to the organism's body plan and protect it against predation, infection, and mechanical injury. The capability to synthesize chitin, which is found in diverse organisms including fungi, sponges, annelids, and arthropods, is thought to have been pivotal in the evolution of arthropods, as it allows survival in diverse aquatic and terrestrial environments. Several chapters in this book deal with the structural properties and modifications of chitin–protein composites that account for the diverse properties of the arthropod/ insect cuticle or other chitin-containing structures. In this chapter, the focus will be exclusively on the enzymes involved in chitin remodeling that contribute to its unique structural properties. In particular, the structures, catalytic properties and enzyme mechanisms will be discussed, with a strong bias toward insect enzymes with which the authors are most familiar.

5.2 Chitin Synthases, the Enzymes that Polymerize Precursors of Chitin

Chitin synthases (CHS) carry out the processive addition of *N*-acetylglucosamine from the activated precursor, UDP-*N*-acetylglucosamine (donor) to the 4-hydroxyl group of the nonreducing end sugar of the growing chitin chain (acceptor) in an SN2 type of reaction in which an aspartate residue acts as the general base and UDP as the leaving group (Merzendorfer 2013). The pKa of this catalytic aspartate is influenced by the presence of other acidic groups near the binding pocket. As expected, these enzymes are integral membrane proteins that secrete the chitin product into the extracellular space, where the chitin chains become incorporated into the cell wall (or to other cell wall polysaccharides in the case of fungi) or into laminae in the cuticular matrix and the lattice-like peritrophic matrices (in arthropods/insects).

5.2.1 A Mechanistic View on Chitin Synthesis, Translocation and Fibrillogenesis

The CHSs belong to GT2 family of processive glycosyltransferases with the characteristic protein folding pattern of GT-A with an open β-sheet surrounded by an α-helix on either side. The insect/arthropod enzymes have two sets of transmembrane segments connected by a large central intracellular loop domain that contains several catalytically critical amino acid residues and motifs. Based on an analogy with bacterial cellulose synthase (BcsA) (Morgan et al. 2016), one set of transmembrane helices next to the catalytic domain lines a channel in the membrane, through which the elongating chitin polymer is thought to be extruded. Energetically, transport and extrusion of the growing chain through this transmembrane channel appears to be driven by the polymerization reaction as long as these processes are coupled (Gohlke et al. 2017).

The intracellular loops are predicted (or shown to) to contain the critical amino acid residues or sequence motifs implicated in catalysis by site-directed mutagenesis in yeast chitin synthases (Yabe et al. 1998) and crystallographic studies of BcsA (Morgan et al. 2016). These are the QRRRW (W residue of this motif sits next to acceptor glucose binding site at the entrance to the transmembrane channel in BcsA), EDR (equivalent to the TED motif at the *N*-terminus of the "finger helix" and the general base for the catalytic process). This finger helix has been found to occupy two different positions ("down" and "up" positions) during pre-translocation and post-translocation, respectively, in cellulose synthases in a process governed by another helix known as "gating helix" or "interfacial helix" that lies at the base of the carbohydrate-conducting channel (Morgan et al. 2016). A similar mechanism has been proposed for yeast CHS3 based on structural homology modeling of the helical regions of this enzyme (Gohlke et al. 2017).

A detailed investigation of another GT2 enzyme, NodC from *Sinorhizobium meliloti*, which synthesizes short chitooligosaccharides (rather than longer chitin chains) was carried out by Dorfmueller et al. (2014). By site-directed mutagenesis and model-building using the cellulose synthase complex associated with cellulose as a template for modeling, they could identify the catalytically critical residues (EDR and QR/QRW motifs) and provide a structural explanation for the inability of NodC to make longer products. The enzyme is only able to form chitooligomers, because the catalytic site is capped by a hydrophilic product-binding pocket and lacks the open transmembrane channel found in processive chitin synthases. They also proposed a biochemical mechanism in which the two terminal sugars of the growing chain rotate during repositioning of the acceptor site for the next round of polymerization. In subsequent steps, the +1 sugar rotates only every other synthetic step to maintain the β-1-4 orientation of the glycosidic bond.

There is evidence that chitin synthases are di/oligomeric complexes that assemble on cell membranes. Using bimolecular fluorescence complementation techniques, Gohlke et al. (2017) demonstrated that yeast CHS3 subunits formed oligomers at the bud neck and lateral plasma membrane. These oligomers

presumably form even in the ER indicating oligomerization prior to their final transport to the Golgi, bud neck, or plasma membrane. In *Manduca sexta*, CHS2 in the midgut appears to be present as an oligomer based on gel filtration studies (presumably a trimer) (Maue et al. 2009). Oligomerization of yeast Chs3 appeared to involve interactions involving the *N*-terminal domain and to involve several proteins of the vesicular transport pathway (Sacristan et al. 2013). It is conceivable that like cellulose synthase, larger assemblies of CHS are involved in the coordinated synthesis of chitin fibrils which spontaneously self-assemble to form chitin microfibrils containing 18–24 strands (Neville et al. 1976). While this mechanism would favor the formation of β-chitin, in which the fibrils are in parallel orientation, it does not explain the formation of α-chitin with antiparallel fibers. Additional mechanisms must be involved in post-synthetic regulation of chitin fiber assembly, which will be discussed later in this chapter.

5.2.2   Specialization Among CHS Isoforms

Sequence searches of genomes of chitin-containing organisms indicate a wide range in the number of genes encoding CHSs (Merzendorfer 2011). In fungi, this number can range widely and reach up to 20 or more isoforms in filamentous fungi. But, in most other organisms including nematodes, insects, and arthropods, this number is either one or two even though the chitinous structures formed are highly differentiated and vary widely in their chitin content. The biochemical basis for this variation is not obvious. In insects where *CHS* gene expression has been studied extensively, it is clear that there is specialization in the type of CHS involved in the synthesis of chitin in the cuticle or in the peritrophic matrix. One CHS (called CHS-A) specializes in cuticular chitin synthesis and the other CHS, CHS-B, is utilized exclusively to make PM chitin. The general domain architectures of these two CHS proteins are nearly the same with a couple of notable differences. While both enzymes have a set of transmembrane segments in the *N*-terminal domain the number of TMS are not identical. This TMS domain is followed by a central catalytic domain with a "finger helix" and "gating helix" as in the yeast CHSs, consistent with a processive mechanism. Both types of insect CHS enzymes have a similar number and organization of TMSs in the *C*-terminal domain, but a predicted coiled coil in insect CHS-A enzyme is absent in CHS-B enzymes. Since the CHS-A products are organized into hydrophobic chitin laminae (α-form) but the products of CHS-B are hydrophilic and consist of a criss-crossing matrix of chitin (presumably β-form), some sequence differences such as the coiled-coil region (or some other unidentified difference(s) in structural features between CHS-A and CHS-B) may determine their oligomerization and/or interaction with other proteins and hence the assembly of the chitin products. It is noteworthy that several proteins that are found in the cuticle that are not expressed in the midgut epithelial cells. These include Knickkopf, CPAP3A, CPAP3C and CDA1 and CDA2. In their absence (or on depletion) the chitin fibers in the procuticle become amorphous, less organized, and fibrillar, which could indicate

that they may be involved in chitin assembly (Chaudhari et al. 2011; Petkau et al. 2012; Pesch et al. 2015; Noh et al. 2018a, b). See also Sect. 7.

Additional complexity may arise from the use of alternate splicing, which is well studied in the case of CHS-A from insects. In all CHS-A enzymes from insects, there are two alternative forms of an exon that encodes 59 amino acids long segment that includes the first of the two TMS segments near the *C*-terminus of CHS proteins. This allows control of expression of CHS-A proteins with either exon-encoded segment in specific tissues or at developmental stages (Tellam et al. 2000; Arakane et al. 2005; Hogenkamp et al. 2005; Zhang et al. 2010; Wang et al. 2012; Yang et al. 2013). The forms with the alternate exon b appear to be expressed predominantly in tracheal tissue. RNA interference (RNAi) to silence expression of these isoforms produce distinctly different phenotypes compared to RNAi of the other CHS-A isoform with exon a.

Another set of alternate exons involving an upstream exon (exon 2) of CHS-A has been reported in two lepidopteran species, *Ostrinia furnacalis* and *Bombyx mori* (Qu and Yang 2011, 2012). This set of alternate exons appears to be preferentially expressed in epidermal cells. The expression of these alternate transcripts of *CHS*s has been explored. All four combinations of transcripts involving these two pairs of exons were shown to be differentially expressed. Transcripts with exon 2a were the predominant forms at all developmental stages. Transcripts with exon 2b were derived from a promoter downstream of the main promoter and missed all of exon 1. Silencing of this alternatively spliced transcript affected only the formation of the head capsule in the third instar larvae. Transcripts with exon 19a were expressed at all developmental stages except pupal day 3. Transcripts with exon 19b were predominant in day 3 embryos and day 3 pupae indicating a special requirement for this transcript at these stages. RNAi of transcripts with either 2a or 19a exon sequences produced similar molting defects. RNAi of exon 2b containing transcripts resulted in "double head" molting phenotype in third instar larvae. Collectively, these results indicate that each of the four transcripts derived from this *CHS-A* gene is uniquely required at particular developmental stages or in specific tissues.

A comparable study with *B. mori* CHS-A, which also has this alternate exon 2 and an alternative promoter has been carried out. Expression analysis of the two alternatively spliced transcripts of exon 2 of *CHS-A* in *B. mori* has indicated differential regulation of these two transcripts during development and in different sexes, with the males showing higher levels of this transcript in the wing at mid-pupal stage. Depletion of this transcript by RNAi resulted in decreased chitin content of the wing and vein crimpling (Xu et al. 2017). The two promoters differed in their expression response when 20-hydroxyecdysone (20HE) was injected into 3-day old fifth instar larvae or when the epidermis tissue was incubated with 20-HE. These results have led to the suggestion that lepidopteran insects may have utilized an alternative promoter to regulate wing development in the mid-pupal stage in this order of insects.

An alternative splice variant of *CHS-B* has also been described in *Heliothis zea*. But the protein product was predicted to be missing the catalytic domain, while retaining the transmembrane segments. The physiological significance of this protein is not yet established (Shirk et al. 2015).

5.3 Chitinases

Chitinases belong to either family 18 or family 19 glycosyl hydrolases (Henrissat 1991) that share no amino acid sequence similarities. Family 18 chitinases are widely distributed in all kingdoms, whereas family 19 enzymes are restricted to plants. In this chapter, we will restrict our attention to family 18 chitinases. They are exclusively endochitinases cleaving internal β-1-4 bonds in chitin polymers. These hydrolytic enzymes yield β-anomeric products at the reducing ends and hence use a retaining mechanism. The analysis of crystal structures of several family 18 chitinases with bound substrates or products has provided clues concerning their catalytic mechanism. It indicated that in addition to the proton donor glutamate, the C2 *N*-acetyl group of the sugar bound in the -1 position of the substrate-binding site has a role in catalysis. This mechanism has been called substrate-assisted catalysis (anchimeric assistance). It involves the formation of a positively charged oxazolinium ion intermediate, in which a covalent bond is formed between the carbonyl oxygen of the acetamido group and the C1 atom of the sugar, thus stabilizing the transition state intermediate (Brameld et al. 1998). Additional support for this mechanism comes from the inhibition of this class of enzymes by allosamidine, which forms a non-hydrolyzable analog of the transition state oxazolinium intermediate, and the fact that an obvious nucleophile near the substrate-binding site in family 18 chitinases is absent (Koga et al. 1997; Tews et al. 1997). The crystal structures of substrate–enzyme complexes also indicate that the reducing end of the sugar at the -1 subsite is in the boat conformation, which is energetically unfavorable. Additionally, as it is close to the catalytically important aspartate residue, this arrangement may provide an explanation for hydrolysis at this site (Chen et al. 2014).

The catalytic domains of family 18 chitinases assume the typical $\beta_8\alpha_8$ TIM barrel structure with eight β-strands in the center of the barrel and eight α-helices constituting the outer surface of the barrel. Four conserved motifs at characteristic positions in this structure have been identified. The conserved motif I is in the third β-strand and has the consensus sequence KXX(V/L/I)A(V/L)GGW. The second motif, FDG (L/F)DLDWE(Y/F)P in the β4 strand contains the catalytically critical glutamate, which has been shown to be the proton donor in the hydrolytic reaction. The other two conserved motifs, MXYDL(R/H)G and GAM(T/V)WA(I/L)DMDD are in the β6 and β8-strand respectively (Arakane and Muthukrishnan 2010). In the crystal structure of a group I chitinase from *O. furnacalis*, a deep and long substrate-binding cleft was observed, which is lined by several aromatic amino acids interacting with the sugar residues of the substrate that is open at both ends. In addition, the surface contains a flat plane with additional aromatic amino acids that are positioned close to the cleft near where the reducing end of the substrate is located. Mutations of any one of these aromatic amino acids, especially the one closest to the binding cleft affected the enzyme's activity on long substrates (but not the shorter substrates) as well as binding affinity to chitin substrate (Chen et al. 2014), emphasizing the role of

stacking interactions between these residues and several sugar residues of long substrates.

Most insects have multiple chitinases with the number of proteins ranging from one to >20. Based on phylogenetic analyses, tissue and/or developmental stage specificity of expression and presence of different protein domains, insect chitinases, and chitinase-like proteins have been divided into as many as 11 groups (Tetreau et al. 2015a, b). These domains include a catalytic domain, chitin-binding domains, a serine–threonine-rich glycosylation domain and other domains. All of them have at least one catalytic domain, though some of them are devoid of chitinase activity (group V proteins, e.g., Zhu et al. 2008b). Almost all of them have signal peptides that get cleaved inside the ER and the rest of the protein packaged in secretory vesicles are transported into the extracellular space via a default secretory pathway. However, there are a couple of exceptions. Members of group III and group VIII chitinases have transmembrane segments and hence are predicted to remain on the plasma membrane with the catalytic domains facing the extracellular space. Overall, the insect chitinases have diverse domain organizations, tissue and developmental patterns of expression, and differences in their catalytic parameters and substrate preferences. This apparently leads to functional specialization among the chitinases, with some of them having roles in chitin degradation during molts, while others may have functions such as cuticle and peritrophic matrix remodeling, digestion and defense or function as growth factors.

5.3.1 Group I Chitinases

The roles of individual groups of chitinases or individual chitinases have been analyzed by RNAi studies in a variety of insects including *T. castaneum*, *D. melanogaster*, *O. nubilalis, Nilaparvata lugens, S. exigua, S. litura,* and others. These studies have indicated that group I chitinases are clearly implicated in the turnover and shedding of the exuvium, the remaining old cuticle, especially at the pupal to the adult stage. Upon RNAi to knockdown specific chitinase genes involved in molting, the insects are trapped in the pupal cuticle and cannot digest away the old cuticle (Zhu et al. 2008a; Pesch et al. 2016a, b; Li et al. 2015; Su et al. 2016). In most insects, there is a single copy of a group I chitinase characterized by the presence of a signal peptide, followed by a catalytic domain, a linker domain, and a chitin-binding domain (CBD) belonging to CBM14 family. However, in mosquito genomes, this gene has duplicated to have four or five copies of the gene (Zhang et al. 2011b). In *Locusta migratoria,* there are two copies of this gene encoding chitinases, with one of them missing the CBD (Li et al. 2015). Even though both genes show similar expression profiles and regulation by 20-hydroxyecdysone, only one is required for molting. A similar situation seems to exist in *Nilaparvata lugens,* where a gene duplication has occurred and only the group I chitinase with CBD appears to be required for molting, whereas the other one devoid of a CBD is not. Curiously, this gene is expressed only in male reproductive tissues and accessory

glands (Xi et al. 2015). The transcripts for this group of genes are generally expressed at all stages of development but are elevated during the pupal stages suggesting a critical role in adult eclosion.

5.3.2 Group II Chitinases

Group II chitinases have an *N*-terminal signal peptide, followed by four or more catalytic domains interspersed with multiple CBDs. Typically, one or two catalytic domains have substitutions of the proton donor, glutamate, in conserved domain II indicating that they may not have catalytic activity, while retaining substrate-binding ability. However, these chitinases do have two or more catalytic domains at the *C*-terminal region that are presumed to have catalytic activity, with all four conserved domains intact and multiple CBDs. In fact, individual domains of this chitinase from *O. furnacalis* have been expressed and purified from yeast cells and crystallized. They possess endochitinase activity and act additively rather than synergistically (Chen et al. 2018a, b). RNAi to silence the expression of this chitinase gene results in inhibition of egg hatch as well as molting arrest at all stages without affecting new cuticle deposition in *T. castaneum* (Zhu et al. 2008c). Down-regulation of the transcripts of group II genes also results in molting phenotypes (double cuticle, entrapment in pupal cuticle and lethality) in other insects including *Chilo suppressalis* and *O. furnacalis* (Su et al. 2006; He et al. 2013). Analyses of molting fluids for fragments of this protein by tryptic peptide analyses have indicated that this protein and a group I chitinase are indeed secreted into the molting fluid (Qu et al. 2014). Since molting defects are seen after RNAi of a group II chitinase gene in multiple insect species, it may have a critical role to play in molting that is not fulfilled by group I chitinases alone. It is likely that both group I and group II chitinases are complementary and essential for cuticle degradation. Possibly, the group II enzyme carries out the initial decrystallization of α-chitin followed by an endo-type of attack along with a group I chitinase.

5.3.3 Group III Chitinases

Group III chitinases have an *N*-terminal transmembrane domain followed by two catalytic domains and ending with a *C*-terminal CBD belonging to CBM14 family (ChtBD2). Chitinases with the same domain organization are found in all insects analyzed and several other subphyla of arthropods suggesting an ancient origin for this group of chitinases (Tetreau et al. 2015a; Liu et al. 2018). Each catalytic domain has the characteristic $\beta_8\alpha_8$ TIM barrel structure of GH18 family of chitinases and the two domains are linked by a spacer domain. They are predicted to be membrane-anchored and, in fact, expression in a baculovirus system supports this notion (Noh et al. 2018a, b). However, this protein has also been localized in distal

parts of the cuticle including the layers immediately below the epicuticle as revealed by TEM studies suggesting the release of this enzyme after insertion into the plasma membrane (Noh et al. 2018a, b). Both domains act as endochitinases and there is no synergism between the two domains as observed in some bacterial systems (Liu et al. 2018). This class of enzymes prefers single-stranded chitin substrates and is devoid of any activity on insoluble chitin.

RNAi to silence group III chitinase genes has been carried out in different insects. In *T. castaneum*, there was no inhibitory effect on molting and adults did emerge with high frequency. However, there was defective abdominal contraction and forewing expansion during the pupal stage. Pupal maturation was also affected with defective leg folding and pronotum was not fully extended. When RNAi was carried out during the prepupal stage, pupation and adult emergence occurred at the normal time, but the elytra were significantly shorter and the hindwings did not fold properly. The elytra had a rough and wrinkled appearance (Zhu et al. 2008c). The structural abnormalities of the cuticle were investigated in much greater detail by Noh et al. (2018a, b). The adults had significantly shorter wings and legs. Even though the insects moved freely, they toppled over frequently and had difficulties in uprighting their bodies. Similarly, Chen et al. (2017) reported that RNAi to silence expression of a group III chitinase gene in white-backed planthopper, (*Sogatella furcifera*) affected molting and wing development, which resulted in multiple phenotypes including "wasp-waisted" adult insects, elongated distal wing pads and thin thorax–abdominal junctions. Similarly a group III chitinase from rice striped stem borer, *Chilo suppresalis* resulted in wings that were curled and did not spread properly (Su et al. 2016).

5.3.4 TEM Analyses of Cuticle

Analyses of larval body wall or pupal procuticle following RNAi for the group III chitinase in *T. castaneum* indicated the defective organization of the alternating electron-dense and electron-lucent layers resulting in fuzzy boundaries between adjacent cuticle layers as well as irregular pore canals (Noh et al. 2018a, b). Both observations suggest a loss of structural integrity of the cuticle. The ultrastructural defects persisted in the adult cuticle, as well as with the laminae becoming less compact compared to controls. The pore canals were rudimentary and devoid of long pore canal fibers. The defects were also seen in cuticles of other tissues such as legs and ventral abdomen. Immunolocalization studies have indicated that even though this protein is predicted to have a transmembrane segment, it is found in distal layers of the procuticle, reaching even the laminae just below the epicuticle (Noh et al. 2018a, b). This enzyme has also been detected in the mesocuticle and endocuticle of adult elytra which have a different morphology and assume a brick-like or "Balken" structure. After down-regulation of this group III chitinase, the cuticular layers that form after adult eclosion are abnormal and contain amorphous fibers. The requirement of a group III chitinase was demonstrated not only

for "hard" cuticles but also for soft cuticles (Noh et al. 2018a, b). Since purified catalytic domains of group III chitinases act only on soluble chitin but not on insoluble chitin (Liu et al. 2018), it has been proposed that they are not involved in chitin degradation during molting but in some step of chitin maturation during cuticular chitin deposition and pore canal formation (Noh et al. 2018a, b; Liu et al. 2018). The presence of a *C*-terminal CBD and two tandem catalytic domains suggests that this enzyme acts on nascent chitin at two places in the same chitin chain, to release chitin fragments of a uniform size and possibly to aid in the formation of antiparallel chitin bundles and/or higher order chitin fiber assemblies.

5.3.5   *Group IV Chitinases*

Unlike members of the group I, II, and III which generally seem to have single copies (with some notable exceptions; see Sect. 2.1), group IV chitinases have numerous representatives in many insects and presumably arose from several gene duplication events. Often they are clustered in the genome. They are generally expressed in the gut tissue at both larval and adult stages (Zhu et al. 2008c; Khajuria et al. 2010; Su et al. 2016). They have a single catalytic domain and some of them lack a CBD. But all of them do have signal peptides and presumably are secreted into the gut lumen. Representative members of this group of chitinases from *D. melanogaster*, one with a CBD and one without a CBD, have been expressed and purified from the culture medium of insect cells and shown to possess catalytic activity. Their catalytic efficiencies are comparable to those of group I enzymes, even though their affinities for long substrates are less than those of the group I chitinases (Zhu et al. 2008b). They all have the 4 conserved sequence motifs of family 18 chitinases including the conserved motif II (FDGLDWEYP), which contains the catalytically critical proton donor, glutamate (Zhu et al. 2004).

   RNAi to silence expression of individual group IV chitinases has yielded mixed results concerning their essentiality for insect survival. Down-regulation of individual group IV chitinases did not result in any noticeable phenotype or molting defects or survival in *T. castaneum* larvae. RNAi of some combinations of these genes also did not result any phenotypes (Zhu et al. 2008c). This observation was attributed to the redundancy of the members of this group and the ability of different members to compensate for the functions of other members of the same group. However, RNAi of Cht2 in *D. melanogaster* did result in larval lethality and a thinning of the procuticle (Pesch et al. 2016a, b). Feeding of dsRNA with a group IV chitinase from *Ostrinia nubilalis* resulted in the elevation of chitin content of the PM and a reduction in body weight (Khajuria et al. 2010). In *Chilo suppresalis*, injection of dsRNA targeting two different group IV chitinases expressed predominantly in larval midgut resulted in the death of more than two-thirds of the larvae. Interestingly, while one of these proteins had the proton donor glutamate in consensus region II, the other enzyme had substitutions of an aspartate as well as the proton donor, glutamate. These data suggest a role for group IV chitinases in

PM chitin turnover, but this needs further examination as the authors also reported an effect on molting (Su et al. 2016).

5.3.6   Group V Chitinases

This family of proteins was initially identified as growth promoting substances in conditioned medium derived from cultures of imaginal discs (Kawamura et al. 1999). A total of six proteins belonging to this family were identified from *D. melanogaster*. All of them have a leader peptide and are secreted into the culture medium. They have a single GH18 domain and do not have a CBD or other domains. A crystal structure of one of these proteins is available for the imaginal disc growth factor 2 (DmIDGF2) (Varela et al. 2002). It has the typical $\beta_8\alpha_8$ TIM barrel structure of GH18 family of hydrolases, but has two prominent insertions, one between the β_4 strand and the α_4 helix and the second insertion is between β_7 and α-7. The first insertion is highly conserved among chitinases of group V and has the consensus sequence, KPRKVGXX(L/I)GSXWKFKKXF(T/S)GDXVVDE. It seems to be devoid of a defined structure as it is not visible in the crystal structure. This solvent exposed structure must interact with some other cellular component because of its conserved sequence. Further, this presumed loop structure undergoes proteolysis at a precise location between the F and T residues in DmIDGF2 (Varela et al. 2002). This cleavage does not seem to affect its function in cell proliferation. There is some evidence for a similar cleavage of *T. castaneum* IDGF4 (Zhu et al. 2008b). Both DmDS47 (a group V chitinase-like protein) and TcIDGF2 do bind to colloidal chitin (Zhu et al. 2008b). The second insertion is of variable length and has been implicated in determining the specificity of bacterial chitinases in acting as an endo- versus exochitinase as well as affecting its processivity (Zees et al. 2009; Li and Greene 2010).

Another notable feature of group V chitinases is that the conserved region II of this type of insect chitinases contains one or more substitutions. Several (but not all) lack the proton donor glutamate. Even some IDGFs with this glutamate residue are devoid of enzymatic activity (Zhu et al. 2008b). Since one of the two insertions found in all group V chitinase-like proteins follows immediately after the consensus sequence II, it is likely that this insertion interferes with the binding of the substrate and/or the catalytic function of this glutamate. The finding that at least some members of this group of proteins do bind to colloidal chitin, but are devoid of catalytic activity, makes the latter possibility more likely.

Group V chitinase-like proteins are expressed at all developmental stages and RNAi studies have indicated that several of them have essential biological functions in insects (Zhu et al. 2008c; Pesch et al. 2016a, b; Xi et al. 2015). In *T. castaneum*, TcIDGF4 did not affect pupation, but did affect adult eclosion. But the precise step affected has not been determined. In *D. melanogaster*, depletion of transcripts for four of the IDGFs resulted in "double cuticle" phenotypes involving the head skeleton and the posterior spiracles in the larval stages indicating molting defects in

larval stages. No molting defects were seen at pupal stages (Pesch et al. 2016a, b). Some IDGFs have been suggested to have a role in cuticle maintenance and in epithelial defense). In *D. melanogaster* stage 18 embryos, IDGFs showed expression in cuticle-forming tissues including epidermis, posterior spiracles and tracheal tubules with differences among different IDGFs. In larval and pupal stages, the expression levels were relatively low, except for IDGF5 in larval stage 3 and high levels of IDGF5 in the adult stages. RNAi of *DmIDGF* genes also led to deformed cuticle with reduced thickness (Pesch et al. 2016a, b). In *T. castaneum,* RNAi to silence expression of *TcIDGF4* resulted in arrest of pupal development and death, whereas RNAi for *TcIDGF2* did not produce any phenotype. In *Nilaparvata lugens,* IDGF was highly expressed in fat body and female reproductive organs but RNAi did not result in any observable effects on molting, survival or fecundity (Xi et al. 2015). In several cases, the precise location of expression (e.g., pupal or adult tracheae) of individual IDGFs have not been determined.

5.3.7 Group VI Chitinases

This group was not recognized as a separate group in earlier classifications, but it deserves a separate grouping. First, the chitinases of this group differ from those of the prominent group I (and group IV) in some significant ways while sharing some characteristics. They have a signal peptide at the *N*-terminus immediately preceding a GH18 chitinase domain with all the signature motifs of active chitinases. The GH18 domain is immediately followed by a CBM14 domain. At the *C*-terminus, there is a long domain rich in serine, threonine, proline and glutamic acid (the so-called PEST domain). It resembles the mucin domain and several members of this family have been classified as mucins or as acidic chitinases. In some members of this family in insects, there is a second CBD close to the *C*-terminus. These proteins are presumed to be heavily *O*-glycosylated. In *T. castaneum*, they are expressed at all developmental stages at nearly constant levels and mostly in the carcass with low-level expression in the posterior midgut. Notably, their expression was barely detectable in the anterior and posterior midgut (Zhu et al. 2008c).

5.3.8 Other Chitinase Families

Not enough studies have been done either to identify the phylogenetic relationship among members of these families involving a large number of insect species or their tissue specificity or developmental patterns of expression. There are one or two RNAi studies with some of these families. But further studies are needed to draw general conclusions about the roles of these chitinases in insects in general or in other arthropods.

5.4 *N*-acetylglucosaminidases

Among the several families of *N*-acetylglucosaminidases (NAG), only the members of the family 20 glycosylhydrolases (GH) are implicated in chitin remodeling. Although GH20 enzymes can act on different substrates such as glycans, glycoproteins, and glycolipids with *N*-acetylhexosamines, we will focus exclusively on chitinolytic glucosaminidases in this review. Along with the endo-acting chitinases, NAGs, which are also secreted into molting fluid (Qu et al. 2014; Zhang et al. 2014) are required for complete depolymerization of polymeric chitin to the monosaccharide, *N*-acetylglucosamine, which can be utilized as building block for new chitin upon activation by UDP. They have been purified from different sources including the molting fluid, hemolymph, integument and gut (Dziadik-Turner et al. 1981; 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). NAGs typically cleave oligosaccharides from the nonreducing end and release *N*-acetylglucosamine or *N*-acetylgalactosamine. They tend to prefer *N*-acetylglucosamine over *N*-acetylgalactosamine, though this preference can be highly variable.

The crystal structure of *O. furnacalis* HEX1 (homolog of TcNAG1) has been resolved at 2.1Å and has provided us with some structural insights (Liu et al. 2012). It is a homodimer with a side-by-side symmetry and *N*-glycosylated at Asn164 and Asn375. Each monomer has two identifiable domains. The *N*-terminal Domain 1, which follows a signal peptide, is about 200 amino acids long and is stabilized by six disulfide bonds. It has a six-stranded antiparallel β-strand conserved among most members of this family and an α-helix and a β-strand at the *N*-terminus, both of which are implicated in dimerization. The second domain of about 300 amino acids comprises the standard $\beta_8\alpha_8$ TIM barrel structure with some structural features unique to GH20 enzymes. The active site consisting of the conserved catalytic triad of Asp249, His303, and Glu368 is flanked by three tryptophans and an aspartate and tyrosine, which form several H-bonds with the inhibitor-substrate (TMG-chitotriomycin) and contains the catalytic water molecule (Liu et al. 2012). The mode of action of this enzyme is a substrate-assisted catalysis that involves the acetamido group of the substrate and the formation of the oxazolinium intermediate as in GH18 chitinases. This gene is highly expressed in the integument at the pharate pupal stage but remained at a nearly constant lower level in the alimentary tract during the last instar mid-larval stage and pharate pupal stage indicating an important role in molting and chitin turnover, presumably in response to rising ecdysteroid titers. Administration of dsRNA in the middle of the feeding stage of the last instar did not seem to affect pupation but caused pupal lethality at different periods of the pupal stage, with a range of phenotypes that differed in the extent of removal of the old pupal cuticle.

Analyses of genomes of insects indicate the presence of multiple GH20 enzymes. In *T. castaneum*, at least four enzymes (called TcNAG1, TcNAG2, TcNAG3, and TcFDL) have been implicated in molting. RNAi to suppress the expression of any one of these four genes affected molting to varying degrees

depending on the timing of injection of the dsRNA for the targeted *NAG* gene (Hogenkamp et al. 2008). Even though all molts are susceptible to RNAi of each of these genes, dsRNA-mediated silencing of *TcNAG1* seems to result in mortality especially at the pupal to adult molt (90%). This gene is most highly expressed in the integument at the pupal stage. Other genes including *TcNAG2* and *TcFDL* had a similar mortality (80%) at the pupal stage, but considerably lower mortality (10-20%) at earlier stages. The morphology of these moribund insects was reminiscent of those caused by RNAi-silencing of the *TcCHT5* gene, encoding a group I chitinase, which is the most prominent chitinolytic enzyme secreted into the molting fluid at the pharate pupal stage (Kramer et al. 1993; Zhu et al. 2008c). The insects failed to get rid of their old cuticle even though the newly formed cuticle was visible under the old cuticle. It is known that NAGs and CHTs are secreted into the molting fluid with similar timings and hormonal control and to synergize the actions of each other (Fukamizo and Kramer 1985). It has been shown that the activity of group I chitinases is inhibited by high concentrations of the product chitooligosaccharides. It is likely that the synergism of the CHT + NAG combination is due to relief from product inhibition. Moreover, it is possible that the substrate specificities and/or kinetic constants of the NAGs for different chitooligosaccharides differ to achieve high-efficiency hydrolysis on crystalline chitin by the binary system consisting of CHTs and NAGs. This would explain why RNAi of each of the *NAGs* can produce some phenotype in at least a percentage of the animals. The other explanation is that the tissue specificity of expression of the different *NAG* genes is different. For example, *TcNAG1* is highly expressed both in the integument and midgut whereas *TcNAG3* is expressed at high levels in the midgut (Hogenkamp et al. 2007).

A phylogenetic analysis of genomes from sixteen insects including the cigarette beetle, *Lasiderma sericorne*, has shown that all insects have the four groups of NAGs (including FDLs) indicating their evolutionary importance (Chen et al. 2018a b). The ortholog of *TcNAG1* in the cigarette beetle, *L. serricorne, LsNAG1,* exhibits periodic peaks of expression at late larval and late pupal stages. RNAi-silencing of this gene by injection of dsRNA, resulted in defective molting and high mortality.

5.5   Molting Fluid-Associated Chitinolytic Enzymes

When insects molt to next stage of development, their old cuticle first separates from the epidermal cells (apolysis) and then a new envelope and epicuticle start to form all over the body plan before deposition of new layers of the chitin–protein-containing procuticle. Molting fluid containing proteolytic and chitinolytic enzymes is secreted in the space between the two cuticular layers for the digestion/dissolution of the innermost layers of the old cuticle, which are presumably not tanned or otherwise cross-linked (Reynolds and Samuels 1996). Since crystalline chitin is not a good substrate for many chitinolytic enzymes and is protected by

being embedded in a matrix of proteins, many of which have a chitin-binding domain (ChtBD2 or the Rebers & Riddiford (R & R) domain, or other types of CBDs), the proteases must first unpack the chitin fibrils from the protein matrix in a process that is the reverse of the assembly of chitin–protein laminae. One can anticipate that the actions of proteases first weaken the bonds between these chitin-binding proteins and chitin bundles leading to the disorganization of the stacked cuticular laminae and the liberation of chitin–protein bundles of ∼300 nm often seen in extracts of cuticle following treatment with harsh solvents (Fabritius et al. 2009; Kaya et al. 2014). Proteins that presumably join short chitin–protein nanofibers into longer fibers of the stacks of laminae may be targeted first. The 18–24 chains of chitin bundles that constitute the α-chitin crystallites first proposed by Neville et al. (1976) are most likely to fray near their ends (or surface-exposed chains) due to thermodynamic fluctuations (Beckham and Crowley 2011) and or the actions of oxidative enzymes (Vaaje-Kolstad et al. 2010) (as in microbial systems) or equivalent chitin depolymerizing enzymes (lytic polysaccharide monooxygenases, LPMOs) in arthropods. Group II chitinase also are plausibly involved in this process, as they possess several CBDs that bind chitin and inactive as well as active GH18 domains capable of endochitinase activity. The synergist actions of chitinases and NAG1 may efficiently depolymerize crystalline chitin to the monomeric GlcNAc sugars, which are likely to be resorbed by epidermal cells by endocytosis. When the old cuticle is nearly completely detached, other physiological processes such as abdominal contractions triggered by neuropeptides lead to sloughing of the old cuticle (Reynolds and Samuels 1996; Arakane et al. 2008a, 2008b).

With the expectation that the composition of the molting fluid proteins will provide some clues about the enzymes and their targets during molting/apolysis, Qu et al. (2014) and Zhang et al. (2014) analyzed the molting fluid from two developmental stages of the silkworm using tandem mass spectrometry. Surprisingly, they found a total of only four different chitinolytic enzymes in these fluids even though the genome encodes more than two dozen enzymes potentially capable of chitin hydrolysis. One group I chitinase, one group II chitinase, and one bacterial-type chitinase along with an exochitinase belonging to GH20 family accounted for most of the chitinolytic activities at either stage. This finding is consistent with the expression of only these chitinolytic enzyme encoding genes in the integumental tissue in many insects. Other enzymes detected in the molting fluid include chitin deacetylases (CDA1, CDA2, and CDA4). Several chitin-binding proteins including six cuticular proteins analogous to peritrophins (CPAP3s) were identified. Also present were several proteins with R & R domains and two belonging to the CPH family of cuticular proteins. In addition, the molting fluid in the pharate adult stage contains Knickkopf, which is known to be a GPI-anchored protein with a role in protecting and organizing chitin into laminae (Moussian et al. 2005; Chaudhari et al. 2011). Whether these proteins were released due to digestion by molting-associated proteases/phospholipases or merely dissociated from the chitin bundles in the cuticular laminae during apolysis is not established.

5.6   Chitin Deacetylases (CDAs)

CDAs (E.C.3.5.1.41) and chitooligosaccharide deacetylases (3.5.1.105) belong to family 4 carbohydrate esterases (CE4; www.cazy.org). CDAs are widely distributed in microorganisms and in arthropods, but only the microbial enzymes have been well characterized. They can act on nascent chitin (in coordination with chitin synthases), soluble forms of chitin such as glycol chitin, or on chitooligosaccharides, but generally have very little activity on crystalline (α or β) or colloidal chitin. But their activity on crystalline chitin can be enhanced by pre-treatment with lytic polysaccharide monooxygenases (LPMOs) that oxidatively cleave the glycoside linkage in chitin and increase the accessibility of the acetyl groups on the surface of the substrate (Liu, et al. 2017; see also Chap. 6 of this book). The CDAs have a $\sim$150 amino acids long NodB homology domain found in the well-characterized NodB chitooligosaccharide deacetylase from *Rhizobium meliloti* (see also Chap. 3 of this book). This domain is a distorted $\beta8\alpha8$ TIM barrel domain that is missing one β/α repeat unit and displays several loops characteristic of particular CDAs. Typically, the CE4 domain contains five conserved motifs. The first motif TFDD contains the aspartate (first asp) that acts as the general base followed by the second aspartate which chelates a Zn^{2+} ion. The two histidine residues in a second motif HS/TXXH are also involved in Zn^{2+} binding forming a loop with the His…His…Asp (Asp from motif 1) triad. The consensus of the third conserved motif is RXPY, which constitutes one side of the active site with motif 4 (DXXDW/Y) representing the other side of the active site. The catalytic acid residue is provided by the His in motif 5 (IV/ILXHD), which forms a hydrophobic pocket binding the methyl group of the C2-acetate (Grifoll-Romero et al. 2018). Recent crystal structures have provided additional insights regarding the roles of small versus large loops in determining the substrate specificity or pattern of deacetylation of chitooligosaccharides. The reaction is a metal-assisted general acid–base reaction that involves a catalytic water molecule bound in the active site pocket. Unfortunately, there is no structural information on insect CDAs. However, it is clear from chemical and spectroscopic analyses of cuticles that only about 10–20% of the total sugars in chitin are deacetylated. But there is no evidence for long stretches of deacetylation in insect chitin chains, because the end products of digestion of insect cuticles with a mixture of endo- and exochitinases are mostly monosaccharides and disaccharides with little evidence for larger chitosan oligosaccharides (Charles Specht, unpublished data).

5.6.1   Arthropod/Insect CDAs

Following the first identification of an insect CDA from *Trichoplusia ni* PM (Guo et al. 2005), a search of the completed genomes of twelve arthropods (10 insects, an arachnid (deer tick), and a crustacean (water flea)) for genes encoding CDAs and related proteins has revealed that all of them have multiple genes encoding CDAs

that can be arranged phylogenetically into five groups with distinct compositions of subdomains. While all five groups of CDAs have the CE4 (NodB) domain, some groups have additional domains including a low density lipoprotein receptor a domain (LDLa; PMID:7548065) domain, a CBM14 chitin-binding domain and a long serine–threonine–proline–glutamine-rich linker domain (Dixit et al. 2008; Tetreau et al. 2015a, b). While all five groups have the five conserved sequences implicated in catalysis, metal or substrate/product binding, some of them have mutations of catalytically critical residues leading to the suggestion that they may not all be enzymatically active. The absence of definitive assays for CDA activity and the varying modes of action among CDAs (multichain, sequential, and random deacetylations) have further complicated functional analyses (Grifoll-Romero et al. 2018). Most assays involve the use of soluble chitin substrates and the detection of chitosan produced, which requires sequential deacetylation of the chitin substrate. This hampers detection of activity using enzymes that randomly deacetylate chitin or deacetylate chitin only at specific positions (at or near the ends of chitin chains, for example). However, there are some limited examples of successful detection of CDA activity as well as several examples of inability to detect deacetylase activity with purified enzymes and chitin substrates. Assays involving chitooligosaccharides as substrates and detection of the product acetate formed have proven to be the most promising assays.

Studies using homozygous mutants of *D. melanogaster* have provided evidence concerning the physiological importance of group I CDAs. These mutations in CDA1 and CDA2 (both belonging to group I CDAs) termed *serpentine* and *vermiform* affected the development of dorsal tracheal trunks during embryonic development resulting in tortuous tracheal tubules (Luschnig et al. 2006; Wang et al. 2006). In *T. castaneum*, injection of dsRNA for *CDA1* and *CDA2*, which are mainly expressed in epidermis and tracheal tissues, resulted in the arrest of every molt and in mortality (Arakane et al. 2009). Similarly, injection of dsRNA for *CDA2* on day 2 of the fifth instar nymphs of *Locusta migratoria* resulted in molting failure (Yu et al. 2016). Administration of dsRNA for *Leptinotarsa decemlineata CDA2* resulted in some larvae being trapped in their exuviae, some pupae with abnormal morphology and death at the pharate adult stage, and adults that were small, wrinkled wings, as well as adult mortality. These results suggest that depletion of CDAs leads to defective molting at all developmental stages and cuticular abnormalities in multiple insects (Wu et al. 2018).

The roles of CDA belonging to the other groups are not clear-cut. RNAi-silencing of *TcCDA6, 7, 8* and *9* (belonging to group V), which are only expressed in the gut tissue, did not reveal any developmental phenotypic abnormalities irrespective of whether one or all of these CDA transcripts were down-regulated (Arakane et al. 2009). However, overexpression of a group V CDA from *Helicoverpa armigera* (using a recombinant baculovirus containing this gene) resulted in the accelerated killing of the host insects, *Spodoptera frugiperda* and *S. exigua* (Jakubowska et al. 2010), when compared with insects infected with the control virus. This result suggests that the permeability of the PM might have been altered by exposure to high concentrations of this CDA.

Detailed studies on the structure of the insect cuticle using TEM analyses of cuticles from control insects and those subjected to RNAi-silencing of *CDA1* and *CDA2* from *Locusta migratoria* and *T. castaneum* have been carried out. In *L. migratoria*, pore canals in the nymphal cuticle appear to follow a crescent path in the cuticular laminae. Such a helicoidal path is consistent with the cuticle containing a stack of chitin fibril planes that are rotated with respect to one another by a small angle. However, after depletion of transcripts for *LmCDA2*, the newly formed pore canals appear to lose this helicoidal appearance and assume a straight appearance consistent with a parallel stack of chitin fibrils. Further, immunolocalization of LmCDA2 placed this protein at the apical surface of the procuticle just below the epicuticle, leading to the hypothesis that as the cuticular layers are being assembled, LmCDA2 is required to organize them in a helicoidal fashion.

Different results were obtained after similar analyses of elytral cuticles of *T. castaneum* following RNAi-silencing of *TcCDA1* or *TcCDA2*. At the larval stage, the body wall cuticle of this beetle is indeed helicoidal, but at the pupal stage when CDAs continue to be expressed at high levels, the pore canals follow a nearly straight path all the way to the epicuticle suggesting a parallel stacking of chitin fibers. In these insects, after RNAi-silencing of either *TcCDA*, the cuticle organization became disorganized, with disruption of the laminae and loss of clear-cut boundaries between the dark and light regions of the cuticular laminae and loss of pore canal integrity (Noh et al. 2018a, b). When both CDAs were down-regulated, there was no evidence of laminae. The pore canals were also distorted with loss of the long electron-lucent (presumably chitin) fibers found in the middle of the pore canals. The chitin fibrils were much smaller and did not form thick and long bundles leading to the hypothesis that CDAs are needed for higher order organization of chitin bundles in the cuticle. Based on gel electrophoresis, some of the TcCDA1 and TcCDA2 proteins appeared to form a dimer. Further, they were both localized in the assembly zone immediately above the plasma membrane, when gold-labeled antibodies were used to detect them in TEM. This observation suggests that CDAs may have a role in assembly of nascent chitin crystallites.

5.7 Chitin-Remodeling Proteins

Besides the enzymes that are known to remodel chitin by acting on the polymer directly, there are other proteins that bind to chitin and participate in organizing chitin fibrils into higher order structures. Examples of this class of proteins are Knickkopf (KNK) and several cuticular proteins belonging to the CPAP families, PMP families, and the R& R families. They have one or more of the chitin-binding motifs belonging to the CBM14 family or they have one of three consensus sequences known as Rebers & Riddiford consensus sequences. It is likely that there are other uncharacterized chitin-binding proteins with distinct biochemical functions with other motifs or domains. In this chapter, we will focus only on those proteins known to affect chitin-containing structures.

5.7.1 Knickkopf Family of Proteins

Knickkopf (German word meaning literally kinked head) was initially identified as a gene that affected cuticle integrity in mutational screens of *D. melanogaster* (Weischaus et al. 1984) and was cloned by Ostrowski et al. (2002). Since then orthologs and paralogs of this gene have been identified in a variety of insects and nematodes. There are three members in this family in insect genomes. All three of them have two DM13 domain, a dopamine monooxygenase *N*-terminal domain (DOMON) and a long *C*-terminal domain whose function remains undetermined. The DM13 and DOMON (Pfam 10517 and 03351 domains, respectively) are associated with electron transport proteins involved in hydroxylation and oxidative cross-linking of proteins. The DOMON domain proteins also contain *C*-terminal domains that are predicted to provide thiol groups as binding sites for a heme prosthetic group associated with cytochrome (Iyer et al. 2007). Other ligands such as dopamine and carbohydrates may also be potentially linked to this *C*-terminal domain. The DM13 domain has a fold rich in β-strands.

In *D. melanogaster*, mutations of the KNK gene result in embryonic death and loss of chitin organization in tracheal tubes which take on a cystic appearance (Moussian et al. 2005). They seem to be deficient in the fibrillar chitin fibers and take on an amorphous appearance. In *T. castaneum*, RNAi-silencing of this major *KNK* that is expressed in the integument (but not in the gut) results in a clear reduction in cuticular chitin, loss of laminar organization of the cuticle, severe molting defects and lethality at all developmental stages (Chaudhari et al. 2011). The other two *KNK* paralogs (*KNK2* and *KNK3*) are essential for adult molting and cause lethality but seem to affect only body wall denticles and tracheal taenidia, indicating their unique functions in specialized cuticles (Chaudhari et al. 2014).

The TcKNK protein has been expressed in a baculovirus system and shown to be a GPI-anchored protein that can be released from insect cells by treatment with phosphoinositide-specific phospholipase C (Chaudhari et al. 2011). In vivo, this protein is, in fact, distributed throughout the procuticle and seems to be co-localized with chitin throughout the procuticle. The purified protein does bind to colloidal chitin and seems to protect it from chitinases in vivo, because the reduction in chitin content of the pharate adult brought about by RNAi of *TcKNK* is ameliorated by the simultaneous down-regulation of the two major chitinase(s) in the molting fluid by RNAi (Chaudhari et al. 2011). But the chitin that accumulates in these insects lacking both KNK and chitinases is not organized in the form of alternating light and dark laminae or long pore canal fibers. Instead smaller, thinner filaments accumulate in the pharate adult elytral cuticle indicating that KNK not only protects chitin fibers but also organizes them into thicker and longer bundles that further assemble into orderly horizontal laminae and long vertical pore canal fibers. The precise mechanism of how KNK (most likely in cooperation with several other proteins) brings about this process has not been elucidated. However, the timing and level of expression of KNK relative to the period (and amount) of chitin synthesis appear to be important in maintaining the structural integrity of the wing

cuticle, as well as other cuticular structures such as bumps in *D. melanogaster* epidermal tissue (Li et al. 2017).

5.7.2 CPAP Proteins

The insect cuticle contains numerous proteins that exhibit substantial variations in number and composition depending on the type of cuticle and the developmental stage. There are compositional differences between larval and adult cuticle and between soft and hard cuticle at the same developmental stage (Dittmer et al. 2012; Zhou et al. 2016). These proteins interact with chitin and alter its physical properties such as viscoelastic properties and permeability characteristics. The CPAP proteins are characterized by the presence of one (CPAP1s) or three (CPAP3s) CBD domains. This domain is characterized by the presence of a 6-cysteine containing chitin-binding motif with a characteristic spacing between successive cysteines. These cysteines are most likely involved in disulfide bond formation and assume well-defined three-dimensional structures. CPAP1 family and CPAP3 families are further subdivided into 16 and 7 subgroups, respectively, based on the sequence conservation between the cysteines (Tetreau et al. 2015b; Jasrapuria et al. 2010). The sequence conservation in the CBD region within each subgroup is quite high, at least for some groups suggesting a biological need for conservation of these sequences. The CBDs have a strong affinity for chitin and the affinity of proteins containing these domains seems to increase with the number of CBDs (Arakane et al. 2003). The evolutionary conservation of these subgroups in several insect orders indicates their essential nature and functional specialization over a long period of insect/arthropod evolution.

A detailed study of the chitin- and chitosan-binding properties of six CPAP3 proteins from *B. mori* has been carried out using purified preparations following expression in *E. coli* (Qu et al. 2017). All of these proteins bound strongly to crystalline chitin and colloidal chitin. However, they differed in their affinity to partially deacetylated chitin. BmCPAP3-D1 had the highest affinity for chitosan (70 or 100% deacetylation). Curiously, this protein was up-regulated during pupal–adult transition suggesting a physiological role for this protein at this developmental stage.

In CPAP1 family, the CBD domain is located generally near the *N*-terminus but may be located in the middle or near the *C*-terminus in others. The CPAP1 proteins show substantial variation in their total length and are expressed mostly in cuticle-forming tissues but not in the gut. In contrast, the CPAP3 proteins have a narrow size variation and the three CBD domains account for almost all of their total length except for the two short spacers between them. They all have a signal peptide, consistent with their being extracellular proteins, where they can interact with chitin. CPAP proteins may have a structural function or an enzymatic function in which the CBD may serve to anchor the protein on chitin.

The roles of CPAP1 and CPAP3 have been investigated by mutational and/or RNAi studies in several insects (Jasrapuria et al. 2012; Petkau et al. 2012; Pesch et al. 2015). These studies have indicated that at least some of these proteins are essential for survival, molting, cuticle integrity, and fecundity and that their unique functions cannot be substituted by other CPAP proteins. Among the CPAP1 family members, only three of them have been shown to have essential functions based on RNAi studies in *T. castaneum*. These include *TcCPAP1-C, TcCPAP1-H*, and *TcCPAP1-J*. Interestingly, these are the subgroups that show the highest levels of sequence conservation in the CBDs among insects (68–85%). Depletion of these transcripts individually leads to lethality at the pharate adult stage, loss of chitin and/or structural integrity of the elytral cuticle or embryonic arrest (Jasrapuria et al. 2012). The functions of the other CPAP1 proteins have not been investigated in detail.

The *CPAP3* genes have been studied in greater detail in several orders of insects. Knocking down *CPAP3* genes of *T. castaneum* individually caused morphological effects that were varied and led to a wide range of phenotypes. The observed phenotypes included molting defects, mortality, depletion of fat body, underdeveloped ovaries, loss of fecundity, joint defects, and rough elytra (Jasrapuria et al. 2012). The two exceptions were *TcCPAP3A2* and *TcCPAP3E*, which did not yield any observable phenotypes after injection of the corresponding dsRNAs.

In *D. melanogaster*, homozygous mutants of the *CPAP3-A* gene (called *Obst-A*) exhibited growth reduction, molting defects, and defective wound healing (Petkau et al. 2012). The corresponding protein was localized (or present) in the assembly zone where it may participate in the assembly of chitin along with other proteins that also localize to (or present) this region including CDA1 and CDA2 and KNK (Noh et al. 2018a, b; Pesch et al. 2015). It is noteworthy that reduction in the amount of any of these proteins results in loss of laminar organization of the epidermal procuticle. The precise roles of each of these chitin-remodeling proteins remain to be established.

5.7.3 Peritrophic Matrix Proteins (PMPs)

Besides being the supporting matrix of the cuticle, chitin is also found in the peritrophic matrix, which is also an extracellular matrix elaborated by the epithelial cells lining the midgut (Hegedus et al. 2009; Merzendorfer et al. 2016). The enzyme responsible for the synthesis of chitin associated with PM is chitin synthase B (CHS-B), which is a paralog of chitin synthase A (CHS-A) which makes cuticular chitin, as discussed in Sect. 5.2.2. CHS-B resides in the apical tips of brush border microvilli of the midgut (Zimoch and Merzendorfer 2002). When the gene encoding CHS-B in *T. castaneum* is silenced by RNAi, the structural integrity and barrier function of the peritrophic matrix is lost (Kelkenberg et al. 2015). In addition, several other proteins that accumulate in the assembly zone (Knickkopf, CDAs, and CPAPs) and are implicated in the higher order assembly of chitin fibers

are not expressed in the midgut-lining cells. In addition, the assortment of proteins expressed in these cells do not include many of the R & R proteins, but consist of another family of proteins, named PMPs. This class of proteins contains CBM14 domains closely related to, but slightly different from, the CBDs found in CPAP1 and CPAP3 proteins of insect cuticles (Jasrapuria et al. 2010; Tetreau et al. 2015b). These dissimilarities probably account for the fundamental differences in the properties of the chitin polymers found in these two locations. While the cuticular chitin is rigid and hydrophobic, the PM-associated chitin is a gel that is highly hydrated, flexible and permeable to small solutes and water.

Unlike cuticular chitin, which exists in close association with the epidermal cells as a multilaminar hydrophobic structure, the PM is a sieve-like structure with criss-crossing chitin fibers that delaminate away from the finger-like protrusions of microvilli of midgut-lining cells (Harper and Hopkins 1997; Hopkins and Harper 2001). Multiple laminations of chitin networks are often found especially in the middle and posterior parts of the midgut. Chitin fibers in PM are mostly arranged as orthogonal or hexagonal lattices or as random felts. There are large pores in the PM that allow for the passage of digestive enzymes secreted by the epithelial cells lining the midgut and for digestion products to pass through the PM in opposite directions (Bolognesi et al. 2008). In *T. castaneum*, the size exclusion decreases along the length of the midgut, allowing larger molecules to cross the PM in the anterior parts (size >40 nm), intermediate in the middle midgut (size 8–9 nm), but much smaller in the posterior parts (size 1–2 nm) (Agrawal et al. 2014). There is evidence suggesting that the permeability may be influenced by particular PMPs that are associated with PM at specific regions of the midgut. The PMPs vary substantially in their sizes depending on the number of CBD domains in each protein. This number varies from 1 to as many as 19, which may allow binding/cross-linking of different chitin chains by a single protein forming a three-dimensional network (Jasrapuria et al. 2010; Dinglassen et al. 2009; Venancio et al. 2009; Toprak et al. 2015; Shen and Jakobs-Lorena 1998, Agrawal et al. 2014). It is likely that the ratio of protein to chitin is not uniform along the length of the PM from the anterior side to the posterior. The posterior parts of the PM are mechanically stronger than the anterior part, which sometimes exists as a thin gel-like substance (also termed peritrophic gel; Terra 2001). The expression of the individual *PMP* genes varies along the midgut in *T. castaneum*, where the expression of the genes encoding smaller PMPs is predominantly in the anterior and middle parts and the expression of genes encoding the largest PMPs is confined to the posterior midgut. The proteins with the most CBDs were also confined to the posterior PM (Jasrapuria et al. 2012; Agrawal et al 2014).

Another function of the PM-associated proteins may also be to influence the permeability characteristics of the PM. Some of the PMPs have large mucin domains that are rich in serine and threonine residues and are indeed glycosylated. (Schorderet et al. 1998; Vuocolo et al. 2001; Agrawal et al. 2014; Toprak et al. 2010, 2015). In *T. castaneum*, RNAi of the two PMPs demonstrated to be glycosylated (TcPMP3 and TcPMP5B) resulted in a substantial increase in exclusion size compared to the other TcPMPs that do not have mucin domains (Agrawal et al.

2014). Whether this is a direct effect on pore size or due to binding of other proteins to the sugar moieties is unclear. In fact, feeding of lectins has been shown to cause lethality of *L. cuprina* larvae (Eisemann et al. 1994). An additional way in which PMPs can affect PM permeability is due to charge effects. It is known that cations pass through the PM more readily than anions in the presence of glycosylated proteins (Miller and Lehane 1993; Barbehenn 2001).

5.7.4 CPR Family Proteins

Besides CPAP proteins, there is a large assortment of cuticular proteins (CPRs) with the Rebers & Riddiford (R & R) chitin-binding sequences. Homology modeling of this consensus sequence of 66 amino acids has revealed the structure of an antiparallel β-sheet half barrel, into which a chitin chain could be fitted, and in which the aromatic amino acid residues are stacked on the sugar planes (Hamodrakas et al. 2005). Some of the CPR proteins have been shown to bind to chitin (Willis et al. 2012). The RR1 proteins are generally localized in the soft procuticle and intersegmental membranes and the RR2 proteins are consistently found in the hard cuticle (Zhou et al. 2016). Depletion of two major cuticular proteins belonging to the RR2 family that is found in the laminae and pore canals of hard cuticles of *T. castaneum* (TcCPR27 and TcCPR18) resulted in wrinkled elytra, unfolded hindwings and death. On the other hand, there were alterations in the dynamic mechanical properties of the elytra that were suggestive of a greater degree of cross-linking of proteins. The horizontal laminar organization was less organized, and the pore canals were distorted; they lacked the thick pore canal fibers (Noh et al. 2014). Another *T.castaneum* RR1 protein, TcCPR4, was found exclusively in the pore canals. RNAi-silencing of this gene results in abnormally shaped pore canals filled with loose fibers rather than well-organized bundles of chitin around the periphery with a central column of a thick chitin bundle (Noh et al. 2015). Taken together, these results indicate that CPR cuticular proteins also contribute to the overall organization of chitin in all cuticles.

5.7.5 Other Chitin Modifying Enzymes

Besides the enzymes and binding proteins that interact with chitin as described above, there are other proteins that modify chitin and participate in chitin remodeling. Most prominent among them are lytic polysaccharide monooxygenases (LPMO) which is the subject of another chapter in this book (Aachman and Eijsink, Chap. 6). Other enzymes including transglutaminases or laccases may be involved in cross-linking of cuticular proteins and possibly chitin and they are also not covered in this chapter.

5.7.6 Overview of Chitin Metabolism and Enzymes Involved

Figure 5.1 summarizes the roles of enzymes involved in chitin metabolism. Starting from the activated precursor UDP-*N*-acetylglucosamine and a growing chitin chain as a primer, chitin is synthesized by either CHS-A or CHS-B. Chitin deacetylases
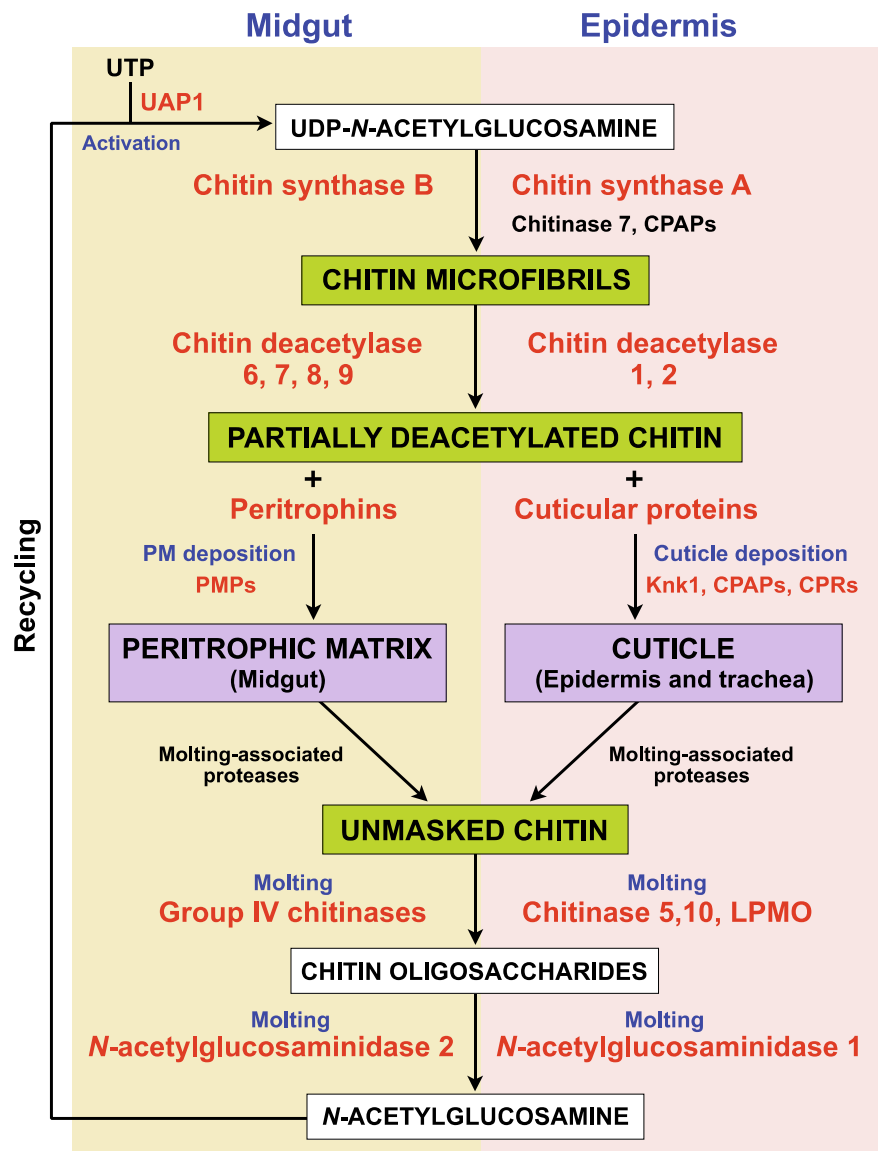

Fig. 5.1 Overview of chitin metabolism in insects

partially deacetylate the nascent chitin in the PM or the cuticle. The partially deacetylated chitin fibers associate with either cuticular proteins or PMPs to form the matrices containing chitin and proteins. At the time of molting, appropriate proteases unmask the chitin chains, which are then acted upon by a mixture of chitinases and *N*-acetylglucosaminidases to yield monomeric *N*-acetylglucosamines, which are then recycled after activation by UTP-*N*-acetylglucosamine pyrophosphorylase (UAP).

5.8 Conclusions

From the time the chitin polymeric chains emerge from the catalytic center of oligomeric assemblies of CHS enzymes, they interact with other nascent chitin chains and a whole assortment of chitin-remodeling enzymes and proteins that determine its final properties such as crystallinity, size, rigidity, elasticity, state of hydration, higher order organization, and cross-linking with other components including phenolics and minerals. These allow the cells that make chitin a variety of choices leading to the production of extracellular matrices with a wide range of physiochemical and biological properties appropriate for the anatomical region and the developmental stage of the organism. Likewise, during the process of disassembly of these matrices, a whole assortment of depolymerizing enzymes and proteins are involved that work in concert to allow remodeling and reutilization of the components of the old matrix. These processes are under a variety of hormonal and developmental controls that offer numerous possibilities for the performance of these organisms in a hostile environment. Further, a better understanding of these complex processes will allow the production of biomimetic agents with desirable properties for industrial and biomedical applications.

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