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Review

Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases

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Summary

Chitin is one of the most important biopolymers in nature. It is mainly produced by fungi, arthropods and nematodes. In insects, it functions as scaffold material, supporting the cuticles of the epidermis and trachea as well as the peritrophic matrices lining the gut epithelium. Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures. For this purpose, insects repeatedly produce chitin synthases and chitinolytic enzymes in different tissues. Coordination of chitin synthesis and its degradation requires strict control of the participating enzymes during development. In this review, we will summarize recent advances in understanding chitin synthesis and its degradation in insects.

Key words: chitin, chitin synthesis, chitin synthase, chitinase, cuticle, peritrophic matrix, insect.

Introduction

Insect cuticles form an exoskeleton that exhibits only a limited capacity to keep pace with body growth because it is a more or less rigid structure due to the presence of chitin and sclerotized proteins. To allow growth and development, insects are therefore periodically forced to replace their old cuticle with a new and looser one during molting (ecdysis). The nascent, non-sclerotized integument underneath the old cuticle is strongly furrowed and can only expand when molting is complete. Ecdysis is initiated by apolysis, the process that separates epidermal cells from the old cuticle by molting fluid secretion and ecdysial membrane formation. The molting fluid contains proteases and chitinases, enzymes that digest the main constituents of the old endocuticle (Reynolds and Samuels, 1996). Shortly before ecdysis, the molting fluid, which has accumulated in the apolysial space, is reabsorbed, allowing the recycling of old cuticle components. Formation of the new cuticle starts after the ecdysial space opens as a result of the secretion of cuticle proteins and chitin fibers through the apical membranes of epidermal cells. Initially, patches of cuticullin, forming later on the outer epicuticle, are secreted, followed by an unsclerotized, chitinous cuticle referred to as procuticle. Afterwards, formation of the epicuticle seals the epidermis and protects it against the digestive enzymes of the molting fluid. Before sclerotization is completed, the insects expand their new cuticle and shed their old envelope, now called exuvia, by performing distinct motor programs and increasing body pressure (Carlson and Bentley, 1977). Pre-ecdysis behavior and ecdysis are controlled by the action of molting hormones such as eclosion hormone, which is secreted in response to falling ecdysteroid titers and causes the release of pre-ecdysis-triggering hormone and ecdysis-triggering hormone (Truman and Riddiford, 1970; Zitnan et al., 1999; Kingan and Adams, 2000).

Chitin, a polymer of *N*-acetyl-β-D-glucosamine, is a major component of the insect cuticle. Solids NMR and gravimetric analysis revealed that the chitin content constitutes up to 40% of the exuvial dry mass depending on the insect species and varies considerably with the different cuticle types even in a single organism (Kramer et al., 1995). Chitin is found in the exo- and endocuticle or in the newly secreted, unsclerotized procuticle but not in the epicuticle, the outermost part of the integument (Andersen, 1979). It functions as light but mechanically strong scaffold material and is always associated with cuticle proteins that mainly determine the mechanical properties of the cuticle. In the migratory locust *Locusta migratoria*, more than a hundred different cuticle proteins have been observed in 2-D electrophoresis (Hojrup et al., 1986). Some of them are highly conserved in various insect orders, some of them are restricted to specific body regions and others contain repeats of hydrophobic residues that seem to be linked with cuticle rigidity (Andersen et al., 1995). One of the best understood cuticle proteins is resilin, a glycine- and proline-rich protein that confers high elasticity to the cuticle of hinge regions (Andersen and Weis-Fogh, 1964).

Chitin is also an integral part of insect peritrophic matrices, which function as a permeability barrier between the food

bolus and the midgut epithelium, enhance digestive processes and protect the brush border from mechanical disruption as well as from attack by toxins and pathogens (Tellam, 1996). Insect peritrophic matrices have been categorized into two classes, based on their mode of synthesis (Wigglesworth, 1930; Peters, 1992). Type I peritrophic matrices are synthesized along the whole midgut and thus form a continuous delamination product. By contrast, type II peritrophic matrices are exclusively produced by specialized cells in the area of the cardia, which is located between the esophagus and the anterior midgut. Peritrophic matrices usually exhibit a chitin content of between 3% and 13% (Peters, 1992). For the peritrophic matrix of the tobacco hornworm *Manduca sexta*, a chitin content of even 40% has been reported (Kramer et al., 1995). The remainder of the peritrophic matrix consists of a complex mixture of proteins, glycoproteins and proteoglycans. The peritrophic matrix is created when the chitin microfibrils associate with the highly hydrated proteoglycan matrix secreted by the gut cells. Further components of the peritrophic matrix, such as peritrophins, may be added during the gelling process. Peritrophins appear to link chitin microfibrils *via* their multiple chitin-binding domains and additionally mediate binding to other glycoproteins. Consequently, they may contribute significantly to the tensile strength of the peritrophic matrix (Lehane, 1997). Variation of peritrophic matrix formation rate is observed frequently in insects, depending on the physiological condition (Locke, 1991). Some insects even completely cease peritrophic matrix production during periods of starvation or molt. The old peritrophic matrix then gets expelled or reabsorbed and regenerates when the animal starts feeding again.

Thus, insect growth and development is strictly dependent on the capability to remodel chitinous structures. Therefore, insects consistently synthesize and degrade chitin in a highly controlled manner to allow ecdysis and regeneration of the peritrophic matrices. Chemical compounds that interfere with chitin metabolism, such as diflubenzuron, have been of special interest for the control of agricultural pests. Moreover, due to its unique properties, chitin itself is attracting more and more interest as a basic material for the chemical and pharmaceutical industry. In this review, we will focus on recent advances in understanding biosynthesis and degradation of chitin in cuticles and peritrophic matrices. In particular, we will address the substantial progress that has been made on chitin synthases and chitinases as a result of identification and sequencing of the insect genes encoding these enzymes.

Chitin structure

Chitin is the most widespread amino polysaccharide in nature and is estimated annually to be produced almost as much as cellulose. It is mainly found in arthropod exoskeletons, fungal cell walls or nematode eggshells. However, derivatives of chitin oligomers have also been implicated as morphogenic factors in the communication between leguminous plants and *Rhizobium* and even in vertebrates, where they may be important during early stages of embryogenesis (Bakkers et al., 1999).

Chitin is composed largely of alternating *N*-acetylglucosamine residues, which are linked by β-(1-4)-glycosidic bonds. Since hydrolysis of chitin by chitinase treatment leads to the release of glucosamine in addition to *N*-acetylglucosamine, it was concluded that glucosamine might be a significant portion of the polymer. However, solids NMR analysis of tobacco hornworm cuticle preparations suggested that little or no glucosamine is present (Kramer et al., 1995). Chitin polymers tend to form microfibrils (also referred to as rods or crystallites) of ~3 nm in diameter that are stabilized by hydrogen bonds formed between the amine and carbonyl groups. Chitin microfibrils of peritrophic matrices may even exceed 0.5 μm in length and frequently associate in bundles containing parallel groups of 10 or more single microfibrils (Peters et al., 1979; Lehane, 1997). X-ray diffraction analysis suggested that chitin is a polymorphic substance that occurs in three different crystalline modifications, termed α-, β- and γ-chitin. They mainly differ in the degree of hydration, in the size of the unit cell and in the number of chitin chains per unit cell (Rudall and Kenchington, 1973; Kramer and Koga, 1986). In the α form, all chains exhibit an anti-parallel orientation; in the β form the chains are arranged in a parallel manner; in the γ form sets of two parallel strands alternate with single anti-parallel strands. In addition, non-crystalline, transient states have also been reported in a fungal system (Vermeulen and Wessels, 1986). All three crystalline modifications are actually found in chitinous structures of insects. The α form is most prevalent in chitinous cuticles, whereas the β and γ forms are frequently found in cocoons (Kenchington, 1976; Peters, 1992). Peritrophic matrices usually consist of α- and β-chitin. Sometimes the presence of β-chitin in cocoons is traced back to the fact that some cocoons are formed from peritrophic matrices; for example, those of Australian spider beetle *Ptinus tectus*, a specialized beetle (Rudall and Kenchington, 1973).

The anti-parallel arrangement of chitin molecules in the α form allows tight packaging into chitin microfibrils, consisting of ~20 single chitin chains that are stabilized by a high number of hydrogen bonds formed within and between the molecules. This arrangement may contribute significantly to the physicochemical properties of the cuticle such as mechanical strength and stability (Giraud-Guille and Bouligand, 1986). By contrast, in the β- and γ-chains, packing tightness and numbers of inter-chain hydrogen bonds are reduced, resulting in an increased number of hydrogen bonds with water. The high degree of hydration and reduced packaging tightness result in more flexible and soft chitinous structures, as are found in peritrophic matrices or cocoons. The picture drawn above is certainly oversimplified and does not explain the physicochemical properties of cuticles and peritrophic matrices adequately because it is reduced to only one component of a complex structure. However, differences in the arrangement of chitin microfibrils between cuticles and peritrophic matrices may help to understand their function. The cuticle is secreted in the form of thin layers by the apical microvilli of epidermal

cells. The chitin microfibrils are embedded into the protein matrix and stabilize it in a way that resembles constructions of steel-reinforced concrete. Since horizontal microfibrils, in parallel with the cuticle plane, rotate either progressively or abruptly from one level to another, complex patterns (e.g. helicoidal) and textures (e.g. plywood-like structures) arise, depending on the degree of rotational displacement (Bouligand, 1972). By contrast, in peritrophic matrices, the microfibrils are normally arranged as a network of randomly organized, felt-like structures embedded in an amorphous matrix, and only in a few cases have higher ordered configurations been reported (Lehane, 1997).

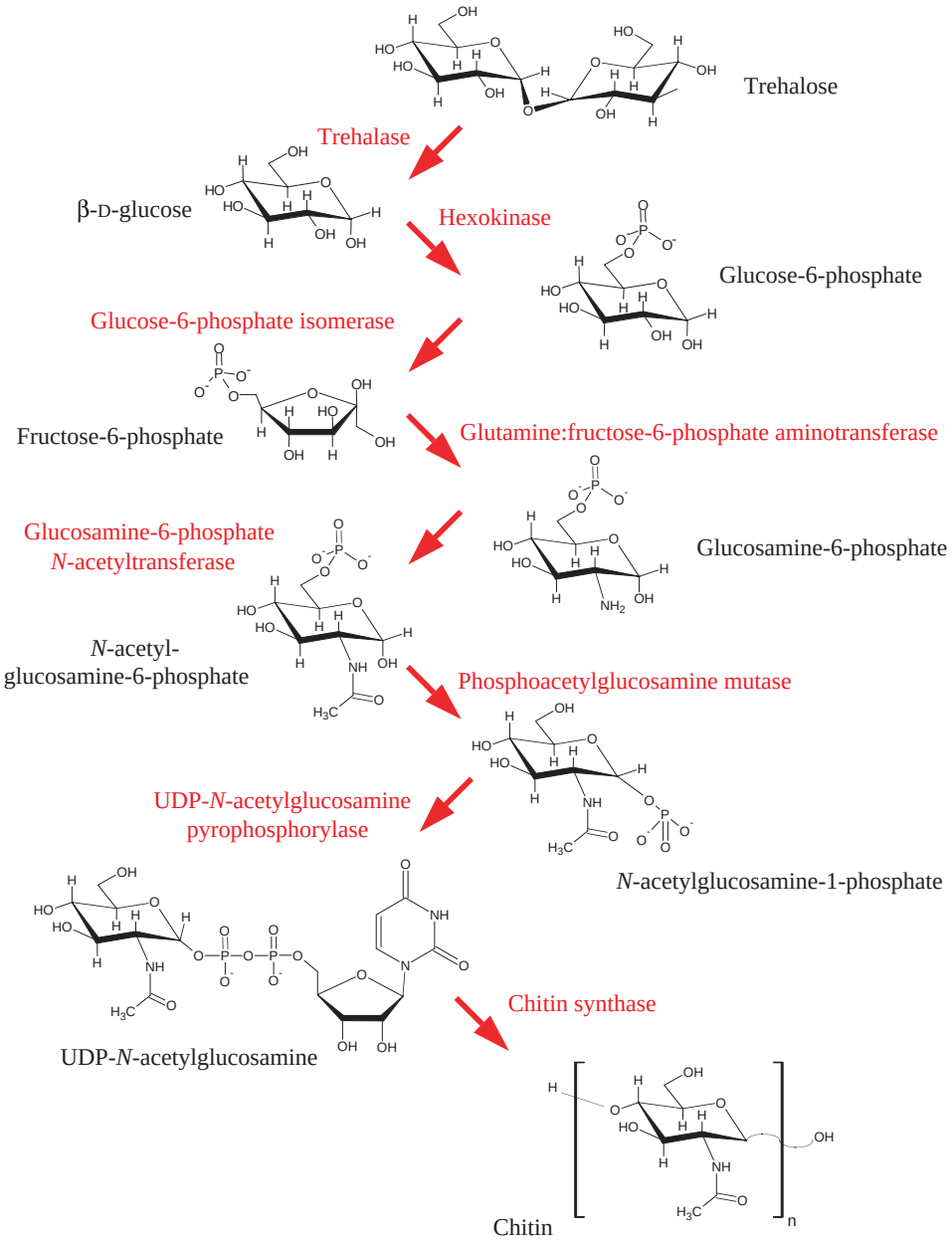

Fig. 1. Biosynthesis of chitin in insects. The pathway starts with trehalose, the main hemolymph sugar in most insects, and ends with the chitin polymer. The diagrammatic representation is based on previously published pathways (Kramer and Koga, 1986; Cohen, 2001).

Chitin formation

Although chitin is one of the most important biopolymers in nature, knowledge of its biosynthesis is still fragmentary. Formation of the different chitin forms is catalyzed by chitin synthase (UDP-*N*-acetyl-D-glucosamine:chitin 4-β-*N*-acetylglucosaminyltransferase; EC 2.4.1.16), a highly conserved enzyme found in every chitin-synthesizing organism. It utilizes UDP-*N*-acetylglucosamine (UDP-GlcNAc) as the activated sugar donor to form the chitin polymer (Glaser and Brown, 1957). Candy and Kilby (1962) were the first to propose a biosynthetic pathway for insect chitin synthesis starting with glucose and ending with UDP-GlcNAc. The pathway from UDP-GlcNAc to chitin was finally established in insects by Jaworski et al. (1963) using cell-free extracts from the southern armyworm *Spodoptera eridania*. Many subsequent studies conducted with preparations from various insects supported this general pathway, which is shown in Fig. 1. Little is known about the final steps in chitin synthesis in any organism. One intermediate step might be the transfer of GlcNAc onto a lipid to form dolichyldiphospho-*N*-acetylglucosamine, as was deduced from studies with microsomal fractions of bugs and brine shrimps (Quesada-Allue et al., 1976; Horst, 1983). However, the current evidence for the occurrence of lipid-GlcNAc intermediates or acceptors during chitin assembly is weak and therefore not included in the depicted pathway. In fungal systems, at least, an acceptor other than UDP-GlcNAc seems not to be required to initiate polymerization (McMurrough and Bartnicki-Garcia, 1971; Orlean, 1987; Merz et al., 1999a; Chang et al., 2003). Chitin synthase is definitely the key enzyme within the biosynthetic pathway, but detailed understanding of its mode of action seems to be a distant prospect, in particular because the enzyme has not yet been purified to homogeneity.

Many basic studies have been performed with fungal systems, and some of the results seem to be valid for the insect

enzymes as well. Common features of most chitin synthases are that enzyme activity is dependent on the presence of divalent cations such as Mg^{2+} or Mn^{2+} and that it is increased by mild proteolysis, suggesting the existence of a zymogenic form (Duran et al., 1975; Mayer et al., 1980; Hardy and Gooday, 1983; Kramer and Koga, 1986; Merz et al., 1999a). Usually, chitin synthase activity can be inhibited by structural UDP-GlcNAc analogues such as polyoxins and nikkomycin (Gooday, 1972; Dahn et al., 1976). Enzyme activity seems to be restricted exclusively to membrane-containing fractions (Ruiz-Herrera and Martinez-Espinoza, 1999). Since chitin synthase has been localized in the membranes of Golgi complexes (Horst and Walker, 1993) and intracellular vesicles (Sentandreu et al., 1984), as well as in plasma membranes (Duran et al., 1975; Vardanis, 1979), it may be concluded that the enzyme follows an exocytotic pathway, accumulating in cytoplasmic vesicles during its transport to the cell surface. This view is supported by studies performed with imaginal discs of Indian mealmoth *Plodia interpunctella*, which showed that chitin synthesis is inhibited when microtubules are disrupted by cytoskeletal poisons such as colchicine or vinblastine (Oberlander et al., 1983).

In fungal systems, substantial data have accumulated indicating that chitin synthase activity of at least one chitin synthase isoform (CHS3p) is associated with specialized intracellular microvesicles, known as chitosomes, which exhibit a special lipid and protein composition (Bracker et al., 1976; Hernandez et al., 1981; Florez-Martinez et al., 1990). Electron microscopy has revealed that, in the presence of UDP-GlcNAc and activators, purified chitosomes synthesize microfibrils that crystallize in the lumen of the vesicles (Bracker et al., 1976). Similar results were obtained when cell-free precipitates resulting from chitin synthase activity in crude extracts of red flour beetle *Tribolium castaneum* were examined. Electron micrographs of the chitin synthase products showed a network of long, parallel-aligned microfibrils that varied in thickness from 10 nm to 80 nm. The microfibrils were associated with particles ranging from approximately 50 nm to 250 nm in diameter, which may be interpreted as ‘insect chitosomes’ (Cohen, 1982). However, final proof for direct involvement of ‘insect chitosomes’ in chitin synthesis is missing. Interestingly, chitosome-like structures do not seem to occur in insect epidermal cells from Brazilian skipper butterfly *Calpodes ethlius* and Australian sheep blowfly *Lucilia cuprina*. Instead, electron microscope studies showed densely stained areas at the tips of microvilli from epidermal cells, referred to as plasma membrane plaques, which were considered as clusters of chitin-synthesizing enzymes. During cuticle formation, these areas undergo hormonally controlled cyclic turnovers (Binnington, 1985; Locke, 1991; Locke and Huie, 1979). In accordance with the predicted site of chitin synthesis, immunohistochemistry using polyclonal antibodies raised against a conserved region of the chitin synthase showed strong labeling within the apical region of the epidermis from the epiproct of the American cockroach *Periplaneta americana* (Fig. 2; H. Merzendorfer and L. Zimoch, unpublished).

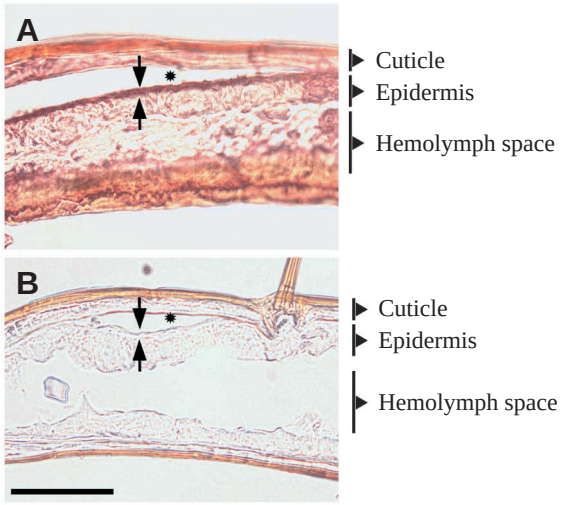

Fig. 2. Localization of chitin synthase in the epiproct of *Periplaneta americana*. (A) Cryosections of 10 μm were stained with a polyclonal antiserum raised against a conserved region of the *Manduca sexta* chitin synthase as described previously (Zimoch and Merzendorfer, 2002). Visualization of primary antibodies was conducted with anti-rabbit antibodies (whole molecules) conjugated to alkaline phosphatase. Detection with 5-bromo-4-chloroindolylphosphate and nitroblue tetrazolium was carried out in the presence of 2 mmol l^{-1} levamisole to block endogenous alkaline phosphatase activity. (B) Control reaction performed in the absence of primary antibodies. Arrows in A and B mark the apical region of epidermal cells, which are in part detached from the endocuticle as a result of the sectioning procedure (asterisks). Scale bar, 50 μm.

Similar results have been obtained for the chitin synthase found in insect intestinal systems to produce chitin for peritrophic membranes, which are thought to be secreted by the microvilli of gut epithelial cells, since in electron microscopy secreted material appears as more or less electron-dense aggregation on top of or in between the microvilli (Peters, 1992). By secreting the peritrophic matrix, the microvilli act as a mold that causes microfibril spacing and, in doing so, contribute to the formation of regular patterns that are sometimes found in peritrophic matrices. Recently, Hopkins and Harper (2001) used transmission electron microscopy and wheat germ agglutinin (WGA)–gold staining to visualize newly secreted chitinous fibers in lepidopteran midgut sections. They found them on the microvillar surface but also within the apical region of microvilli. In line with this view, immunohistochemistry conducted with polyclonal antibodies raised against a conserved polypeptide of the *Manduca sexta* chitin synthase demonstrated that the enzyme is restricted to the apical tips of microvilli from columnar cells, one major cell type found in larval midgut (Zimoch and Merzendorfer, 2002). However, as may also be the case for epidermal cells, it is not yet clear whether chitin synthase is actually integrated into the plasma membrane or resides in vesicles enriched underneath the plasma membrane. Confocal laser scanning microscopy, at least, unveiled vesicular

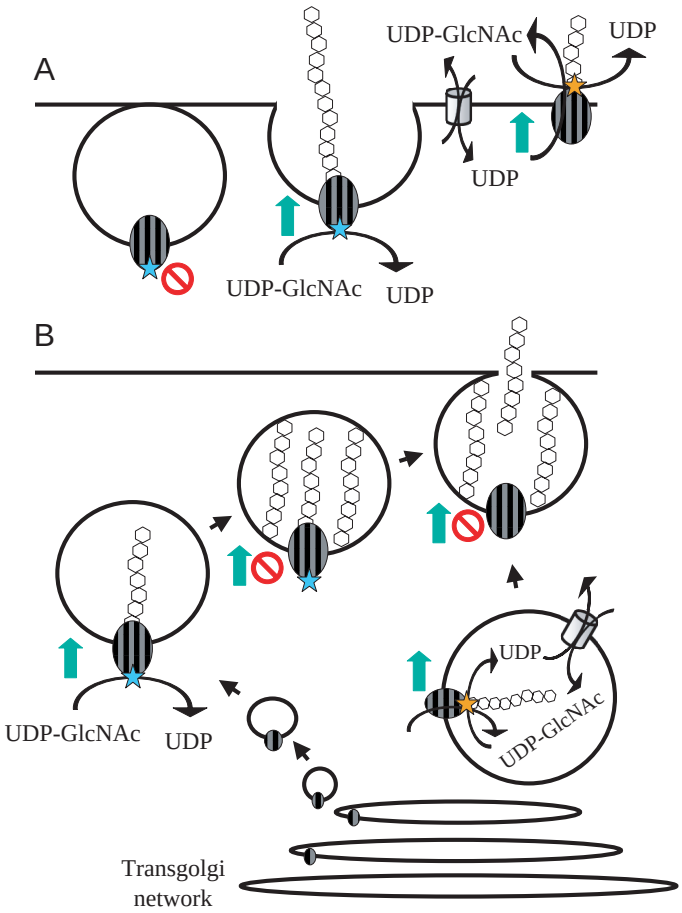

Fig. 3. Alternative models of chitin formation in insects. (A) Chitin synthase-loaded vesicles are transported from the transgolgi network to the apical region of epithelial cells by a constitutive secretory pathway and subsequently fuse with the plasma membrane. Upon fusion, they may get activated by proteolytic enzymes present in the molting fluid or the gut lumen. Blue and orange stars indicate the catalytic site facing either the cytoplasm or the extraplasmic space, respectively. (B) In this more speculative model, chitin synthesis has already occurred before the vesicles have fused with the plasma membrane and may continue or cease upon fusion. If the catalytic domain faces the cytoplasm (blue star), nascent chitin polymers have to be transported across the vesicular membrane, presumably involving transmembrane segments of the chitin synthase. By contrast, intravesicular arrangement of the catalytic domain (orange star) would require some uptake mechanism for UDP-GlcNAc.

structures within the cytoplasm of columnar cells that immunoreacted with the anti-chitin synthase antibodies and, hence, may represent ‘insect chitosomes’ on their way from the Golgi complex to the apical tips of microvilli (Zimoch and Merzendorfer, 2002).

The specific mechanism by which chitin is produced is still unknown. However, evidence suggests that chitin is synthesized through an asymmetric mechanism, accepting GlcNAc units from the cytosolic UDP-GlcNAc pool and releasing the nascent chain into the extraplasmic phase (Ruiz-Herrera and Martinez-Espinoza, 1999). Indeed, from predictive analysis it seems likely that the catalytic site of the chitin synthase that binds UDP-GlcNAc faces the cytoplasm (Tellam et al., 2000). On the basis of the presented data, one can propose two alternative models for insect chitin synthesis (Fig. 3). In one model, intracellular vesicles merely function as exocytotic conveyors responsible for the transport of chitin synthase to the plasma membrane. After membrane fusion, the chitin synthase may be activated and subsequently secretes chitin into the extracellular space. This model requires some regulatory step, which controls enzyme activity, keeping the enzyme switched off until the vesicles fuse with the plasma membrane. Since proteolytic activation of chitin synthesis is observed in microsomal preparations from stable fly *Stomoxys calcitrans* pupae (Mayer et al., 1980), onset of chitin synthase activity upon vesicle fusion might be achieved by extracellular proteases present in the midgut or in the molting fluid (Law et al., 1977; Reynolds and Samuels, 1996; Terra et al., 1996).

In a more speculative model, chitin is secreted into the lumen of specialized vesicles, which accumulate underneath the terminal web and fuse with the plasma membrane when chitin needs to be released. This model allows storage of chitin polymers and their rapid release, which may be important for peritrophic matrix secretion upon feeding of blood-sucking mosquitoes. However, the length of chitin polymers may be restricted due to the limited volume of the vesicles.

If the catalytic site really faces the cytoplasm, UDP-GlcNAc could directly bind from the cytoplasmic pool. Consequently, in both presented models, nascent chitin has to be transported across the membrane, possibly involving transmembrane regions of the chitin synthase. If the catalytic domain should, contrary to the predictions, face the extraplasmic site, UDP-GlcNAc would need to be transported either into the extracellular environment or into the lumen of the vesicles. Substrate transport might be achieved either by the chitin synthase itself or by transmembrane proteins similar to the UDP-GlcNAc transporters that reside in the endoplasmic reticulum or the Golgi vesicles (Perez and Hirschberg, 1985; Cecchelli et al., 1986; Segawa et al., 2002). Although no biochemical data that support intravesicular catalysis are currently available, it would cleverly circumvent the unsolved problem of how to translocate the nascent chitin polymer across the membrane, because chitin would already be synthesized on the side of its subsequent release.

Chitin synthase can be assayed readily and some progress has been made in purifying active components in fungal systems (Duran and Cabib, 1978; Kang et al., 1984; Machida and Saito, 1993; Uchida et al., 1996). However, despite all efforts that have been made during the past decades, the enzyme has still not been purified to homogeneity. Therefore, we have only a vague image of the molecular mechanism of chitin synthesis. In contrast to fungi, only few studies have been conducted using chitin synthase-containing preparations from insects. *In vivo* studies, as well as *in vitro* studies using insect organ and cell cultures, first provided insights into insect chitin synthesis (Candy and Kilby, 1962; Marks and Leopold, 1971; Marks, 1972; Surholt, 1975; Vardanis, 1976). More

detailed knowledge emerged from investigations performed in cell-free systems, although preservation of enzyme activity turned out to be difficult. Quesada-Allue et al. (1976) were among the first to measure chitin synthase activity in cell-free extracts of insects. For this purpose, they used crude extracts from the kissing bug *Triatoma infestans* integument and monitored [^{14}C]*N*-acetylglucosamine incorporation into the polymer. Chitin synthase activity exhibited a pH optimum of about 7.2 and was dependent on the presence of Mg^{2+} and GlcNAc. Interestingly, radioactivity was also found concomitantly with chitin synthesis in a liposoluble fraction. Chromatographic analysis of this fraction suggested the involvement of *N*-acetylglucosaminyl-phospholipid in insect chitin synthesis, which was supported by the finding that chitin synthesis was blocked by tunicamycin, an inhibitor of UDP-*N*-acetylglucosamine:dolichyl-phosphate *N*-acetylglucosamine-phosphotransferase (Heifetz et al., 1979; Quesada-Allue, 1982). Supporting evidence came from studies performed with microsomes from brine shrimps (*Artemia salina*), which catalyzed the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to a lipid acceptor. The resulting dolichyldiphosphate-linked chito-oligomer may act as a GlcNAc acceptor for chitin synthesis (Horst, 1983). By contrast, from kinetic studies it was concluded that chitin synthesis generally occurs without the need for soluble or lipid GlcNAc acceptors functioning as primers for chain assembly (Horsch et al., 1996; Merz et al., 1999a). In line with this interpretation, some groups have reported that chitin synthesis was not affected significantly by tunicamycin in several insect systems (Mayer et al., 1981; Fristrom et al., 1982; Bade, 1983). The inconsistency regarding the published data, together with the fact that chain assembly occurs without the need of an initial acceptor other than UDP-GlcNAc in fungal systems, however, raises doubt about the significance of lipid intermediates or primers in arthropod systems.

Chitin synthesis is influenced in different ways by other effectors as well, depending on the particular enzyme source. For instance, GlcNAc has been reported to stimulate chitin synthesis in fungi and also in some insects (Keller and Cabib, 1971; Quesada-Allue et al., 1976; Cohen and Casida, 1980a,b, 1982). By contrast, studies with microsomal fractions from *Stomoxys* showed almost complete inhibition of chitin synthesis with 1 mmol l^{-1} GlcNAc (Mayer et al., 1980). Even more confusing, the activity of classical inhibitors of chitin synthesis such as polyoxin, nikkomycin and diflubenzuron also seems to depend on the insect system used for the particular study. Cohen and Casida (1982), for instance, reported different effects of polyoxins and nikkomycin on chitin synthesis in cecropia moth *Hyalophora cecropia* and cabbage looper *Trichoplusia ni*. Mayer et al. (1980, 1981) observed polyoxin D inhibition in microsomal preparations from *Stomoxys* only at high concentrations but no inhibitory effect for diflubenzuron, whereas Turnbull and Howells (1983) showed for crude homogenates of larval integuments from *Lucilia* that chitin synthesis was inhibited by both polyoxin D and diflubenzuron. However, due to the crude character of the investigated preparations, care has to be taken not to jump to conclusions. Besides cell-free extracts, chitin synthesis has also been reported for several insect cell lines. For instance, Marks et al. (1984) demonstrated chitin synthase activity in MRRL-CH cells, a continuous cell line from *Manduca* embryos. Londershausen and colleagues showed chitin synthesis in an epithelial-like cell line from the non-biting midge, *Triatoma infestans* as well as in K_c cell lines from *Drosophila melanogaster*. In cell cultures from *Chironomus*, incorporation of radiolabeled glucosamine was partially inhibited by the acyl urea SIR 8514, polyoxin D and nikkomycin (Londershausen et al., 1988).

Insect chitin synthases

Tellam and colleagues were the first to sequence the complete cDNA of a putative arthropod chitin synthase (*LsCHS-1*; Tellam et al., 2000). The cDNA was derived from *Lucilia*, the Australian sheep blowfly. It encoded a presumed protein with a molecular mass of approximately 180 kDa, and predictive analysis suggested the existence of 15–18 transmembrane helices, indicating that the enzyme is an integral membrane protein. Analysis of the *LsCHS-1* expression pattern by RT-PCR and *in situ* hybridization revealed that the transcripts are detectable in all developmental stages of the fly and that they localize in the epidermal cells underlying the procuticle.

Meanwhile, progress has been made in investigating insect chitin synthases due to the availability of an increasing number of gene and cDNA sequences deposited in sequence databases or published within the past three years, although final proof that the deduced proteins synthesize chitin is pending (Ibrahim et al., 2000; Tellam et al., 2000; Gagou et al., 2002; Zhu et al., 2002).

In contrast to fungi, which possess multiple genes encoding chitin synthase isoforms (Munro and Gow, 2001), molecular analysis of nematode and insect chitin synthase genes (*CHS*) has so far revealed a limited number of gene copies. Genome sequencing projects have shown that *Caenorhabditis elegans*, *Drosophila* and *Anopheles gambiae* possess two different *CHS* genes, and recently cDNA sequencing or genomic Southern blotting also provided evidence for two gene copies in *Lucilia*, *Manduca* and *Tribolium* (Gagou et al., 2002; Tellam et al., 2000; Zhu et al., 2002; Zimoch and Merzendorfer, 2002; Y. Arakane, D. Hogenkamp, Y. C. Thu, C. A. Specht, R. W. Beeman, K. J. Kramer, M. Kanost and S. Muthukrishnan, unpublished results). Comparison of amino acid sequences from fungal, nematode and insect chitin synthases has revealed that insect enzymes are more closely related to those of nematodes than those of fungi.

Insect chitin synthases are large transmembrane proteins with theoretical molecular masses ranging from 160 kDa to 180 kDa and exhibit a slightly acidic isoelectric point between 6.1 and 6.7. Alignments of the amino acid sequences from *Lucilia*, *Drosophila* and *Caenorhabditis* revealed a tripartite domain structure (Tellam et al., 2000; see also Fig. 4A).

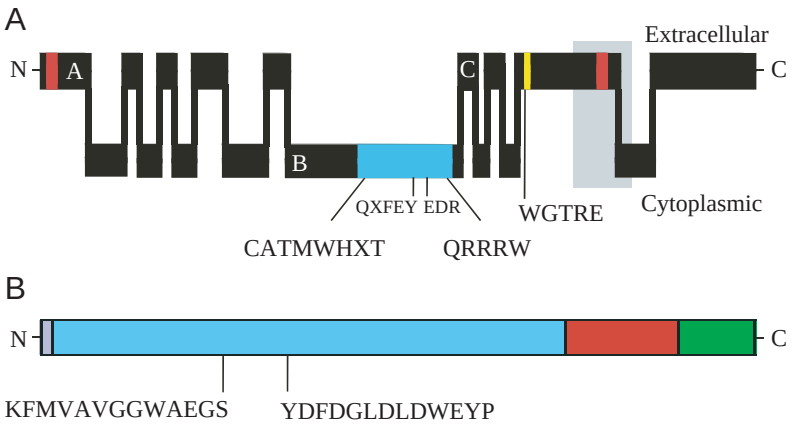

Fig. 4. Schematic representation of the domain structures of insect enzymes involved in chitin metabolism. (A) Domain structure and membrane topology of the *Manduca sexta* chitin synthase 1 (accession no. AY062175). Transmembrane helices are depicted as vertical bars, cytoplasmic or extracellular regions are depicted as horizontal bars. The N-terminal, catalytic and C-terminal domains are marked with A, B and C, respectively, following a previously suggested nomenclature (Tellam et al., 2000). Highly conserved blocks are tagged with the respective consensus sequences, with EDR and QRRRW being the chitin synthase signature sequences. The gray-shaded box highlights the region that is affected by alternate exon usage of class A genes. The red, blue and yellow boxes indicate supposed sites of *N*-glycosylation, catalysis and coiled-coils, respectively. (B) Domain structure of the *Manduca sexta* chitinase (accession no. A56596). Highly conserved blocks are tagged with the respective consensus sequence, with YDFDGLDLDWEYP being the insect signature sequence, which is consistent with that of family 18 chitinases. The pink box represents the signal peptide preceding the amino acid sequence of mature chitinases. The blue, red and green boxes indicate the catalytic, serine/threonine-rich and chitin-binding domains, respectively. Note that the serine/threonine-rich region is extensively processed by *O*-glycosylation.

Domain A is found in the N-terminal region, has varying numbers of transmembrane helices and shows the least sequence similarity among any of the species. Depending on the number of predicted transmembrane helices in the A domain, the N-terminus appears to be located at different sides of the membrane, facing either the extracellular environment or the cytoplasm. However, this may also reflect shortcomings regarding the computer-based prediction of transmembrane helices.

Domain B is found in the center of chitin synthases, comprises ~400 amino acids and contains the catalytic center of the protein. The B domain is highly conserved and contains two unique motifs, E**D**R and **Q**RR**RW**, that are present in all types of chitin synthases and therefore can be regarded as signature sequences. Some of the conserved residues have been implicated to be essential for the catalytic mechanism, since they may be involved in protonation of the substrate (Sinnott, 1990; Breton et al., 2001). In particular, even conservative substitutions of those residues that have been highlighted in bold above drastically decrease chitin synthase activity in yeast, although they do not significantly affect the apparent K_m values for the substrate (Nagahashi et al., 1995). Similar sequences have been found in bacterial and vertebrate hyaluron synthases (Rosa et al., 1988; DeAngelis et al., 1994; Pummill et al., 1998), cellulose synthases (Saxena et al., 2001) and *N*-actelyglucosaminyltransferases such as the NodC protein (Geremia et al., 1994). An aspartic acid residue at position 441 of the yeast chitin synthase 2 protein (CHS2p) was also suggested to be conserved in all chitin synthases. Its substitution by glutamic acid led to a severe loss of chitin synthase activity in the resulting *CHS2* mutant (Nagahashi et al., 1995). This aspartic acid residue is nevertheless replaced by glutamate in some insect chitin synthases at a corresponding position, supporting the necessity of at least an acidic residue at this position. However, a highly conserved aspartic acid is also found at position 344 of the yeast CHS2p. Unfortunately, this position has not been addressed by *in vitro* mutagenesis so far. Zhu et al. (2002) described three additional highly conserved blocks in insect chitin synthases, CATMWHXT, QXFEY and WGTRE (at positions 583–590, 794–798 and 1076–1080 of the *Drosophila* CHS-1 protein, respectively; see Fig. 4A), with most of the amino acids also conserved in fungal or nematode chitin synthases.

Domain C comprises the C-terminal part of the enzyme and contains two amino acids that might also be involved in catalysis, since site-directed mutagenesis performed with CHS2p of yeast showed that enzyme activity was diminished when W803 or T805 were exchanged for alanine (Yabe et al., 1998). Both residues are conserved in insects at positions comparable to those of the yeast enzyme, immediately following transmembrane helix five of the C domain. Although this domain is far less conserved than the catalytic domain, it exhibits seven transmembrane helices as a common feature.

As has been reported for several fungal chitin synthases, insect enzymes may also be glycosylated because they exhibit several putative *N*-glycosylation sites of which one is conserved in every insect chitin synthase (Table 1). In fungal systems, the affinity of lectins such as concanavalin A or wheat germ agglutinin to the sugar portion of *N*-glycosylated residues has already been used for the purification of active components of chitin synthases (Machida and Saito, 1993; Merz et al., 1999a,b).

Based on relative sequence differences, chitin synthases have been grouped into two classes, class CHS-A and class CHS-B enzymes. So far, most insects seem to have one gene copy for each enzyme. Since both genes are located at one chromosome in both *Drosophila* and *Anopheles*, it is likely that they have evolved from a common ancestor by gene duplication (Gagou et al., 2002; Y. Arakane, D. Hogenkamp, Y. C. Thu, C. A. Specht, R. W. Beeman, K. J. Kramer, M. Kanost and S. Muthukrishnan, unpublished results). Gene expression studies performed in *Lucilia*, *Tribolium* and

Table 1. *Characteristics of some insect chitin synthases*

	MsCHS-1	*LcCHS-1*	*AaCHS-1*	*DmCHS-1, DmeChSB, kkv*	*DmCHS-2, DmeChSA*	*AgCHS-2*	*AgCHS-1*
Organism	*Manduca sexta*	*Lucilia cuprina*	*Aedes aegypti*	*Drosophila melanogaster*	*Drosophila melanogaster*	*Anopheles gambiae*	*Anopheles gambiae*
Accession no.	AY062175	AF221067	AF223577	NM_079509	NM_079485	XM_321337	AY056833*
Class	A	A	B	A	B	A	B
Molecular mass (kDa)	178.56	180.72	179.39	182.83	161.39	186.40	181.27
pI	6.56	6.39	6.13	6.37	6.66	6.24	6.37
TMH	16	17	14	17	16	14	16
N-terminus	Outside	Inside	Outside	Inside	Outside	Outside	Outside
PEST sites	9–33 1178–1205	7–39	1147–1179	7–39	n.p.	7–30	n.p.
Coiled-coils	1060–1094	1065–1099	n.p.	1078–1108	n.p.	1066–1098	n.p.
N-glycosylation sites							
Total number	9	5	6	7	3	11	7
Conserved position	905	910	895	921	912	909	891

Prediction of putative transmembrane helices, PEST sites, coiled-coils and *N*-glycosylation sites was performed with the programs TMHMM, PESTfind, PAIRCOIL and PROSCAN, respectively (Rogers et al., 1986; Berger et al., 1995, Bairoch et al., 1997; Hansen et al., 1997; Krogh et al., 2001).

pI, isoelectric point; TMH, transmembrane helices; n.p., not predicted.

*This accession number refers to a sequence that reveals a truncated version of the protein. For calculations, the N-terminus was elongated according to 5′ upstream sequences found in the corresponding mosquito gene, which is located in region 7B of chromosome 2R.

Manduca indicated that class A chitin synthases are specifically expressed in the epidermis and related ectodermal cells such as tracheal cells, while expression of class B chitin synthases may be restricted to gut epithelial cells that produce peritrophic matrices (Y. Arakane, D. Hogenkamp, Y. C. Thu, C. A. Specht, R. W. Beeman, K. J. Kramer, M. Kanost and S. Muthukrishnan, unpublished results). In *Lucilia*, LcCHS-1, a class A chitin synthase, was found in the carcass, which is free of internal tissues, but not in the midgut (Tellam et al., 2000). In *Aedes aegypti*, RT-PCR with a probe to AaCHS-1, a class B chitin synthase, resulted in products that were detectable in midgut or whole mosquitoes but not in the carcass (Ibrahim et al., 2000). Moreover, RT-PCR that was conducted with mRNA preparations from *Manduca* using isoform-specific primers suggests that expression of class B chitin synthases is restricted, since MsCHS-2-specific products can only be observed in the midgut but not in other tissues (D. Hogenkamp and S. Muthukrishnan, personal communication; K. Gerdemann and H. Merzendorfer, unpublished). Besides homology criteria, class A insect chitin synthases are characterized by the presence of a coiled-coil region immediately following the five transmembrane helices of the C domain (Tellam et al., 2000; Y. Arakane, D. Hogenkamp, Y. C. Thu, C. A. Specht, R. W. Beeman, K. J. Kramer, M. Kanost and S. Muthukrishnan, unpublished results; Fig. 4A; Table 1). The coiled-coil region is predicted to face the extracellular space and may be involved in protein–protein interaction, vesicle fusion or oligomerization (Skehel and Wiley, 1998; Burkhard et al., 2001). Interestingly, cellulose synthases from mosses, ferns, algae and vascular plants, which have some similarities with chitin synthases, are organized in rosettes consisting of six subunits, which in turn may each contain six single polypeptides (Doblin et al., 2002). Rosette assembly may involve oxidative dimerization between single cellulose synthase polypeptide subunits *via* zinc finger domains (Kurek et al., 2002). It is therefore tempting to speculate that oligomerization may be important for chitin synthases too, possibly mediated by the coiled-coil region.

It seems that class A chitin synthases are encoded by a gene that is differentially spliced, resulting in the expression of an alternate exon comprising 59 amino acids and encoding transmembrane helix six and adjacent regions of the C domain (Tellam et al., 2000; Y. Arakane, D. Hogenkamp, Y. C. Thu, C. A. Specht, R. W. Beeman, K. J. Kramer, M. Kanost and S. Muthukrishnan, unpublished results). The alternate exons share 70%, 72% and 78% identical amino acids in TcCHS-1, DmCHS-1 and MsCHS-1, respectively. Recently, Arakane and colleagues demonstrated that both exons are actually expressed in *Tribolium* (Y. Arakane, D. Hogenkamp, Y. C. Thu, C. A. Specht, R. W. Beeman, K. J. Kramer, M. Kanost and S. Muthukrishnan, unpublished results). Although their expression pattern differs to some extent during development, the functional significance of alternate exon usage is not yet clear.

Regulation of chitin synthases

As discussed above, chitin formation and degradation are essential for insect development. Not surprisingly, malfunction of chitin synthesis leads to developmental disorders that are

already observable during embryogenesis. In *Drosophila*, it was shown that mutations in the *CHS-1* gene are allelic with *kkv (krotzkopf verkehrt)*, a gene that was originally identified in a screen for severe disruptions of the head cuticle (Ostrowski et al., 2002). Therefore, the expression of the involved genes has to be precisely controlled during each molt. Insect metamorphosis is known to be regulated by the release of ecdysone, a steroid hormone secreted into the hemolymph by the prothoracic gland, which could be involved in the regulation of chitin synthesis. For *Drosophila* imaginal disks, it has been shown that the presence of ecdysone inhibits chitin synthesis as well as the expression of procuticle proteins (Apple and Fristrom, 1991; Hiruma et al., 1991). Converse data were obtained for cultured wing discs from *Plodia*, where ecdysone stimulated uptake and incorporation of radiolabeled glucosamine (Oberlander, 1976). These opposite effects on chitin synthesis are not necessarily conflicting; rather, they may reflect a dual effect of ecdysteroids, since these steroids are known to act as both positive and negative regulators (Apple and Fristrom, 1991; Spindler et al., 2001). Upregulation of transcriptional activities by ecdysteroids during insect development was found in a number of cases. The *Drosophila* genes *Eips 28* and *Eips 29*, for instance, are controlled tissue- and stage-specifically by ecdysone-responsive elements present in the upstream and downstream flanking regions (Andres and Cherbas, 1994). Further examples of this mode of ecdysteroid-mediated transcriptional regulation are the *Drosophila* genes encoding the yolk protein (Bownes et al., 1996), the heat-shock proteins hsp23 and hsp27 (Luo et al., 1991), the caspase DRONC (Dorstyn et al., 1999; Hawkins et al., 2000) and the *Manduca* genes *EcR-A* and *EcR-B1*, which encode two ecdysone receptor (EcR) isoforms (Jindra et al., 1996). Downregulation of transcriptional activities by ecdysteroids was observed too, but these effects may be due to indirect actions. For instance, the ecdysteroid-regulated gene *esr20*, which is expressed in the trachea of *Manduca*, has been suggested to be downregulated at ecdysis. In this case, downregulation may be caused by a decline in transcript stability triggered indirectly by 20-hydroxyecdysone (Meszaros and Morton, 1997). Moreover, transcription of the dopa decarboxylase-encoding gene from *Manduca* may be indirectly suppressed by 20-hydroxyecdysone *via* an ecdysteroid-induced transcription factor that itself suppresses dopa decarboxylase transcription (Hiruma et al., 1995).

Analysis of chitin synthase expression during *Drosophila* metamorphosis indicates that ecdysone has a regulatory role on CHS-1 (DmeChSB) and CHS-2 (DmeChSA) transcript levels (Gagou et al., 2002). In third instar larvae and shortly after pupariation CHS transcripts were barley detectable. However, in response to the first ecdysone pulse, both transcripts were drastically upregulated, although at different points in time. CHS-1 transcripts were upregulated first, coinciding with the formation of pupal inner epicuticle, whereas CHS-2 transcripts were upregulated a few hours later, concurrent with pupal procuticle formation. The progression of transcript upregulation may suggest that ecdysone activates transcription of the *CHS* genes by activating a nuclear receptor heterodimer consisting of the EcR and the *Drosophila* retinoid X receptor homologue USP, the ultraspiracle protein (Yao et al., 1993). Indeed, computational scanning of the 'transfac database' revealed that both genes contain putative ecdysone responsive elements (EcREs) in their upstream regions. The regulatory elements correspond with the consensus sequences (G/T)NTCANTNN(A/C)(A/C) and (A/G)G(G/T)T(G/C) ANTG(A/C)(A/C)(C/T)(C/T), deduced from promoters of *hsp23*, *hsp27* and *Fbp1*, which encode two *Drosophila* heat-shock proteins and a fat body protein, respectively (Luo et al., 1991; Antoniewski et al., 1993; Wingender et al., 1997; Tellam et al., 2000). Somewhat different results were obtained when *MsCHS-1* expression was investigated in *Manduca* 5th instar larvae and pupae (Zhu et al., 2002). During feeding, transcript levels were observed to be relatively constant, but dropped drastically when feeding ceased and gradually increased again in the wandering stage to a maximum at pupal molt. Correlation with ecdysteroid titers in the *Manduca* hemolymph suggests that the *MsCHS-1* gene is negatively controlled by ecdysteroids, because ecdysteroid titers increase prior to wandering and decrease before pupation (Bollenbacher et al., 1981; Baker et al., 1987).

Transcriptional or post-transcriptional regulation also seems to occur for the midgut-specific chitin synthase isoform encoded by class B genes. *In situ* hybridization performed with midgut sections from the mosquito *Aedes* showed that the amount of transcripts was upregulated in response to a bloodmeal (Ibrahim et al., 2000). Interestingly, transcripts were localized to the apical region of epithelial cells. Similar results were obtained by *in situ* hybridization of cryosections from the anterior midgut of *Manduca* 5th instar larvae (Zimoch and Merzendorfer, 2002). The observed apical localization may reflect the site of CHS-2 biosynthesis because, in *Manduca*, columnar cell apical regions with large whorls of rough endoplasmic reticulum and Golgi complexes are found beneath the terminal web (Cioffi, 1979). This interpretation is also supported by the observation that in the basal region of the anterior midgut both rough endoplasmic reticulum and Golgi complexes are missing but are present in the basal region of the median and posterior midgut. Correspondingly, CHS-2 transcripts are evenly spread throughout the cytoplasm of the columnar cells in the median and posterior midgut (Zimoch and Merzendorfer, 2002). The only cell organelles that have been observed in the region of the *Manduca* columnar cells' terminal web were interpreted as small Golgi vesicles with electron-dense contents that appeared to be collected at the apical border of the cell (Cioffi, 1979). Are these vesicles loaded with chitin that will be released upon a secretory signal? In any case, inactive chitin synthases also have to be transported to the apical plasma membrane, and vesicle transport may be regulated as well. This notion may be supported by the finding that microtubule disruptans interfere with chitin synthesis (Oberlander et al., 1983).

Since insect chitin synthase activity is increased by limited proteolysis, it is tempting to speculate about the existence of a

cellular pool of inactive proenzymes being activated by specific signals. However, even in fungal systems, the significance of this phenomenon has not yet been elucidated (Merz et al., 1999a). Besides proteases, further regulatory factors that affect chitin synthase activity may exist in insects. In yeast, several proteins that are involved in the regulation of chitin synthesis have been described. Yeast CHS4p, for instance, seems to stimulate chitin synthase III (CHS3p) activity by a direct protein–protein interaction and may be needed for septin-dependent, localized chitin deposition in the yeast cell wall (Ono et al., 2000). SHC1p is a protein homologous to CHS4p and functions in cell wall ascospore assembly but regulates CHS3p activity exclusively during the sporulation process (Sanz et al., 2002). Another protein that is required for fusion and mating, CHS5p, has been implicated in regulation of chitin synthase, since chitin synthase III targeting to cortical sites in yeast is dependent on both CHS5p and the actin cytoskeleton/Myo2p (Santos and Snyder, 1997). Further proteins have been discovered by genetic screens, including CHS6p, which is necessary for the anterograde transport of CHS3p from the chitosome to the plasma membrane (Ziman et al., 1998), and CHS7p, which regulates CHS3p export from the endoplasmic reticulum (Trilla et al., 1999). So far, no orthologs have been described in insects. However, future experiments with two- or three-hybrid systems may reveal interaction partners that regulate chitin synthase activity in insects.

Chitin degradation

Chitin synthases and chitinolytic enzymes work hand in hand in remodeling chitinous structures. So far, we have discussed those enzymes that are relevant for chitin synthesis. The degrading enzymes include the chitinases {poly[1,4-(*N*-acetyl-β-D-glucosaminide)] glycanohydrolase; EC 3.2.1.14} and β-*N*-acetylglucosaminidases (β-*N*-acetyl-β-D-hexosaminide *N*-acetylhexosaminohydrolase; EC 3.2.1.52). All of them catalyze the hydrolysis of β-(1-4)-glycosidic bonds of chitin polymers and oligomers. Some of them, including one insect enzyme, additionally catalyze transglycosylation reactions (Usui et al., 1987; Fukamizo, 2000; Kondo et al., 2002). Since chitin-degrading enzymes can be used to convert chitin-containing raw material into biotechnologically utilizable components, they are of significant interest for the chemical and pharmaceutical industry. Moreover, chitinases and their inhibitors may be adopted as insecticides to combat pests or as fungicides for the treatment of microbial infections (Kramer and Muthukrishnan, 1997; Herrera-Estrella and Chet, 1999). In chitin-producing organisms, chitinolytic enzymes are essential for maintaining normal life cycle functions such as morphogenesis of arthropods or cell division and sporulation of yeast and other fungi (Passonneau and Williams, 1953; Elango et al., 1982; Kuranda and Robbins, 1991). They are also found in organisms that do not contain chitin themselves but utilize chitin as a nutrient source. Several bacterial genera, such as *Streptomyces* spp., convert insoluble chitin into soluble, metabolizable compounds by means of different chitinolytic enzymes and chitin-binding proteins that act as a glue for adherence on chitinous substrates (Charpentier and Percheron, 1983; Schrempf, 2001). Chitinase genes have been identified in the genome of the *Autographa californica* nucleopolyhedrovirus, a member of the virus family Baculoviridae, which is restricted to arthropod hosts (Hawtin et al., 1995). The baculoviral chitinase may play a crucial role in viral infectivity (Thomas et al., 2000; Saville et al., 2002). For plant and vertebrate chitinases, including those produced by human macrophages, it is proposed that they act in defense against chitin-containing pathogens or pests (Leah et al., 1991; Boot et al., 1995, 1998; Gooday, 1999; Carlini and Grossi-de-Sa, 2002).

In insects, chitin-degrading enzymes play a crucial role in postembryonic development, especially during larval molt and pupation. During the molt, proteases and chitinases are synthesized by epidermal cells and accumulate in the molting fluid between the epidermis and the old cuticle (Dziadik-Turner et al., 1981; Samuels and Reynolds, 1993; Samuels and Paterson, 1995; Reynolds and Samuels, 1996). Most of the digestion products are transported *via* the molting fluid to the mouth and anal openings and are subsequently accumulated in the midgut (Reynolds and Samuels, 1996; Yarema et al., 2000). However, direct reabsorption by the epidermis may also occur. In any case, the reincorporated constituents seem to be recycled and used to produce the new procuticle (Surholt, 1975; Reynolds and Samuels, 1996; Kaznowski et al., 1986). In addition, some larvae ingest the shed exuvia to regain its constituents. This behavior coincides with the period of chitinase expression in the gut (Kramer et al., 1993). Moreover, the midgut chitinases seem to be involved in the formation, perforation and degradation of the midgut peritrophic matrix, which protects the gut epithelium from damaging factors (Peters, 1992; Shen and Jacobs-Lorena, 1997; Filho et al., 2002). Chitinolytic enzymes are also found in some hymenopteran venoms and in the digestive fluid of spiders, where they may facilitate the entry of harmful ingredients through the cuticle of the prey (Mommsen, 1980; Krishnan et al., 1994; Jones et al., 1996). Recently, a fat body-specific chitinase that is detected in milk gland tissue and could therefore be important for the development of intrauterine larvae was characterized in the viviparous tsetse fly *Glossina morsitans* (Yan et al., 2002).

Since chitin is hard to break due to its physicochemical properties, its degradation usually requires the action of more than one enzyme type. Endo-splitting chitinases produce chito-oligomers that are subsequently converted to monomers by exo-splitting β-*N*-acetylglucosaminidases. The latter enzyme cleaves off *N*-acetylglucosamine units from non-reducing ends and prefers smaller substrates than chitinases (Koga et al., 1982, 1983, 1997; Fukamizo and Kramer, 1985a,b; Kramer and Koga, 1986; Kramer et al., 1993; Zen et al., 1996; Filho et al., 2002). As a consequence of these properties, the overall rate of chitin hydrolysis is limited by the action of the chito-oligomer-producing chitinase, which drastically increases

the effective substrate concentration for the β-*N*-acetylglucosaminidase.

The mechanism of catalysis seems to be quite similar to that postulated for the cellulase complex and other multi-enzyme systems hydrolyzing linear polymers (Easterby, 1973; Klesov and Grigorash, 1982). The first enzyme of the 'cellulosome', a multiple cellulase-containing protein complex, is an endocellulase that limits monosaccharide formation, because exocellulases are inefficient in degrading insoluble polysaccharides. In contrast to the cellulolytic enzymes, however, chitinolytic enzymes are not believed to assemble into corresponding 'chitinosomes', although evidence excluding their existence is lacking.

Interestingly, the appearance and activity of both chitinolytic enzymes seem to be in reverse order as they function in chitin degradation. In *Manduca*, the silkworm *Bombyx mori* and *Locusta*, the exo-splitting β-*N*-acetylglucosaminidase appears earlier in the molt than the endo-splitting chitinase. This was verified by activity assays and immunoblot analysis with polyclonal antibodies raised against both enzymes (Kimura, 1973a, 1977; Zielkowski and Spindler, 1978; Fukamizo and Kramer, 1987; Koga et al., 1989). Since the cuticle is a complex matrix of chitin and tightly bound proteins, enzyme accessibility is restricted, and free non-reducing ends are limited. Thus, further mechanisms of cuticle degradation exist, including degradation by proteases that are also present in the molting fluid (Law et al., 1977).

Table 2. *Characteristics of some insect chitinases*

	MsCHT-1	*BmCHT-1*	*AaCHT-1*
Organism	*Manduca sexta*	*Bombyx mori*	*Aedes aegypti*
Accession no.	A56596	AAB47538	AAB81849
Molecular mass (kDa)			
Deduced from cDNA	62.20	63.39	64.27
Zymogenic forms*	119	215	
Active forms	50, 62, 75, 97	54, 65, 88	33, 40
pI	5.32	5.15	4.83
PEST sites	404–437 474–508	417–440 471–499	394–408 413–436 451–471
Total number of *N*-glycosylation sites	3	3	2
Total number of *O*-glycosylation sites	24	23	25

Prediction of putative PEST sites and *N*- and *O*-glycosylation sites was performed with the programs PESTfind, PROSCAN and NetOGlyc 2.0, respectively (Rogers et al., 1986; Bairoch et al., 1997; Hansen et al., 1997).

*Putative zymogenic forms were suggested based on their immunoreactivity with anti-chitinase antibodies after SDS-PAGE and western blots (Koga et al., 1989, 1992).

pI, isoelectric point.

Insect chitinases

Insect chitinases belong to family 18 of the glycohydrolase superfamily and share a high degree of amino acid similarity. A characteristic of the family 18 chitinases is their multi-domain structure, which is consistently found in all primary structures deduced from insect genes encoding these enzymes. Substantial biochemical and kinetic data are available, and primary structures of different enzymes have been determined by nucleotide sequencing. Insect chitinases have theoretical molecular masses ranging between 40 kDa and 85 kDa and also vary with respect to their pH optima (pH 4–8) and isoelectric points (pH 5–7) (Table 2). The basic structure consists of three domains that include (1) the catalytic region, (2) a PEST-like region, enriched in the amino acids proline, glutamate, serine and threonine, and (3) a cysteine-rich region (Kramer and Muthukrishnan, 1997; Fig. 4B). The last two domains, however, do not seem to be necessary for chitinase activity because naturally occurring chitinases that lack these regions are still enzymatically active (Girard and Jouanin, 1999; Feix et al., 2000; Yan et al., 2002). In agreement with these observations, C-terminus-truncated versions of the recombinant *Manduca* chitinase still exhibit catalytic activity (Wang et al., 1996; Zhu et al., 2001).

In all insect chitinases sequenced so far, a hydrophobic signal peptide is predicted to precede the N-terminal region of the mature protein (Kramer et al., 1993; Koga et al., 1997; Choi et al., 1997; Nielsen et al., 1997; Shen and Jacobs-Lorena, 1997; Kim et al., 1998; Mikitani et al., 2000; Royer et al., 2002). The signal peptide presumably mediates secretion of the enzyme into the endoplasmic reticulum and it is cleaved off by signal peptidases after the protein has been transported across the membrane (von Heijne, 1990; Müller, 1992).

The catalytic domain of family 18 chitinases comprises the N-terminal half of the enzyme. It was suggested that the N-terminal part of this domain influences the binding or the hydrolysis of the substrate (Perrakis et al., 1996). Sequence alignments revealed two highly conserved regions within the catalytic domain, the second one including the catalytic center (Henrissat, 1991; Coutinho et al., 2003; see also Fig. 4B). The catalytic domain of family 18 chitinases has a TIM-barrel structure (Lasters et al., 1988) that forms a groove on the enzyme's surface. This groove is considered as the active center, which binds sugar units of chitin, possibly $(GlcNAc)_6$ moieties, that are subsequently cleaved by a retaining mechanism discussed later on (Armand et al., 1994; Drouillard et al., 1997). The hallmarks of the chitinase structure are eight parallel β-strands, forming the barrel's core, which is surrounded by eight α-helices connected to the barrel by linkers of different length and form. The two consensus sequences lie along β-strands three and four of the α/β barrel and represent the substrate-binding site (Aronson et al., 1997). So far, no crystal structure of an insect chitinase is available, but homology modeling using crystal structures of bacterial and plant chitinases has revealed three-dimensional models of the catalytic domain from the *Manduca* chitinase showing

striking similarities with the α/β barrel structure described above (Kramer and Muthukrishnan, 1997; Huang et al., 2000). Although the models lack a well defined $(\alpha/\beta)_8$ folding, they predict eight β-sheets and four complete and several incomplete α-helices. In some insects, the catalytic region is followed by a less conserved domain containing a putative PEST-like region that is also found near the C-terminus of the yeast chitinase (Kim et al., 1998; Kuranda and Robbins, 1991; Kramer et al., 1993; Royer et al., 2002). As already mentioned, insect chitinases without a PEST-like region have also been described in the literature (Girard and Jouanin, 1999; Feix et al., 2000; Yan et al., 2002). PEST-like regions presumably increase the susceptibility of the enzyme to proteolysis by a calcium-dependent protease or to degradation *via* the 26S proteasome (Rogers et al., 1986; Rechsteiner and Rogers, 1996). Therefore, these regions could play a role in enzyme turnover or activation of zymogenic chitinases.

Like some fungal chitinases, the chitinases found in insect molting fluids are extensively glycosylated. Thus, insect chitinases can be easily detected by carbohydrate staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotting. Several putative *N*-linked glycosylation sites that may be necessary for the secretion of the protein and maintenance of its stability are found within the deduced amino acid sequences of insect chitinases (Gopalakrishnan et al., 1995; Kramer and Muthukrishnan, 1997; Kim et al., 1998; Fig. 4B). Moreover, the serine/threonine-rich PEST-like region of the *Manduca* chitinase is extensively modified by *O*-glycosylation (Kramer et al., 1993; Arakane et al., 2003). Previous determination of the carbohydrate composition of the *Manduca* chitinases revealed *N*-acetylglucosamine and several neutral hexoses as part of the sugar portion (Koga et al., 1983, 1997). The attachment of oligosaccharides probably increases solubility and protects the peptide backbone against proteases.

Insect chitinases are anchored to their substrate through the C-terminal chitin-binding domain, which is characterized by a six-cysteine motif that is also found in nematode chitinases (Venegas et al., 1996). It functions in targeting of the enzyme to its substrate and thereby facilitates catalysis. The six-cysteine motif is also found in several peritrophic matrix proteins, as well as in receptors and other proteins that are involved in cellular adhesion (Tellam et al., 1992; Tellam, 1996; Kramer and Muthukrishnan, 1997; Shen and Jacobs-Lorena, 1999).

Individual chitinases possess different combinations of these three basic domains. While the chitinases from *Manduca*, *Bombyx* and fall webworm moth *Hyphantria cunea* exhibit the typical tripartite domain structure (Kramer et al., 1993; Kim et al., 1998), some other chitinases lack PEST-like or the typical chitin-binding regions (Girard and Jouanin, 1999; Feix et al., 2000; Yan et al., 2002). Other insects, in turn, may express multi-modular enzymes. In *Aedes*, for example, chitinases are encoded by two different genes. Nucleotide sequencing has revealed that one of the genes contains tandemly arranged open reading frames that encode three separate chitinases, each containing a catalytic- and also a chitin-binding domain. The gene arrangement suggests co-regulated transcription resembling bacterial operons (Niehrs and Pollet, 1999). Post-transcriptional splicing, however, may also lead to a single, multi-modular protein with three catalytic- and chitin-binding domains each (de la Vega et al., 1998; Henrissat, 1999). *TmChit5*, the gene that encodes chitinase 5 of the beetle *Tenebrio molitor*, also exhibits an unusual structure, since it contains five chitinase units of approximately 480 amino acids that are separated by putative PEST-like, chitin-binding and mucin-like domains (Royer et al., 2002). It is speculated that multi-modular chitinases may be expressed as zymogens that are subsequently cleaved by proteolysis to reveal multiple active enzymes.

The occurrence of conserved acidic residues seems to be a common characteristic for the active site of glycohydrolases (Bourne and Henrissat, 2001; Henrissat, 1990). Since the tertiary structure of family 18 chitinases is similar to that of other glycohydrolases, a common mechanism of hydrolysis involving conserved acidic amino acids was postulated (Henrissat and Bairoch, 1993). The signature sequence FDxxDxDxE is found in the active sites of family 18 chitinases, including a glutamate residue that is essential for catalysis. The highly conserved sequence YDFDGLDLDWEYP found in insect chitinases is consistent with the family 18 chitinase signature (Terwisscha van Scheltinga et al., 1994; Choi et al., 1997; de la Vega et al., 1998). Consequently, site-directed mutagenesis of the essential glutamate of the insect chitinase active site results in a loss of enzymatic activity (Huang et al., 2000; Lu et al., 2002; Royer et al., 2002). Based on crystallographic data and theoretical models, the catalytic reaction of family 18 chitinases might take place through a substrate-assisted, double displacement mechanism with a geometrically deformed oxocarbonium intermediate, more conveniently referred to as a ‘retention mechanism’ (Sinnott, 1990; Hart et al., 1995; Robertus and Monzingo, 1999). It is postulated that the active site has a binding cleft for a hexamer of *N*-acetylglucosamine. Following a convention developed for hen egg-white lysozyme, the single sugar binding sites were termed A–F (Blake et al., 1967; Kelly et al., 1979). In a first step, the sugar in binding site D is distorted to a boat conformation. Subsequently, the catalytic glutamate breaks the glycosidic bond between the sugars in sites D and E by protonation of the leaving group. This leads to a positively charged oxocarbonium intermediate that is stabilized by a covalent bond between the carboxyl oxygen of the *N*-acetyl-group and the C1 atom of the sugar. The cleaving group then leaves the active site and a water molecule enters and attacks the C1 carbon from the β-side and protonates the glutamate. This reaction results in the retention of the stereochemistry at the anomeric carbon of the product, in contrast to the inverting mechanism of family 19 chitinases (Robertus and Monzingo, 1999). The soluble products of this catalytic mechanism are chitotetraose, chitotriose and chitobiose, the latter chito-oligosaccharide being predominant (Terwisscha

van Scheltinga et al., 1995; Kramer and Muthukrishnan, 1997; Brameld et al., 1998; Robertus and Monzingo, 1999; Fukamizo, 2000; Abdel-Banat et al., 2002). The functional importance of active site residues has also been demonstrated for an insect chitinase (Lu et al., 2002). Site-directed mutagenesis of the *Manduca* chitinase revealed that E146 may function as an acid/base catalyst while D142 may influence the pK_a values of the catalytic residue E146 but also that of D144; the latter residue may be an electrostatic stabilizer of the positively charged transition state. Moreover, W145, which is also present in all family 18 chitinases, might extend the alkaline pH range in which the enzyme is active and may increase affinity to the substrate (Huang et al., 2000).

Regulation of chitin degradation

Due to the vital role of chitin in insect development, its degradation must be as tightly regulated as its synthesis. By monitoring transcripts or enzyme activities, it has been shown that the expression or activity of integumental chitinases is restricted to periods of molt and pupation whereas that of gut chitinases is induced by feeding (Koga et al., 1982, 1983, 1989, 1991, 1992; Kramer et al., 1993; Zen et al., 1996; Shen and Jacobs-Lorena, 1997; Kim et al., 1998; Filho et al., 2002). Interestingly, chitinase expression in the molting fluid is delayed compared with β-*N*-acetylglucosaminidase expression (Koga et al., 1991; Kramer et al., 1993). In epidermal cells, 20-hydroxyecdysone stimulates the secretion of the molting fluid containing chitinolytic enzymes and proteases, indicating that this hormone may also be involved in regulating enzyme expression and activity (Reynolds and Samuels, 1996).

In the course of the molting process, the ecdysteroid titer increases continuously and reaches its maximum shortly before apolysis (Bollenbacher et al., 1981; Riddiford, 1994). Juvenile hormone allows larval molting in response to ecdysteroids but prevents the switching of gene expression necessary for metamorphosis (Riddiford, 1996). Therefore, the delayed chitinase expression may be caused by differential sensing of hormone titers during molting. Both secretion and activation of chitinolytic enzymes are clearly controlled by ecdysteroids (Reynolds and Samuels, 1996). Concordantly, Kimura (1973b) showed some 30 years ago that the activity of molting fluid enzymes can be stimulated by ecdysteroid injections.

The chitinases of *Bombyx* and *Manduca* are induced at high hemolymph levels of 20-hydroxyecdysone, while β-*N*-acetylglucosaminidase is already induced at low levels of the steroid (Fukamizo and Kramer, 1987; Koga et al., 1991, 1992; Kramer et al., 1993; Zen et al., 1996). As a consequence, β-*N*-acetylglucosaminidase expression was found to start before that of the chitinase. Although it is obvious that the expression of chitinolytic enzymes is hormonally coordinated, responsive elements that would affect gene transcription have not been identified so far. Nevertheless, the chitinase may be an early responsive gene and a direct target of the ecdysone receptor because protein synthesis is not required for its induction (Royer et al., 2002).

Degradation of cuticles by chitinolytic enzymes certainly needs the assistance of molting fluid proteases to degrade proteinaceous components (Law et al., 1977). However, these proteases may also function in the proteolytic activation of inactive chitinase precursors, as was suggested for *Manduca* or *Tenebrio* (Kramer et al., 1993; Samuels and Reynolds, 1993). Proteolytic activation of chitinases may also occur in insect gut systems, where, in the case of blood-sucking insects, the activities of gut chitinase and β-*N*-acetylglucosaminidase are found to rise shortly after feeding (Filho et al., 2002). Indeed, the gut-specific chitinase of *Anopheles* was shown to be secreted into the gut lumen as an inactive pro-enzyme that needs to be trypsinized in order to develop chitinolytic activity (Shen and Jacobs-Lorena, 1997). Interestingly, the malaria parasite *Plasmodium* also utilizes the protease-rich environment of the mosquito midgut to increase the enzymatic activity of its own chitinase, which facilitates penetration of the peritrophic matrix (Shahabuddin et al., 1993).

Proteolytic activation of chitinases has been extensively investigated in *Manduca* and *Bombyx*. A probable zymogenic form of the chitinase with a molecular mass of 215 kDa was observed during the spinning period of *Bombyx*. Two to three days later, when enzyme activity is detectable, three active fragments of 88 kDa, 65 kDa and 54 kDa appear, which may be the result of successive, proteolytic processing (Koga et al., 1989, 1992, 1997; Abdel-Banat et al., 1999). The three active forms have a common N-terminal sequence, indicating that they differ in length at the C-terminus. It was suggested that the 88 kDa enzyme still contains a potent chitin-binding domain. This domain, however, may get gradually lost by further proteolysis from the C-terminal side, resulting in chitinase variants that are more active on shorter substrates. Consistent with this, when the chitin-binding domain is attached to the catalytic domain, the resulting recombinant fusion protein exhibits increased activity towards the insoluble polymer but not towards the soluble chitin oligosaccharide (Arakane et al., 2003). Thus, it seems that the domain structure of insect chitinases has evolved to optimize degradation of insoluble polysaccharides to soluble oligosaccharides, thereby accelerating the overall chitin degradation rate in addition to the presence of β-*N*-acetylglucosaminidases (Koga et al., 1997; Abdel-Banat et al., 1999).

Similar results were observed in the *Manduca* integument, where a 119 kDa protein might be interpreted as a zymogenic precursor and several smaller proteins as proteolytically activated fragments (Koga et al., 1992). In general, studies on the zymogenic nature of insect chitinases are complicated by the observed discrepancies between theoretical molecular masses deduced from obviously complete cDNA sequences and apparent molecular masses estimated from SDS-PAGE. Since these discrepancies cannot be explained by post-translational processing, it has been concluded that at least some insect chitinases may exhibit an anomalous electrophoretic migration behavior (Kramer et al., 1993).

The chitinase PEST sequences could possibly enhance the activation of chitinase zymogens (Royer et al., 2002). Consistent with this, activity of the fat body-specific chitinase from the tsetse fly, which lacks a PEST-like region, is not increased by trypsinization (Yan et al., 2002). In contrast to chitinases, the exo-cleaving β-*N*-acetylglucosaminidases of *Manduca* and *Bombyx* do not seem to be zymogens (Koga et al., 1991, 1992, 1997).

Inhibition of chitin metabolism

Since chitin metabolism is crucial for fungal and arthropod development, inhibition or deregulation of the key enzymes are important objectives for the development of fungicides and insecticides, including anti-malarial agents, not least because chitin polymers are absent in vertebrates.

Inhibitors of chitin synthesis have been classified into three major groups: peptidyl nucleosides, acyl ureas and substances interfering with hormonal control. Peptidyl nucleosides isolated from diverse *Streptomyces* species act as substrate analogues and include polyoxins and nikkomycins (Zhang and Miller, 1999). They competitively inhibit both fungal and insect chitin synthases. It is believed that inhibition occurs *via* binding to the catalytic site (Ruiz-Herrera and San-Blas, 2003). Polyoxins have found some applications in the control of phytopathogens, whereas the commercial application of nikkomycins is pending, although they seem to be more potent inhibitors than polyoxins (Cohen and Casida, 1980a; Zhang and Miller, 1999; Tellam et al., 2000). Generally, the application of peptidyl nucleosides is complicated by low permeability, hydrolytic lability, varying susceptibility of fungal species and the multitude of responses found in animals (Zhang and Miller, 1999; Ruiz-Herrera and San-Blas, 2003).

In contrast to the peptidyl nucleosides, acyl ureas play an important role in integrated pest management. Although it is well established that acyl ureas such as diflubenzuron and teflubenzuron affect chitin synthesis (Post et al., 1974; van Eck, 1979), their mode of action is still puzzling. However, several lines of experiment argue against a direct interaction of these inhibitors with the chitin synthase. For instance, in cell-free systems, acyl ureas do not inhibit chitin synthesis (Cohen and Casida, 1980a; Mayer et al., 1981; Cohen, 1985). Instead of directly blocking chitin synthase activity, they may alter either vesicle transport or fusion, inhibit the translocation of chitin fibrils across the plasma membrane (Nakagawa and Matsumura, 1994; Cohen, 2001) or interfere with the hormonal regulation of chitin synthesis by influencing ecdysteroid production (Fournet et al., 1995).

The third group of inhibitors evidently affects hormonal regulation of insect growth and development. One of the manifold effects of these substances is certainly deregulation of chitin synthesis, probably by preventing the expression of the chitin synthase or regulating factors. Hormonal regulation can already be disturbed at the level of hormone biosynthesis. Some synthetic imidazole and cholesterol derivates have been shown to prevent ecdysteroid biosynthesis (Kadano-Okuda et al., 1987; Roussel, 1994; Lorenz et al., 1995). By contrast, the heterocyclic compound brevioxime and the alkaloid arborine show significant blocking of juvenile hormone synthesis (Moya et al., 1997; Muthukrishnan et al., 1999). The auxiliary application of isolated molting hormones or their synthetic agonists and antagonists leads to abnormalities in insect development as well. The ecdysteroid agonist tebufenozide manifests its effect by interacting with the ecdysone receptor, the juvenile hormone agonists fenoxycarb, methoprene and pyriproxyfen mimic the hormone action, and the juvenile hormone antagonists precocene I and II act *via* their cytotoxicities on the corpora allata (Schooneveld, 1979; Mulla et al., 1985; Dhadialla et al., 1998; Hoffmann and Lorenz, 1998; Kostyukovsky et al., 2000; Retnakaran et al., 2001).

Chitinase inhibitors can generally be grouped into two major classes: they mimic either carbohydrate substrates or the oxocarbonium reaction intermediate of family 18 chitinases. The most-studied chitinase inhibitor is allosamidin, a pseudotrisaccharide. It was isolated from the mycelium of *Streptomyces* sp. and exhibits a strong inhibitory activity against family 18 chitinases of insects and fungi with a K_i in the nano- to micromolar range (Sakuda et al., 1987; Blattner et al., 1996; Berecibar et al., 1999). Most strikingly, it blocks malaria parasite transmission by inhibiting the chitinase of *Plasmodium* that is essential to penetrate the host's peritrophic matrix (Shahabuddin et al., 1993; Tsai et al., 2001; Filho et al., 2002). The structural basis of interactions between the inhibitor and several family 18 chitinases has been solved by x-ray crystallography (Terwisscha van Scheltinga et al., 1995; van Aalten et al., 2001). As a result of these studies, it was proposed that allosamidin mimics the catalytic transition state. Allosamidin consists of two *N*-acetylallosamine sugars linked to an allosamizoline that may resemble the catalytic intermediate and cannot be hydrolyzed because it lacks the pyranose oxygen (Bortone et al., 2002; Fusetti et al., 2002; Rao et al., 2003).

Allosamidin is a potent chitinase inhibitor; however, its production is expensive because it is difficult to synthesize. A new, alternative class of inhibitors includes the cyclopentapeptides argifin and argadin. These molecules are as potent inhibitors as allosamidin but synthesis by peptide chemistry is less expensive (Arai et al., 2000; Omura et al., 2000; Houston et al., 2002b). While the cyclopentapeptides are carbohydrate mimics, the small peptide CI-4, which was recently identified in the marine bacterium *Pseudomonas*, functions like allosamidin as a mimic of the family 18 chitinases' catalytic transient state (Izumida et al., 1996; Houston et al., 2002a).

Conclusion and outlook

The use of insecticides is indispensable for the control of a wide range of crop, public hygiene, amenity and veterinary pests. However, efficacy of commercially applied insecticides is increasingly compromised by the occurrence of resistance,

which leads to compound detoxification or altered target sites. To overcome problems arising from insufficient pest control in the long term, novel compounds acting on new target sites have to be identified. As we have outlined in this review, chitin synthesis and degradation are crucial for insect growth and development and are thus ideal targets for disruption and perturbation by novel insecticides. Despite all new insights resulting from cDNA cloning and sequencing, however, we are still far away from understanding the detailed modes of action of the enzymes involved, especially chitin synthases and chitinases. Moreover, we do not actually know the regulatory mechanisms that control and coordinate enzyme biosynthesis and activity during development. One major problem in studying insect chitin synthases is that we still lack homogenous enzyme preparations from native sources, possibly due to intrinsic instability. Heterologous expression and site-directed mutagenesis of chitin synthase or its domains in baculovirus-infected insect cells may pave the way for a better understanding of this enzyme. This strategy has already provided new insights into the domain structure and the role of highly conserved amino acid residues of insect chitinases (Zhu et al., 2001; Lu et al., 2002; Zhang et al., 2002). Moreover, recombinant baculoviruses expressing mutant insect chitinase genes may themselves constitute potent biocides since they could perturb peritrophic matrix function of infected insects, leading to enhanced susceptibility to other harmful compounds. Insect chitinases have even been alienated to induce insect resistance in plants by expressing a truncated but active version of the enzyme in transgenic tobacco (Ding et al., 1998). Plants expressing an insect chitinase gene may have agronomic potential for insect control. Heterologous expression and subsequent purification may also lead to insect enzyme preparations that are suitable for crystallization to obtain three-dimensional structures of the chitin synthase catalytic domain or the chitinase, the latter already having been successfully crystallized from different non-insect sources (Vorgias et al., 1992; Hart et al.,1992, 1995; Rao et al., 2003).

So far, no cellular interaction partners for chitin synthases or chitinases are known in insects. Since protein–protein interactions are presumably essential for the regulation of enzyme biosynthesis, targeting and activity, identification of interacting proteins would provide new insights into cellular control mechanisms. To obtain first clues, the application of yeast two- or three-hybrid systems may yield putative binding partners, which have to be further analyzed regarding their binding capability by biochemical or cytological methods. Those proteins that interact either with chitin synthases or chitinases may again turn out to be suitable target sites for future biocides. In conclusion, understanding of the basic principles underlying insect chitin metabolism and its regulation will open up new vistas in pest management.

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References

Abdel-Banat, B. M., Kameyama, Y., Yoshioka, T. and Koga, D. (1999). Purification and characterization of a 54 kDa chitinase from *Bombyx mori*. *Insect Biochem. Mol. Biol.* **29**, 537-547.

Abdel-Banat, B. M., Zhou, W., Karasuda, S. and Koga, D. (2002). Analysis of hydrolytic activity of a 65-kDa chitinase from the silkworm, *Bombyx mori*. *Biosci. Biotechnol. Biochem.* **66**, 1119-1122.

Andersen, S. O. (1979). Biochemistry of the insect cuticle. *Annu. Rev. Entomol.* **24**, 29-61.

Andersen, S. O., Hojrup, P. and Roepstorff, P. (1995). Insect cuticular proteins. *Insect Biochem. Mol. Biol.* **25**, 153-176.

Andersen, S. O. and Weis-Fogh, T. (1964). Resilin. A rubberlike protein in arthropod cuticle. *Adv. Insect Physiol.* **2**, 1-66.

Andres, A. J. and Cherbas, P. (1994). Tissue-specific regulation by ecdysone: distinct patterns of Eip28/29 expression are controlled by different ecdysone response elements. *Dev. Genet.* **15**, 320-331.

Antoniewski, C., Laval, M. and Lepesant, J. A. (1993). Structural features critical to the activity of an ecdysone receptor binding site. *Insect Biochem. Mol. Biol.* **23**, 105-114.

Apple, R. T. and Fristrom, J. W. (1991). 20-Hydroxyecdysone is required for, and negatively regulates, transcription of *Drosophila* pupal cuticle protein genes. *Dev. Biol.* **146**, 569-582.

Arai, N., Shiomi, K., Yamaguchi, Y., Masuma, R., Iwai, Y., Turberg, A., Kolbl, H. and Omura, S. (2000). Argadin, a new chitinase inhibitor, produced by *Clonostachys* sp. FO-7314. *Chem. Pharm. Bull.* **48**, 1442-1446.

Arakane, Y., Zhu, Q., Matsumiya, M., Muthukrishnan, S. and Kramer, K. J. (2003). Properties of catalytic, linker and chitin-binding domains of insect chitinase. *Insect Biochem. Mol. Biol.* **33**, 631-648.

Armand, S., Tomita, H., Heyraud, A., Gey, C., Watanabe, T. and Henrissat, B. (1994). Stereochemical course of the hydrolysis reaction catalyzed by chitinases A1 and D from Bacillus circulans WL-12. *FEBS Lett.* **343**, 177-180.

Aronson, N. N., Blanchard, C. J. and Madura, J. D. (1997). Homology modeling of glycosyl hydrolase family 18 enzymes and proteins. *J. Chem. Inf. Comput. Sci.* **37**, 999-1005.

Bade, M. L. (1983). Chitin biosynthesis: does it involve a lipid bound intermediate. *J. Appl. Polym. Sci.* **37**, 165-178.

Bairoch, A., Bucher, P. and Hofmann, K. (1997). The PROSITE database, its status in 1997. *Nucleic Acids Res.* **25**, 217-221.

Baker, F. C., Tsai, L. W., Reuter, C. C. and Schooley, D. A. (1987). *In vivo* fluctuation of JH, JH acid, and ecdysteroid titer, and JH esterase activity during development of fifth stadium *Manduca sexta*. *Insect Biochem.* **17**, 989-996.

Bakkers, J., Kijne, J. W. and Spaink, H. P. (1999). Function of chitin oligosaccharides in plant and animal development. *EXS* **87**, 71-83.

Berecibar, A., Grandjean, C. and Siriwardena, A. (1999). Synthesis and biological activity of natural aminocyclopentitol glycosidase inhibitors: mannostatins, trehazolin, allosamidins, and their analogues. *Chem. Rev.* **99**, 779-844.

Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M. and Kim, P. S. (1995). Predicting coiled coils by use of pairwise residue correlations. *Proc. Natl. Acad. Sci. USA* **92**, 8259-8263.

Binnington, K. C. (1985). Ultrastructural changes in the cuticle of the sheep blowfly, *Lucilia*, induced by certain insecticides and biological inhibitors. *Tissue Cell* **17**, 131-140.

Blake, C. C., Johnson, L. N., Mair, G. A., North, A. C., Phillips, D. C. and Sarma, V. R. (1967). Crystallographic studies of the activity of hen egg-white lysozyme. *Proc. R. Soc. Lond. B Biol. Sci.* **167**, 378-388.

Blattner, R., Furneaux, R. H. and Lynch, G. P. (1996). Synthesis of allosamidin analogues. *Carbohydr Res.* **294**, 29-39.

Bollenbacher, W. E., Smith, S. L., Goodman, W. and Gilbert, L. I. (1981). Ecdysteroid titer during larval–pupal–adult development of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* **44**, 302-306.

Boot, R. G., Renkema, G. H., Strijland, A., van Zonneveld, A. J. and Aerts, J. M. (1995). Cloning of a cDNA encoding chitotriosidase, a human chitinase produced by macrophages. *J. Biol. Chem.* **270**, 26252-26256.

Boot, R. G., Renkema, G. H., Verhoek, M., Strijland, A., Bliek, J., de Meulemeester, T. M., Mannens, M. M. and Aerts, J. M. (1998). The human chitotriosidase gene. Nature of inherited enzyme deficiency. *J. Biol. Chem.* **273**, 25680-25685.

Bortone, K., Monzingo, A. F., Ernst, S. and Robertus, J. D. (2002). The structure of an allosamidin complex with the *Coccidioides immitis* chitinase defines a role for a second acid residue in substrate-assisted mechanism. *J. Mol. Biol.* **320**, 293-302.

Bouligand, Y. (1972). Twisted fibrous arrangements in biological materials and cholesteric mesophases. *Tissue Cell* **4**, 189-217.

Bourne, Y. and Henrissat, B. (2001). Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr. Opin. Struct. Biol.* **11**, 593-600.

Bownes, M., Ronaldson, E. and Mauchline, D. (1996). 20-Hydroxyecdysone, but not juvenile hormone, regulation of yolk protein gene expression can be mapped to cis-acting DNA sequences. *Dev. Biol.* **173**, 475-489.

Bracker, C. E., Ruiz-Herrera, J. and Bartnicki-Garcia, S. (1976). Structure and transformation of chitin synthetase particles (chitosomes) during microfibril synthesis *in vitro*. *Proc. Natl. Acad. Sci. USA* **73**, 4570-4574.

Brameld, K. A., Shrader, W. D., Imperiali, B. and Goddard, W. A., III (1998). Substrate assistance in the mechanism of family 18 chitinases: theoretical studies of potential intermediates and inhibitors. *J. Mol. Biol.* **280**, 913-923.

Breton, C., Mucha, J. and Jeanneau, C. (2001). Structural and functional features of glycosyltransferases. *Biochimie* **83**, 713-718.

Burkhard, P., Stetefeld, J. and Strelkov, S. V. (2001). Coiled coils: a highly versatile protein folding motif. *Trends Cell Biol.* **11**, 82-88.

Candy, D. J. and Kilby, B. A. (1962). Studies on chitin synthesis in the desert locust. *J. Exp. Biol.* **39**, 129-140.

Carlini, C. R. and Grossi-de-Sa, M. F. (2002). Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides. *Toxicon* **40**, 1515-1539.

Carlson, J. R. and Bentley, D. (1977). Ecdysis: neural orchestration of a complex behavioral performance. *Science* **195**, 1006-1008.

Cecchelli, R., Cacan, R. and Verbert, A. (1986). Mechanism of UDP-sugar transport into intracellular vesicles. Occurrence of UDP-GlcNAc/UDP and UDP-Gal/UDP antiports. *FEBS Lett.* **208**, 407-412.

Chang, R., Yeager, A. R. and Finney, N. S. (2003). Probing the mechanism of a fungal glycosyltransferase essential for cell wall biosynthesis. UDP-chitobiose is not a substrate for chitin synthase. *Org. Biomol. Chem.* **1**, 39-41.

Charpentier, M. and Percheron, F. (1983). The chitin-degrading enzyme system of a *Streptomyces* species. *Int. J. Biochem.* **15**, 289-292.

Choi, H. K., Choi, K. H., Kramer, K. J. and Muthukrishnan, S. (1997). Isolation and characterization of a genomic clone for the gene of an insect molting enzyme, chitinase. *Insect Biochem. Mol. Biol.* **27**, 37-47.

Cioffi, M. (1979). The morphology and fine structure of the larval midgut of a moth (*Manduca sexta*) in relation to active ion transport. *Tissue Cell* **11**, 467-479.

Cohen, E. (1982). *In vitro* chitin synthesis in an insect: formation and structure of microfibrils. *Eur. J. Cell Biol.* **26**, 289-294.

Cohen, E. (1985). Chitin synthetase activity and inhibition in different insect microsomal preparations. *EXS* **41**, 470-472.

Cohen, E. (2001). Chitin synthesis and inhibition: a revisit. *Pest Manag. Sci.* **57**, 946-950.

Cohen, E. and Casida, J. E. (1980a). Inhibition of *Tribolium* gut chitin synthetase. *Pestic. Biochem. Physiol.* **13**, 129-136.

Cohen, E. and Casida, J. E. (1980b). Properties of *Tribolium* gut chitin synthetase. *Pestic. Biochem. Physiol.* **13**, 121-128.

Cohen, E. and Casida, J. E. (1982). Properties and inhibition of insect integumental chitin synthetase. *Pestic. Biochem. Physiol.* **17**, 301-306.

Coutinho, P. M., Deleury, E., Davies, G. J. and Henrissat, B. (2003). An evolving hierarchical family classification for glycosyltransferases. *J. Mol. Biol.* **328**, 307-317.

Dahn, U., Hagenmaier, H., Hohne, H., Konig, W. A., Wolf, G. and Zahner, H. (1976). Stoffwechselprodukte von Mikroorganismen. 154. Mitteilung. Nikkomycin, ein neuer Hemmstoff der Chitinsynthese bei Pilzen. *Arch. Microbiol.* **107**, 143-160.

de la Vega, H., Specht, C. A., Liu, Y. and Robbins, P. W. (1998). Chitinases are a multi-gene family in *Aedes*, *Anopheles* and *Drosophila*. *Insect Mol. Biol.* **7**, 233-239.

DeAngelis, P. L., Yang, N. and Weigel, P. H. (1994). The *Streptococcus pyogenes* hyaluronan synthase: sequence comparison and conservation among various group A strains. *Biochem. Biophys. Res. Commun.* **199**, 1-10.

Dhadialla, T. S., Carlson, G. R. and Le, D. P. (1998). New insecticides with ecdysteroidal and juvenile hormone activity. *Annu. Rev. Entomol.* **43**, 545-569.

Ding, X., Gopalakrishnan, B., Johnson, L. B., White, F. F., Wang, X., Morgan, T. D., Kramer, K. J. and Muthukrishnan, S. (1998). Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic Res.* **7**, 77-84.

Doblin, M. S., Kurek, I., Jacob-Wilk, D. and Delmer, D. P. (2002). Cellulose biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol.* **43**, 1407-1420.

Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA* **96**, 4307-4312.

Drouillard, S., Armand, S., Davies, G. J., Vorgias, C. E. and Henrissat, B. (1997). *Serratia marcescens* chitobiase is a retaining glycosidase utilizing substrate acetamido group participation. *Biochem. J.* **328**, 945-949.

Duran, A., Bowers, B. and Cabib, E. (1975). Chitin synthetase zymogen is attached to the yeast plasma membrane. *Proc. Natl. Acad. Sci. USA* **72**, 3952-3955.

Duran, A. and Cabib, E. (1978). Solubilization and partial purification of yeast chitin synthetase. Confirmation of the zymogenic nature of the enzyme. *J. Biol. Chem.* **253**, 4419-4425.

Dziadik-Turner, C., Koga, D., Mai, M. S. and Kramer, K. J. (1981). Purification and characterization of two β-*N*-acetylhexosaminidases from the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera:Sphingidae). *Arch. Biochem. Biophys.* **212**, 546-560.

Easterby, J. S. (1973). Coupled enzyme assays: a general expression for the transient. *Biochim. Biophys. Acta* **293**, 552-558.

Elango, N., Correa, J. U. and Cabib, E. (1982). Secretory character of yeast chitinase. *J. Biol. Chem.* **257**, 1398-1400.

Feix, M., Gloggler, S., Londershausen, M., Weidemann, W., Spindler, K. D. and Spindler-Barth, M. (2000). A cDNA encoding a chitinase from the epithelial cell line of C*hironomus tentans* (Insecta, diptera) and its functional expression. *Arch. Insect Biochem. Physiol.* **45**, 24-36.

Filho, B. P., Lemos, F. J., Secundino, N. F., Pascoa, V., Pereira, S. T. and Pimenta, P. F. (2002). Presence of chitinase and beta-*N*-acetylglucosaminidase in the *Aedes aegypti*: a chitinolytic system involving peritrophic matrix formation and degradation. *Insect Biochem. Mol. Biol.* **32**, 1723-1729.

Florez-Martinez, A., Lopez-Romero, E., Martinez, J. P., Bracker, C. E., Ruiz-Herrera, J. and Bartnicki-Garcia, S. (1990). Protein composition of purified chitosomes of *Mucor rouxii*. *Exp. Mycol.* **14**, 160-168.

Fournet, F., Sannier, C., Moriniere, M., Porcheron, P. and Monteny, N. (1995). Effects of two insect growth regulators on ecdysteroid production in *Aedes aegypti* (Diptera: Culicidae). *J. Med. Entomol.* **32**, 588-593.

Fristrom, J. W., Doctor, J., Fristrom, D. K., Logan, W. R. and Silvert, D. J. (1982). The formation of the pupal cuticle by *Drosophila* imaginal discs *in vitro*. *Dev. Biol.* **91**, 337-350.

Fukamizo, T. (2000). Chitinolytic enzymes: catalysis, substrate binding, and their application. *Curr. Protein Pept. Sci.* **1**, 105-124.

Fukamizo, T. and Kramer, K. J. (1985a). Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. *Insect Biochem.* **15**, 141-145.

Fukamizo, T. and Kramer, K. J. (1985b). Mechanism of chitin oligosaccharide hydrolysis by the binary enzyme chitinase system in insect moulting fluid. *Insect Biochem.* **15**, 1-7.

Fukamizo, T. and Kramer, K. J. (1987). Effect of 20-hydroxyecdysone on chitinase and β-*N*-acetylglucosaminidase during the larval–pupal transformation of *Manduca sexta* (L.). *Insect Biochem.* **17**, 547-550.

Fusetti, F., von Moeller, H., Houston, D., Rozeboom, H. J., Dijkstra, B. W., Boot, R. G., Aerts, J. M. and van Aalten, D. M. (2002). Structure of human chitotriosidase. Implications for specific inhibitor design and function of mammalian chitinase-like lectins. *J. Biol. Chem.* **277**, 25537-25544.

Gagou, M. E., Kapsetaki, M., Turberg, A. and Kafetzopoulos, D. (2002). Stage-specific expression of the chitin synthase *DmeChSA* and *DmeChSB* genes during the onset of *Drosophila* metamorphosis. *Insect Biochem. Mol. Biol.* **32**, 141-146.

Geremia, R. A., Mergaert, P., Geelen, D., Van Montagu, M. and Holsters,

M. (1994). The NodC protein of *Azorhizobium caulinodans* is an *N*-acetylglucosaminyltransferase. *Proc. Natl. Acad. Sci. USA* **91**, 2669-2673.
Girard, C. and Jouanin, L. (1999). Molecular cloning of a gut-specific chitinase cDNA from the beetle *Phaedon cochleariae*. *Insect Biochem. Mol. Biol.* **29**, 549-556.
Giraud-Guille, M. M. and Bouligand, Y. (1986). Chitin–protein molecular organization in arthropods. In *Chitin in Nature and Technology* (ed. R. Muzzarelli, C. Jeuniaux and G. W. Gooday), pp. 29-35. New York: Plenum Press.
Glaser, L. and Brown, D. H. (1957). The synthesis of chitin in cell-free extracts of *Neurospora crassa*. *J. Biol. Chem.* **228**, 729-742.
Gooday, G. W. (1972). The effect of polyoxin D on morphogenesis in *Coprinus cinereus*. *Biochem. J.* **129**, 17P-18P.
Gooday, G. W. (1999). Agressive and defensive roles for chitinases. In *Chitin and Chitinases* (ed. R. A. A. Muzzarelli and P. Jolles), pp. 157-169. Basel: Birkhäuserverlag.
Gopalakrishnan, B., Muthukrishnan, S. and Kramer, K. J. (1995). Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem. Mol. Biol.* **25**, 255-265.
Hansen, J. E., Lund, O., Rapacki, K. and Brunak, S. (1997). O-GLYCBASE version 2.0: a revised database of O-glycosylated proteins. *Nucleic Acids Res.* **25**, 278-282.
Hardy, J. C. and Gooday, G. W. (1983). Stability and zymogenic nature of chitin synthase from *Candida albicans*. *Curr. Microbiol.* **9**, 51-54.
Hart, P. J., Ready, M. P. and Robertus, J. D. (1992). Crystallization of an endochitinase from *Hordeum vulgare* L. seeds. *J. Mol. Biol.* **225**, 565-567.
Hart, P. J., Pfluger, H. D., Monzingo, A. F., Hollis, T. and Robertus, J. D. (1995). The refined crystal structure of an endochitinase from *Hordeum vulgare* L. seeds at 1.8 Å resolution. *J. Mol. Biol.* **248**, 402-413.
Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y. and Hay, B. A. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem.* ***275***, 27084-27093.
Hawtin, R. E., Arnold, K., Ayres, M. D., Zanotto, P. M., Howard, S. C., Gooday, G. W., Chappell, L. H., Kitts, P. A., King, L. A. and Possee, R. D. (1995). Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology* **212**, 673-685.
Heifetz, A., Keenan, R. W. and Elbein, A. D. (1979). Mechanism of action of tunicamycin on the UDP-GlcNAc:dolichyl-phosphate Glc-NAc-1-phosphate transferase. *Biochemistry* **18**, 2186-2192.
Henrissat, B. (1990). Weak sequence homologies among chitinases detected by clustering analysis. *Protein Seq. Data Anal.* **3**, 523-526.
Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **280**, 309-316.
Henrissat, B. (1999). Classification of chitinases modules. *EXS* **87**, 137-156.
Henrissat, B. and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* **293**, 781-788.
Hernandez, J., Lopez-Romero, E., Cerbon, J. and Ruiz-Herrera, J. (1981). Lipid analysis of chitosomes, chitin synthesizing microvesicles from *Mucor rouxii*. *Exp. Mycol.* **5**, 349-356.
Herrera-Estrella, A. and Chet, I. (1999). Chitinases in biological control. *EXS* **87**, 171-184.
Hiruma, K., Carter, M. S. and Riddiford, L. M. (1995). Characterization of the dopa decarboxylase gene of *Manduca sexta* and its suppression by 20-hydroxyecdysone. *Dev. Biol.* **169**, 195-209.
Hiruma, K., Hardie, J. and Riddiford, L. M. (1991). Hormonal regulation of epidermal metamorphosis *in vitro*: control of expression of a larval-specific cuticle gene. *Dev. Biol.* **144**, 369-378.
Hoffmann, K. H. and Lorenz, M. W. (1998). Recent advances on hormones in insect pest control. *Phytoparasitica* **26**, 323-330.
Hojrup, P., Andersen, S. O. and Roepstorff, P. (1986). Isolation, characterization, and *N*-terminal sequence studies of cuticular proteins from the migratory locust, *Locusta migratoria*. *Eur. J. Biochem.* **154**, 153-159.
Hopkins, T. L. and Harper, M. S. (2001). Lepidopteran peritrophic membranes and effects of dietary wheat germ agglutinin on their formation and structure. *Arch. Insect Biochem. Physiol.* **47**, 100-109.
Horsch, M., Mayer, C. and Rast, D. M. (1996). Stereochemical requirements of chitin synthase for ligand binding at the allosteric site for *N*-acetylglucosamine. *Eur. J. Biochem.* **237**, 476-482.
Horst, M. N. (1983). The biosynthesis of crustacean chitin. Isolation and characterization of polyprenol-linked intermediates from brine shrimp microsomes. *Arch. Biochem. Biophys.* **223**, 254-263.
Horst, M. N. and Walker, A. N. (1993). Crustacean chitin synthesis and the role of the Golgi apparatus: *in vivo* and *in vitro* studies. In *Chitin Enzymology*, vol. 1 (ed. R. A. A. Muzzarelli), pp. 109-118. Atec: Grottammare.
Houston, D. R., Eggleston, I., Synstad, B., Eijsink, V. G. and van Aalten, D. M. (2002a). The cyclic dipeptide CI-4 [cyclo-(l-Arg-d-Pro)] inhibits family 18 chitinases by structural mimicry of a reaction intermediate. *Biochem. J.* **368**, 23-27.
Houston, D. R., Shiomi, K., Arai, N., Omura, S., Peter, M. G., Turberg, A., Synstad, B., Eijsink, V. G. and van Aalten, D. M. (2002b). High-resolution structures of a chitinase complexed with natural product cyclopentapeptide inhibitors: mimicry of carbohydrate substrate. *Proc. Natl. Acad. Sci. USA* **99**, 9127-9132.
Huang, X., Zhang, H., Zen, K. C., Muthukrishnan, S. and Kramer, K. J. (2000). Homology modeling of the insect chitinase catalytic domain–oligosaccharide complex and the role of a putative active site tryptophan in catalysis. *Insect Biochem. Mol. Biol.* **30**, 107-117.
Ibrahim, G. H., Smartt, C. T., Kiley, L. M. and Christensen, B. M. (2000). Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*. *Insect Biochem. Mol. Biol.* **30**, 1213-1222.
Izumida, H., Imamura, N. and Sano, H. (1996). A novel chitinase inhibitor from a marine bacterium, *Pseudomonas* sp. *J. Antibiot.* **49**, 76-80.
Jaworski, E., Wang, L. and Margo, G. (1963). Synthesis of chitin in cell-free extracts of *Prodenia eridania*. *Nature* **198**, 790.
Jindra, M., Malone, F., Hiruma, K. and Riddiford, L. M. (1996). Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **180**, 258-272.
Jones, D., Wache, S. and Chhokar, V. (1996). Toxins produced by arthropod parasites: salivary gland proteins of human body lice and venom proteins of chelonine wasps. *Toxicon* **34**, 1421-1429.
Kadano-Okuda, K., Kuwano, E., Eto, M. and Yamashita, O. (1987). Inhibitory action of an imidazole compound on ecdysone synthesis in prothoracic glands of the silkworm, *Bombyx mori*. *Growth Diff.* **29**, 527-533.
Kang, M. S., Elango, N., Mattia, E., Au-Young, J., Robbins, P. W. and Cabib, E. (1984). Isolation of chitin synthetase from *Saccharomyces cerevisiae*. Purification of an enzyme by entrapment in the reaction product. *J. Biol. Chem.* **259**, 14966-14972.
Kaznowski, C., Schneiderman, H. A. and Bryant, P. J. (1986). The incorporation of precursors into *Drosophila* larval cuticle. *J. Insect Physiol.* **32**, 133-142.
Keller, F. A. and Cabib, E. (1971). Chitin and yeast budding. Properties of chitin synthetase from *Saccharomyces carlsbergensis*. *J. Biol. Chem.* **246**, 160-166.
Kelly, J. A., Sielecki, A. R., Sykes, B. D., James, M. N. and Phillips, D. C. (1979). X-ray crystallography of the binding of the bacterial cell wall trisaccharide NAM-NAG-NAM to lysozyme. *Nature* **282**, 875-578.
Kenchington, W. (1976). Adaption of insect peritrophic membranes to form cocoon fabrics. In *The Insect Integument* (ed. H. R. Hepburn), pp. 497-513. Amsterdam: Elsevier Science.
Kim, M. G., Shin, S. W., Bae, K. S., Kim, S. C. and Park, H. Y. (1998). Molecular cloning of chitinase cDNAs from the silkworm, *Bombyx mori* and the fall webworm, *Hyphantria cunea*. *Insect Biochem. Mol. Biol.* **28**, 163-171.
Kimura, S. (1973a). Chitinolytic enzymes in the larval development of the silkworm, *Bombyx mori*. *Appl. Ent. Zool.* **8**, 234-236.
Kimura, S. (1973b). The control of chitinase activity by ecdysterone in larvae of *Bombyx mori*. *J. Insect Physiol.* **19**, 115-123.
Kimura, S. (1977). Exo-β-*N*-acetylglucosaminidase and chitobiase in *Bombyx mori*. *Insect Biochem.* **7**, 237-245.
Kingan, T. G. and Adams, M. E. (2000). Ecdysteroids regulate secretory competence in Inka cells. *J. Exp. Biol.* **203**, 3011-3018.
Klesov, A. A. and Grigorash, S. Y. (1982). Kinetic mechanisms of hydrolysis of insoluble cellulose by multienzyme cellulase systems under nonsteady-state reaction conditions. *Biokhimiia* **42**, 198-213.
Koga, D., Fujimoto, H., Funakoshi, T., Utsumi, T. and Ide, A. (1989). Appearance of chitinolytic enzymes in integument of *Bombyx mori* during the larval-pupal transformation. Evidence for zymogenic forms. *Insect Biochem.* **19**, 123-128.
Koga, D., Funakoshi, T., Fujimoto, H., Kuwano, E., Eto, M. and Ide, A.

(1991). Effects of 20-hydroxyecdysone and KK-42 on chitinase and β-*N*-acetylglucosaminidase during the larval–pupal transformation of *Bombyx mori*. *Insect Biochem.* **21**, 277-284.

Koga, D., Funakoshi, T., Mizuki, K., Ide, A., Kramer, K. J., Zen, K. C., Choi, H. and Muthukrishnan, S. (1992). Immunoblot analysis of chitinolytic enzymes in integument and molting fluid of the silkworm, *Bombyx mori*, and the tobacco hornworm, *Manduca sexta*. *Insect Biochem. Mol. Biol.* **22**, 305-311.

Koga, D., Jilka, J. and Kramer, K. J. (1983). Insect endochitinases: glycoproteins from moulting fluid, integument and pupal haemolymph of *Manduca sexta* L. *Insect Biochem.* **13**, 295-305.

Koga, D., Mai, M. S., Dziadik-Turner, C. and Kramer, K. J. (1982). Kinetics and Mechanism of exochitinase and β-*N*-acetylhexosaminidase from the tobacco hornworm, *Manduca sexta* L. (Lepidoptera: Sphingidae). *Insect Biochem.* **12**, 493-499.

Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y. and Nagamatsu, Y. (1997). Purification and characterization of *Bombyx mori* chitinases. *Insect Biochem. Mol. Biol.* **27**, 757-767.

Kondo, K., Matsumoto, M., Kojo, A. and Mauda, R. (2002). Purification and characterization of chitinase from pupae *Pieris rapae crucivora* (Boiduval). *J. Chem. Eng. Jap.* **35**, 241-246.

Kostyukovsky, M., Chen, B., Atsmi, S. and Shaaya, E. (2000). Biological activity of two juvenoids and two ecdysteroids against three stored product insects. *Insect Biochem. Mol. Biol.* **30**, 891-897.

Kramer, K. J., Corpuz, L., Choi, H. K. and Muthukrishnan, S. (1993). Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem. Mol. Biol.* **23**, 691-701.

Kramer, K. J., Hopkins, T. L. and Schaefer, J. (1995). Applications of solids NMR to the analysis of insect sclerotized structures. *Insect Biochem. Mol. Biol.* **25**, 1067-1080.

Kramer, K. J. and Koga, D. (1986). Insect chitin: physical state, synthesis, degradation and metabolic regulation. *Insect Biochem.* **16**, 851-877.

Kramer, K. J. and Muthukrishnan, S. (1997). Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem. Mol. Biol.* **27**, 887-900.

Krishnan, A., Nair, P. N. and Jones, D. (1994). Isolation, cloning, and characterization of new chitinase stored in active form in chitin-lined venom reservoir. *J. Biol. Chem.* **269**, 20971-20976.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567-580.

Kuranda, M. J. and Robbins, P. W. (1991). Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266**, 19758-19767.

Kurek, I., Kawagoe, Y., Jacob-Wilk, D., Doblin, M. and Delmer, D. (2002). Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. *Proc. Natl. Acad. Sc.i USA* **99**, 11109-11114.

Lasters, I., Wodak, S. J., Alard, P. and van Cutsem, E. (1988). Structural principles of parallel beta-barrels in proteins. *Proc. Natl. Acad. Sci. USA* **85**, 3338-3342.

Law, J. H., Dunn, P. E. and Kramer, K. J. (1977). Insect proteases and peptidases. *Adv. Enzymol. Relat. Areas Mol. Biol.* **45**, 389-425.

Leah, R., Tommerup, H., Svendsen, I. and Mundy, J. (1991). Biochemical and molecular characterization of three barley seed proteins with antifungal properties. *J. Biol. Chem.* **266**, 1564-1573.

Lehane, M. J. (1997). Peritrophic matrix structure and function. *Annu. Rev. Entomol.* **42**, 525-550.

Locke, M. (1991). Insect epidermal cells. In *Physiology of the Insect Epidermis* (ed. K. Binnington and A. Retnakaran), pp. 1-22. Melbourne: CRISCO Publ.

Locke, M. and Huie, P. (1979). Apolysis and the turnover of plasma membrane plaques during cuticle formation in an insect. *Tissue Cell* **11**, 277-291.

Londershausen, M., Kammann, V., Spindler-Barth, M., Spindler, K. D. and Thomas, H. (1988). Chitin synthesis in insect cell lines. *Insect Biochem.* **18**, 631-636.

Lorenz, J., Lenz, M. and Hoffmann, K. H. (1995). Effects of pharmacological agents on ecdysteroid synthesis *in vitro* in ovaries and abdominal integument from female adult crickets, *Gryllus bimaculatus* de Geer (Ensifera, Gryllidae). *Z. Naturforsch.* **50**, 286-293.

Lu, Y. M., Zen, K. C., Muthukrishnan, S. and Kramer, K. J. (2002). Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase. *Insect Biochem. Mol. Biol.* **32**, 1369-1382.

Luo, Y., Amin, J. and Voellmy, R. (1991). Ecdysterone receptor is a sequence-specific transcription factor involved in the developmental regulation of heat shock genes. *Mol. Cell. Biol.* **11**, 3660-3675.

Machida, S. and Saito, M. (1993). Purification and characterization of membrane-bound chitin synthase. *J. Biol. Chem.* **268**, 1702-1707.

Marks, E. P. (1972). Effects of ecdysterone on the deposition of cockroach cuticle *in vitro*. *Biol. Bull.* **142**, 293-301.

Marks, E. P., Balke, J. and Klosterman, H. (1984). Evidence for chitin synthesis in an insect cell line. *Arch. Insect Biochem. Physiol.* **1**, 225-230.

Marks, E. P. and Leopold, R. A. (1971). Deposition of cuticular substances *in vitro* by leg regenerates from the cockroach, *Leucophaea maderae*. *Biol. Bull.* **140**, 73-83.

Mayer, R. T., Chen, A. C. and DeLoach, J. R. (1980). Characterization of a chitin synthase from the stable fly, *Stomoxys calcitrans* (L.). *Insect Biochem.* **10**, 549-556.

Mayer, R. T., Meola, S. M. and DeLoach, J. R. (1981). Chitin synthesis inhibiting insect growth regulators do not inhibit chitin synthetase. *EXS* **37**, 337-338.

McMurrough, I. and Bartnicki-Garcia, S. (1971). Properties of a particulate chitin synthetase from *Mucor rouxii*. *J. Biol. Chem.* **246**, 4008-4016.

Merz, R. A., Horsch, M., Nyhlen, L. E. and Rast, D. M. (1999a). Biochemistry of chitin synthase. *EXS* **87**, 9-37.

Merz, R. A., Horsch, M., Ruffner, H. P. and Rast, D. M. (1999b). Interaction between chitosomes and concanavalin A. *Phytochemistry* **52**, 213-226.

Meszaros, M. and Morton, D. B. (1997). Up- and downregulation of esr20, an ecdysteroid-regulated gene expressed in the tracheae of *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **34**, 159-174.

Mikitani, K., Sugasaki, T., Shimada, T., Kobayashi, M. and Gustafsson, J. A. (2000). The chitinase gene of the silkworm, *Bombyx mori*, contains a novel Tc-like transposable element. *J. Biol. Chem.* **275**, 37725-37732.

Mommsen, T. P. (1980). Chitinase and beta-*N*-acetylglucosaminidase from the digestive fluid of the spider, *Cupiennius salei*. *Biochim. Biophys. Acta* **612**, 361-372.

Moya, P., Castillo, M., Primo-Yufera, E., Couillaud, F., Martinez-Manez, R., Garcera, M. D., Miranda, M. A., Primo, J. and Martinez-Pardo, R. (1997). Brevioxime: a new juvenile hormone biosynthesis inhibitor isolated from *Penicillium brevicompactum*. *J. Org. Chem.* **62**, 8544-8545.

Mulla, M. S., Darwazeh, H. A., Ede, L. and Kennedy, B. (1985). Laboratory and field evaluation of the IGR fenoxycarb against mosquitoes. *J. Am. Mosq. Control Assoc.* **1**, 442-448.

Müller, M. (1992). Proteolysis in protein import and export: signal peptide processing in eu- and prokaryotes. *EXS* **48**, 118-129.

Munro, C. A. and Gow, N. A. R. (2001). Chitin synthesis in human pathogenic fungi. *Med. Myc.* **39**, 41-53.

Muthukrishnan, J., Seifert, K., Hoffmann, K. H. and Lorenz, M. W. (1999). Inhibition of juvenile hormone biosynthesis in *Gryllus bimaculatus* by *Glycosmis pentaphylla* leaf compounds. *Phytochemistry* **50**, 249-254.

Nagahashi, S., Sudoh, M., Ono, N., Sawada, R., Yamaguchi, E., Uchida, Y., Mio, T., Takagi, M., Arisawa, M. and Yamada-Okabe, H. (1995). Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. Implication of two highly conserved domains as possible catalytic sites. *J. Biol. Chem.* **270**, 13961-13967.

Nakagawa, Y. and Matsumura, F. (1994). Diflubenzuron affects gamma-thio GTP stimulated Ca^{2+} transport *in vitro* in intracellular vesicles from the integument of the newly molted American cockroach, *Periplaneta americana* L. *Insect Biochem. Mol. Biol.* **24**, 1009-1015.

Niehrs, C. and Pollet, N. (1999). Synexpression groups in eukaryotes. *Nature* **402**, 483-487.

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**, 1-6.

Oberlander, H. (1976). Hormonal control of growth and differentiation of insect tissues cultured *in vitro*. *In Vitro* **12**, 225-235.

Oberlander, H., Lynn, D. A. and Leach, C. A. (1983). Inhibition of cuticle production in imaginal disks *Plodia interpunctella* (cultured *in vitro*): Effects of colcemid and vinblastine. *J. Insect Physiol.* **29**, 47-53.

Omura, S., Arai, N., Yamaguchi, Y., Masuma, R., Iwai, Y., Namikoshi, M., Turberg, A., Kolbl, H. and Shiomi, K. (2000). Argifin, a new chitinase inhibitor, produced by *Gliocladium* sp. FTD-0668. I. Taxonomy, fermentation, and biological activities. *J. Antibiot.* **53**, 603-608.

Ono, N., Yabe, T., Sudoh, M., Nakajima, T., Yamada-Okabe, T., Arisawa, M. and Yamada-Okabe, H. (2000). The yeast Chs4 protein stimulates the trypsin-sensitive activity of chitin synthase 3 through an apparent protein-protein interaction. *Microbiology* **146**, 385-391.
Orlean, P. (1987). Two chitin synthases in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **262**, 5732-5739.
Ostrowski, S., Dierick, H. A. and Bejsovec, A. (2002). Genetic control of cuticle formation during embryonic development of *Drosophila melanogaster*. *Genetics* **161**, 171-182.
Passonneau, J. V. and Williams, C. M. (1953). The moulting fluid of the *Cecropia* silkmoth. *J. Exp. Biol.* **246**, 124-131.
Perez, M. and Hirschberg, C. B. (1985). Translocation of UDP-*N*-acetylglucosamine into vesicles derived from rat liver rough endoplasmic reticulum and Golgi apparatus. *J. Biol. Chem.* **260**, 4671-4678.
Perrakis, A., Ouzounis, C., Wilson, K. S. and Vorgias, C. (1996). Implications of the tertiary structure determination of chitinase A. Similarities with Fibronectin III domains, oviductal proteins and narbonin. In *Advances in Chitin Science*, vol. 1 (ed. A. Domard C. Jeuniaux, R. A. A. Muzzarelli and G. Roberts), pp. 34-41. Lyon: Jacques André.
Peters, W. (1992). Peritrophic membranes. In *Zoophysiology*, vol. 30 (ed. S. D. Bradshaw, W. Burggren, H. C. Heller, S. Ishii, H. Langer, G. Neuweiler and D. J. Randall), pp. 1-238. Berlin: Springer.
Peters, W., Heitmann, S. and D'Haese, J. (1979). Formation and fine structure of peritrophic membranes in the earwig, *Forficula auricularia*. *Entomol. Gen.* **5**, 241-254.
Post, L. V., LJong, B. J. and Vincent, W. R. (1974). 1-(2,6-Disubsituted benzoyl)-3-phenylurea insecticides: inhibitors of chitin synthesis. *Pestic. Biochem. Physiol.* **4**, 473-483.
Pummill, P. E., Achyuthan, A. M. and DeAngelis, P. L. (1998). Enzymological characterization of recombinant Xenopus DG42, a vertebrate hyaluronan synthase. *J. Biol. Chem.* **273**, 4976-4981.
Quesada-Allue, L. A. (1982). The inhibition of insect chitin synthesis by tunicamycin. *Biochem. Biophys. Res. Commun.* **105**, 312-319.
Quesada-Allue, L. A., Marechal, L. R. and Belocopitow, E. (1976). Chitin synthesis in *Triatoma infestans* and other insects. *Acta Physiol. Lat. Am.* **26**, 349-363.
Rao, F. V., Houston, D. R., Boot, R. G., Aerts, J. M., Sakuda, S. and Van Aalten, D. M. (2003). Crystal structures of allosamidin derivatives in complex with human macrophage chitinase. *J. Biol. Chem.* **14**, 20110-20116.
Rechsteiner, M. and Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* **21**, 267-271.
Retnakaran, A., Gelbic, I., Sundaram, M., Tomkins, W., Ladd, T., Primavera, M., Feng, Q., Arif, B., Palli, R. and Krell, P. (2001). Mode of action of the ecdysone agonist tebufenozide (RH-5992), and an exclusion mechanism to explain resistance to it. *Pest Manag. Sci.* **57**, 951-957.
Reynolds, S. E. and Samuels, R. I. (1996). Physiology and biochemistry of insect moulting fluid. *Adv. Insect Physiol.* **26**, 157-232.
Riddiford, L. M. (1994). Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. *Adv. Insect Physiol.* **24**, 211-274.
Riddiford, L. M. (1996). Juvenile hormone: the status of its "status quo" action. *Arch. Insect Biochem. Physiol.* **32**, 271-286.
Robertus, J. D. and Monzingo, A. F. (1999). The structure and action of chitinases. *EXS* **87**, 125-135.
Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364-368.
Rosa, F., Sargent, T. D., Rebbert, M. L., Michaels, G. S., Jamrich, M., Grunz, H., Jonas, E., Winkles, J. A. and Dawid, I. B. (1988). Accumulation and decay of DG42 gene products follow a gradient pattern during *Xenopus* embryogenesis. *Dev. Biol.* **129**, 114-123.
Roussel, J.-P. (1994). Synthetic molecules designed as potential inhibitors in ecdysone biosynthesis. *Entomol. Exp. Appl.* **71**, 193-199.
Royer, V., Fraichard, S. and Bouhin, H. (2002). A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis. *Biochem. J.* **366**, 921-928.
Rudall, K. M. and Kenchington, W. (1973). The chitin system. *Biol. Rev.* **48**, 597-636.
Ruiz-Herrera, J. and Martinez-Espinoza, A. D. (1999). Chitin biosynthesis and structural organization *in vivo*. *EXS* **87**, 39-53.
Ruiz-Herrera, J. and San-Blas, G. (2003). Chitin synthesis as target for antifungal drugs. *Curr. Drug Targets Infect. Disord.* **3**, 77-91.
Sakuda, S., Isogai, A., Matsumoto, S. and Suzuki, A. (1987). Search for microbial insect growth regulators. II. Allosamidin, a novel insect chitinase inhibitor. *J. Antibiot.* **40**, 296-300.
Samuels, R. and Reynolds, S. E. (1993). Moulting fluid enzymes of the tobacco hornworm, *Manduca sexta*: timing of proteolytic and chitinolytic activity in relation to pre-ecdysial development. *Arch Insect Biochem. Physiol.* **24**, 33-44.
Samuels, R. I. and Paterson, I. C. (1995). Cuticle degrading proteases from insect moulting fluid and culture filtrates of entomopathogenic fungi. *Comp. Biochem. Physiol. B* **110**, 661-669.
Santos, B. and Snyder, M. (1997). Targeting of chitin synthase 3 to polarized growth sites in yeast requires Chs5p and Myo2p. *J. Cell Biol.* **136**, 95-110.
Sanz, M., Trilla, J. A., Duran, A. and Roncero, C. (2002). Control of chitin synthesis through Shc1p, a functional homologue of Chs4p specifically induced during sporulation. *Mol. Microbiol.* **43**, 1183-1195.
Saville, G. P., Thomas, C. J., Possee, R. D. and King, L. A. (2002). Partial redistribution of the *Autographa californica* nucleopolyhedrovirus chitinase in virus-infected cells accompanies mutation of the carboxy-terminal KDEL ER-retention motif. *J. Gen. Virol.* **83**, 685-694.
Saxena, I. M., Brown, R. M., Jr and Dandekar, T. (2001). Structure–function characterization of cellulose synthase: relationship to other glycosyltransferases. *Phytochemistry* **57**, 1135-1148.
Schooneveld, H. (1979). Precocene-induced necrosis and haemocyte-mediated breakdown of corpora allata in nymps of the locust *Locusta migratoria*. *Cell Tissue Res.* **203**, 25-33.
Schrempf, H. (2001). Recognition and degradation of chitin by streptomycetes. *Antonie Van Leeuwenhoek* **79**, 285-289.
Segawa, H., Kawakita, M. and Ishida, N. (2002). Human and *Drosophila* UDP-galactose transporters transport UDP-*N*-acetylgalactosamine in addition to UDP-galactose. *Eur. J. Biochem.* **269**, 128-138.
Sentandreu, R., Martinez-Ramon, A. and Ruiz-Herrera, J. (1984). Localization of chitin synthase in *Mucor rouxii* by an autoradiographic method. *J. Gen. Microbiol.* **130**, 1193-1199.
Shahabuddin, M., Toyoshima, T., Aikawa, M. and Kaslow, D. C. (1993). Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. *Proc. Natl. Acad. Sci. USA* **90**, 4266-4270.
Shen, Z. and Jacobs-Lorena, M. (1999). Evolution of chitin-binding proteins in invertebrates. *J. Mol. Evol.* **48**, 341-347.
Shen, Z. C. and Jacobs-Lorena, M. (1997). Characterization of a novel gut-specific chitinase gene from the human malaria vector *Anopheles gambiae*. *J. Biol. Chem.* **272**, 28895-28900.
Sinnott, M. L. (1990). Catalytic mechanisms of enzymatic glycosyl transfer. *Chem. Rev.* **90**, 1171-1202.
Skehel, J. J. and Wiley, D. C. (1998). Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* **95**, 871-874.
Spindler, K.-D., Przibilla, S. and Spindler-Barth, M. (2001). Moulting hormones of arthropods: molecular mechanisms. *Zoology* **103**, 189-201.
Surholt, B. (1975). Studies *in vitro* and *in vivo* on chitin synthesis during larval–adult moulting cycle of the migratory locust, *Locusta migratoria* L. *J. Comp. Physiol.* **102**, 135-142.
Tellam, R. L. (1996). The peritrophic matrix. In *Biology of the Insect Midgut* (ed. M. J. Lehane and P. F. Billingsley), pp. 86-114. Cambridge: Chapman and Hall.
Tellam, R. L., Smith, D. H. and Willadsen, P. (1992). Vaccination against ticks. In *Animal Parasite Control Utilizing Biotechnology* (ed. W. K. Yong), pp. 303-331. Boca Raton: CRC Press.
Tellam, R. L., Vuocolo, T., Johnson, S. E., Jarmey, J. and Pearson, R. D. (2000). Insect chitin synthase cDNA sequence, gene organization and expression. *Eur. J. Biochem.* **267**, 6025-6043.
Terra, W. R., Ferreira, C., Jordao, B. P. and Dillon, R. J. (1996). Digestive enzymes. In *Biology of the insect midgut* (ed. M. J. Lehane and P. F. Billingsley), pp. 153-194. Cambridge: Chapman and Hall.
Terwisscha van Scheltinga, A. C., Armand, S., Kalk, K. H., Isogai, A., Henrissat, B. and Dijkstra, B. W. (1995). Stereochemistry of chitin hydrolysis by a plant chitinase/lysozyme and X-ray structure of a complex with allosamidin: evidence for substrate assisted catalysis. *Biochemistry* **34**, 15619-15623.
Terwisscha van Scheltinga, A. C., Kalk, K. H., Beintema, J. J. and Dijkstra, B. W. (1994). Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. *Structure* **2**, 1181-1189.
Thomas, C. J., Gooday, G. W., King, L. A. and Possee, R. D. (2000). Mutagenesis of the active site coding region of the *Autographa californica* nucleopolyhedrovirus *chiA* gene. *J. Gen. Virol.* **81**, 1403-1411.

Trilla, J. A., Duran, A. and Roncero, C. (1999). Chs7p, a new protein involved in the control of protein export from the endoplasmic reticulum that is specifically engaged in the regulation of chitin synthesis in *Saccharomyces cerevisiae*. *J. Cell Biol.* **145**, 1153-1163.

Truman, J. W. and Riddiford, L. M. (1970). Neuroendocrine control of ecdysis in silkmoths. *Science* **167**, 1624-1626.

Tsai, Y. L., Hayward, R. E., Langer, R. C., Fidock, D. A. and Vinetz, J. M. (2001). Disruption of *Plasmodium falciparum* chitinase markedly impairs parasite invasion of mosquito midgut. *Infect. Immun.* **69**, 4048-4054.

Turnbull, I. F. and Howells, A. J. (1983). Integumental chitin synthase activity in cell-free extracts of larvae of the Australian sheep blowfly, *Lucilia cuprina*, and two other species of diptera. *Aust. J. Biol. Sci.* **36**, 251-262.

Uchida, Y., Shimmi, O., Sudoh, M., Arisawa, M. and Yamada-Okabe, H. (1996). Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. II. Both full size and processed enzymes are active for chitin synthesis. *J. Biochem.* **119**, 659-666.

Usui, T., Hayashi, Y., Nanjo, F., Sakai, K. and Ishido, Y. (1987). Transglycosylation reaction of a chitinase purified from *Nocardia orientalis*. *Biochim. Biophys. Acta* **923**, 302-309.

van Aalten, D. M., Komander, D., Synstad, B., Gaseidnes, S., Peter, M. G. and Eijsink, V. G. (2001). Structural insights into the catalytic mechanism of a family 18 exo- chitinase. *Proc. Natl. Acad. Sci. USA* **98**, 8979-8984.

van Eck, W. H. (1979). Mode of action of two benzoylphenyl ureas as inhibitors of chitin synthesis in insects. *Insect Biochem.* **9**, 295-300.

Vardanis, A. (1976). An *in vitro* assay system for chitin synthesis in insect tissue. *Life Sci.* **19**, 1949-1956.

Vardanis, A. (1979). Characteristics of the chitin-synthesizing system of insect tissue. *Biochim Biophys Acta* **588**, 142-147.

Venegas, A., Goldstein, J. C., Beauregard, K., Oles, A., Abdulhayoglu, N. and Fuhrman, J. A. (1996). Expression of recombinant microfilarial chitinase and analysis of domain function. *Mol. Biochem. Parasitol.* **78**, 149-159.

Vermeulen, C. A. and Wessels, J. G. (1986). Chitin biosynthesis by a fungal membrane preparation. Evidence for a transient non-crystalline state of chitin. *Eur. J. Biochem.* **158**, 411-415.

von Heijne, G. (1990). The signal peptide. *J. Membr. Biol.* **115**, 195-201.

Vorgias, C. E., Kingswell, A. J., Dauter, Z. and Oppenheim, A. B. (1992). Crystallization of recombinant chitinase from the cloned *chiA* gene of *Serratia marcescens*. *J. Mol. Biol.* **226**, 897-898.

Wang, X., Ding, X., Gopalakrishnan, B., Morgan, T. D., Johnson, L., White, F., Muthukrishnan, S. and Kramer, K. J. (1996). Characterization of a 46 kDa insect chitinase from transgenic tobacco. *Insect Biochem. Mol. Biol.* **26**, 1055-1064.

Wigglesworth, V. B. (1930). The formation of the peritrophic membrane in insects, with special reference to the larvae of mosquitoes. *Q. J. Microsc. Sci.* **73**, 593-616.

Wingender, E., Kel, A. E., Kel, O. V., Karas, H., Heinemeyer, T., Dietze, P., Knuppel, R., Romaschenko, A. G. and Kolchanov, N. A. (1997). TRANSFAC, TRRD and COMPEL: towards a federated database system on transcriptional regulation. *Nucleic Acids Res.* **25**, 265-268.

Yabe, T., Yamada-Okabe, T., Nakajima, T., Sudoh, M., Arisawa, M. and Yamada-Okabe, H. (1998). Mutational analysis of chitin synthase 2 of *Saccharomyces cerevisiae*. Identification of additional amino acid residues involved in its catalytic activity. *Eur. J. Biochem.* **258**, 941-947.

Yan, J., Cheng, Q., Narashimhan, S., Li, C. B. and Aksoy, S. (2002). Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans*. *Insect Biochem. Mol. Biol.* **32**, 979-989.

Yao, T. P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J. D., McKeown, M., Cherbas, P. and Evans, R. M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366**, 476-479.

Yarema, C., McLean, H. and Caveney, S. (2000). L-Glutamate retrieved with the moulting fluid is processed by a glutamine synthetase in the pupal midgut of *Calpodes ethlius*. *J. Insect Physiol.* **46**, 1497-1507.

Zen, K. C., Choi, H. K., Krishnamachary, N., Muthukrishnan, S. and Kramer, K. J. (1996). Cloning, expression, and hormonal regulation of an insect beta-*N*-acetylglucosaminidase gene. *Insect Biochem. Mol. Biol.* **26**, 435-444.

Zhang, D. and Miller, M. J. (1999). Polyoxins and nikkomycins: progress in synthetic and biological studies. *Curr. Pharm. Des.* **5**, 73-99.

Zhang, H., Huang, X., Fukamizo, T., Muthukrishnan, S. and Kramer, K. J. (2002). Site-directed mutagenesis and functional analysis of an active site tryptophan of insect chitinase. *Insect Biochem. Mol. Biol.* **32**, 1477-1488.

Zhu, X., Zhang, H., Fukamizo, T., Muthukrishnan, S. and Kramer, K. J. (2001). Properties of *Manduca sexta* chitinase and its C-terminal deletions. *Insect Biochem. Mol. Biol.* **31**, 1221-1230.

Zhu, Y. C., Specht, C. A., Dittmer, N. T., Muthukrishnan, S., Kanost, M. R. and Kramer, K. J. (2002). Sequence of a cDNA and expression of the gene encoding a putative epidermal chitin synthase of *Manduca sexta*. *Insect Biochem. Mol. Biol.* **32**, 1497-1506.

Zielkowski, R. and Spindler, K. (1978). Chitinase and chitobiase from the integument of *Locusta migratoria*: characteristics and titer during the fith instar. *Insect Biochem.* **8**, 67-71.

Ziman, M., Chuang, J. S., Tsung, M., Hamamoto, S. and Schekman, R. (1998). Chs6p-dependent anterograde transport of Chs3p from the chitosome to the plasma membrane in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **9**, 1565-1576.

Zimoch, L. and Merzendorfer, H. (2002). Immunolocalization of chitin synthase in the tobacco hornworm. *Cell Tissue Res.* **308**, 287-297.

Zitnan, D., Ross, L. S., Zitnanova, I., Hermesman, J. L., Gill, S. S. and Adams, M. E. (1999). Steroid induction of a peptide hormone gene leads to orchestration of a defined behavioral sequence. *Neuron* **23**, 523-535.