



## Mini-review

# Insect Chitinases: Molecular Biology and Potential Use as Biopesticides

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**Chitin, an insoluble structural polysaccharide that occurs in the exoskeletal and gut linings of insects, is a metabolic target of selective pest control agents. One potential biopesticide is the insect molting enzyme, chitinase, which degrades chitin to low molecular weight, soluble and insoluble oligosaccharides. For several years, our laboratories have been characterizing this enzyme and its gene. Most recently, we have been developing chitinase for use as a biopesticide to control insect and also fungal pests. Chitinases have been isolated from the tobacco hornworm, *Manduca sexta*, and several other insect species, and some of their chemical, physical, and kinetic properties have been determined. Also, cDNA and genomic clones for the chitinase from the hornworm have been isolated and characterized. Transgenic plants that express hornworm chitinase constitutively have been generated and found to exhibit host plant resistance. A transformed entomopathogenic virus that produces the enzyme displayed enhanced insecticidal activity. Chitinase also potentiated the efficacy of the toxin from the microbial insecticide, *Bacillus thuringiensis*. Insect chitinase and its gene are now available for biopesticidal applications in integrated pest management programs. Current knowledge regarding the molecular biology and biopesticidal action of insect and several other types of chitinases is described in this mini-review. © 1998 Elsevier Science Ltd. All rights reserved**

Chitinase Chitin Insect Molting Biopesticide Tobacco hornworm Transgene Transgenic plant  
 Transgenic virus Exoskeleton Cuticle Peritrophic membrane Host plant resistance Biological control  
*Bacillus thuringiensis* toxin Baculovirus Enzyme biotechnology

### INTRODUCTION

Transgenic plants and biological control agents offer many opportunities to manipulate genes from a variety of sources for the enhancement of host plant resistance and pathogenicity, respectively, against insects and other types of pests (Bonning and Hammock, 1996; Estruch *et al.*, 1997). Sources of opportunity include genes for proteins that interact with chitin, a linear homopolymer of 2-acetamido-2-deoxy-D-glucopyranoside (N-acetylglucosamine, GlcNAc) connected by  $\beta$ -1 $\rightarrow$ 4-linkages, which is utilized as a structural polysaccharide in nature. Because the occurrence of this carbohydrate as a struc-

tural component is limited to a few organs, such as the integuments of arthropods, nematodes, and molluscs, the gut linings of insects, and the cell walls of fungi and some algae, chitin metabolism is an excellent target for selective pest control strategies (Kramer and Koga, 1986; Cohen, 1987; Kramer *et al.*, 1997). The chitin content of an individual tissue varies markedly depending on the type of structure and species and must be maintained at precise levels for the proper development of the organism. The polysaccharide can occur in a number of crystalline forms, but only one,  $\alpha$ -chitin, has been identified in insects (Salmon and Hudson, 1997). In this form, neighboring poly-GlcNAc chains run antiparallel to each other (Neville *et al.*, 1976).

Proteins that interact with chitin include various types of chitin-binding lectins such as wheat germ agglutinin (Harper *et al.*, 1995; Peumans and Van Damme, 1995; Zhu *et al.*, 1996) and seed storage proteins such as the vicilins (Firmino *et al.*, 1996; Sales *et al.*, 1996; Xavier-Filho *et al.*, 1996), as well as cuticle proteins and several

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types of chitin-catabolic enzymes including chitinase,  $\beta$ -N-acetylglucosaminidase, and chitin deacetylase (Baker and Kramer, 1996; Kramer *et al.*, 1997). Because of the importance of chitin and its metabolic enzymes in insect growth and development, chitinolytic enzymes and their genes have received a great deal of attention in recent years. Their chemical, physical, kinetic, and regulatory properties, as well as their potential for development as biopesticides or chemical defense proteins in transgenic plants and microbial biological control agents are being explored (Kramer *et al.*, 1997). This mini-review focuses primarily on the molecular biology of one type of insect chitinolytic enzyme, namely chitinase, and its potential applications as a biopesticide or as an enhancing agent for biocontrol agents. Recent progress in the areas of: (1) cloning and characterization of genes encoding insect chitinolytic enzymes; and (2) gene expression in insect and transgenic plant tissues, as well as in cell lines infected with a recombinant baculovirus containing the insect chitinase gene is summarized. A similar use of a number of genes encoding plant chitinases primarily for fungal control has been described previously (Broglie *et al.*, 1991; Vierheilig *et al.*, 1993; Lin *et al.*, 1995).

#### ENZYMOLGY AND PHYSIOLOGY

Chitinases are enzymes (EC 3.2.1.14) with a specific hydrolytic activity directed towards chitin. However, some chitinases can also hydrolyse related polymers, such as cell wall polysaccharides containing not only  $\beta$ -1 $\rightarrow$ 4-linked N-acetylglucosamine but also N-acetylmuramate. Enzymatic cleavages generally occur randomly at internal locations over the entire length of the polymer. The final products from chitinase catalysis are soluble, low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and chitobiose, with the smallest oligosaccharides being predominant (Kramer and Koga, 1986; Reynolds and Samuels, 1996). These oligosaccharides then become substrates for another type of chitinolytic enzyme,  $\beta$ -N-acetylglucosaminidase (EC 3.2.1.30), which cleaves off GlcNAc units sequentially from the non-reducing end of the substrate (Fukamizo and Kramer, 1985). Both types of enzymes have been detected in a variety of organisms, including those that do contain chitin, such as insects, crustaceans, yeasts and other fungi, and also in organisms that do not, such as bacteria, plants, and vertebrates.

In bacteria, chitinases are probably involved in processing and digesting GlcNAc-containing macromolecules as a nutrient source. In plants, they appear to play a role as defensive-response proteins targeted towards pathogenic or pestiferous organisms (Boller, 1987). In vertebrates, the role of chitinase-like proteins is uncertain, but may include resisting infections caused by fungi or nematodes (Boot *et al.*, 1995).

In arthropods, chitinases are involved in cuticle turnover and mobilization and in nutrient digestion. The enzymes have been found in molting fluids, venom

glands, and midguts of several insects, but only chitinases from the former two sources have been characterized extensively (Kramer and Koga, 1986; Fukamizo and Kramer, 1987; Samuels and Reynolds, 1993; Krishnan *et al.*, 1994; Terra and Ferreira, 1994; Reynolds and Samuels, 1996; Terra *et al.*, 1996). Insects periodically shed their old exoskeletons and either continuously or periodically shed their peritrophic membranes and resynthesize new ones (Lehane, 1997). This process is mediated by the elaboration of chitinases in the molting fluid that accumulates in the space between the old cuticle and the epidermis and in the gut tissue. The GlcNAc-containing products of hydrolysis are ultimately recycled for the synthesis of a new cuticle. Often, the larvae will ingest and digest the old cuticle or exuvium, the components of which are also recycled. This behavior coincides with the period of chitinase expression in the gut (Kramer *et al.*, 1993). Apparently, chitinases found in the gut have a digestive function in addition to their role in breaking down chitin present in the gut lining or peritrophic membrane. In the venom of some Hymenoptera, chitinases may expedite the spread of venomous compounds from the site of delivery. In microfilarial infections, chitinases may facilitate infection by breaking down chitin-containing structures of the nematode and/or the insect host. For organisms such as fungi, chitinases apparently help to degrade and mobilize organic matter and possibly to antagonize the growth of competitors. In yeast, chitinases are important for cell separation (Sakuda *et al.*, 1990; Kuranda and Robbins, 1991).

#### BIOCHEMISTRY OF INSECT CHITINASES

Chitinases have been purified from the molting fluid and integument of the tobacco hornworm, *Manduca sexta*, and the silkworm, *Bombyx mori*, as well as the venom gland of the endoparasitic wasp, *Chelonus* (Kramer and Koga, 1986; Koga *et al.*, 1992; Krishnan *et al.*, 1994). The chemical, physical, and kinetic properties of these and several other chitinases have been characterized (Kramer and Koga, 1986; Koga *et al.*, 1992; Krishnan *et al.*, 1994; Reynolds and Samuels, 1996). These enzymes have a size range of 40–85 kDa, which is typically larger than the masses of plant (~25–40 kDa) and bacterial (~20–60 kDa) chitinases. They can be active in the pH range of ~4–8 and their isoelectric points range from pH ~5–7. Insect chitinases have also been purified after expressing cDNAs encoding these enzymes in insect cell lines or transgenic plants (Gopalakrishnan *et al.*, 1995; Wang *et al.*, 1996). Except for the protein obtained from transgenic plants, these enzymes, as well as the molting fluid enzymes, are glycosylated extensively. Some of the smaller enzymes found in molting fluid and transgenic plants appear to arise from proteolysis of larger forms.

## CDNA CLONES FOR INVERTEBRATE CHITINASES

In 1993, the first cDNA encoding an insect chitinase was isolated from *M. sexta* and analysed (Kramer *et al.*, 1993). Cloning of two other insect chitinolytic enzyme cDNAs has been reported (Krishnan *et al.*, 1994; Koga *et al.*, submitted), as well as two nematode chitinase cDNAs (Huber *et al.*, 1991; Fuhrman, 1995; Adam *et al.*, 1996). As expected for secreted proteins, the conceptual proteins encoded hydrophobic leader peptides 19–22 amino acids long, followed by a sequence corresponding to the mature N-terminus of the corresponding chitinase. The lengths of the mature conceptual proteins were 554 amino acids corresponding to a molecular mass of approximately 62 kDa for the *Manduca* enzyme, 482 amino acids corresponding to a mass of 55 kDa for the *Brugia malayi* chitinase, and 464 amino acids with a molecular mass of 52 kDa for the *Chelonus* venom protein. Glycosylation results in native proteins with apparent molecular masses of 70–85 kDa for the hornworm and nematode enzymes, but the venom protein is apparently not glycosylated.

## STRUCTURAL CLASSIFICATION OF INSECT CHITINASES

In the software and databases available on the Internet (Structural Classification of Proteins (SCOP; <http://pdb.weizmann.ac.il/scop/index.html>), SWISS-PROT (<http://expasy.hcuge.ch/sprot/sprot-top.html>), and BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>)), which are designed to search for amino acid sequence and structural similarities and also to classify proteins in terms of both structural and evolutionary relatedness to other proteins, *Manduca* chitinase is classified as a beta/alpha-barrel protein consisting of mainly parallel beta sheets similar to proteins in the superfamily of glycosyltransferases and in family 18 of hydrolytic enzymes, the members of which are noted for two conserved regions of amino acids that contain putative active site residues (Altschul *et al.*, 1990; Henrissat and Bairoch, 1993; Murzin *et al.*, 1995; Bairoch and Apweiler, 1996). Included in this family are viral, bacterial, fungal, insect, reptile, and certain plant proteins (Muzzarelli, 1997), such as *Serratia marcescens* chitinase, *Streptomyces plicatus* endoglycosidase, *Hevea brasiliensis* chitinase/lysozyme, and several seed storage proteins (narbonin, jack bean lectin, and concanavalin). A model three-dimensional ribbon structure of the central portion of the *Manduca* chitinase polypeptide backbone is shown in Fig. 1. This structure is based on that of *Serratia* chitinase A, whose three-dimensional crystal structure is known (Perrakis *et al.*, 1994). Residues 80–387 of the *Manduca* chitinase were aligned with residues 227–534 of *Serratia* chitinase to generate the model insect enzyme structure using Swiss-Model software, an automated knowledge-based protein modelling server available on the Internet (<http://expasy.hcuge.ch/swissmod/SWISS-MODEL.html>). These two regions exhibit 81% similarity

in amino acid sequence. The tertiary structure of family 18 glycosyl hydrolases is characterized by a  $(\beta\alpha)_8$  barrel fold, with the two consensus active-site regions roughly corresponding to the third and fourth barrel strands that form the substrate-binding site. A common example of this type of protein structure is the TIM-barrel, named after the archetype of this fold, triose phosphate isomerase. In TIM-barrel proteins, the internal barrel consists of eight parallel  $\beta$ -strands and the outer shell contains eight  $\alpha$ -helices oriented with an external angle relative to the axis of the barrel (Levitt and Chothia, 1976; Hubbard *et al.*, 1997). All enzymes in this family are expected to show a common tertiary structure, have a common mechanism of hydrolysis (Henrissat and Bairoch, 1993) and have an aspartic acid and a glutamic acid in two of the active-site peptide locations (Van Scheltinga *et al.*, 1996). Site-directed mutagenesis of analogous putative active-site residues in a chitinase from *Bacillus circulans* resulted in either loss of activity or drastic reductions in  $k_{cat}$  values (Watanabe *et al.*, 1993; Watanabe *et al.*, 1994), supporting the notion that these residues are very important for catalysis. The homologous residues in *Manduca* chitinase are aspartic acid 321 and glutamic acid 325 (Fig. 2).

## CHITINASE GENE ORGANIZATION

One of the structural features observed in chitinases from several animals and microorganisms is a multidomain architecture that includes catalytic domains, a cysteine-rich chitin-binding domain distinct from the catalytic domains, and a serine/threonine-rich domain that is glycosylated (Tellam, 1996). The similarity of sequences in fungal, bacterial, and insect chitinases suggests comparable catalytic domains in all of these chitinases (Alam *et al.*, 1995). The 6-cysteine C-terminal structural motif found in insect and nematode chitinases is also found in several peritrophic membrane proteins (Tellam, 1996) and a range of receptors and other proteins controlling cellular adhesion (Tellam *et al.*, 1992). Some chitinases from plants and microbes as well as several other proteins with no chitinase activity have a similar chitin-binding domain containing several cysteines, but this domain may be located near either the N- or the C-terminal ends of these proteins (Blaxter, 1996). The function of these domains in the chitinases is presumably to anchor the enzyme tightly onto the substrate, thereby facilitating the hydrolytic process.

The structure of the insect chitinase gene is consistent with the concept of a multi-domain architecture of the protein. Analysis of the genomic clone of *M. sexta* chitinase revealed that it is organized into at least 11 exons in a region spanning more than 11 kb (Choi *et al.*, 1997). A series of exons correspond to identifiable structural/functional regions of the protein. For example, exons individually encode the entire signal peptide; the mature amino-terminal sequence of the enzyme; either of two conserved motifs found in many chitinases, which



FIGURE 1. Model ribbon structure of *M. sexta* chitinase (amino acid residues 80–387). This structure is based on the three-dimensional structure of *S. marsecens* chitinase residues 227–534 and the amino acid sequence similarity (81%) with *M. sexta* chitinase residues 80–387. It was obtained using SWISS-Model software available on the Internet at <http://expasy.hcuge.ch/www/expasy-top.html>. Note that regions corresponding to amino terminal residues 1–79 and carboxyl terminal residues 388–554 of *M. sexta* chitinase are not included in this structure because there is not a high enough degree of similarity in those regions with the sequence of *S. marsecens* chitinase. The yellow-colored ribbons show beta-sheet structures, red ribbons show alpha-helical segments, and blue ribbons show turn segments. The cleft visible at the bottom of the structure may be part of the active site of the enzyme. The green-colored ribbons show conserved regions 1 (right) and 2 (left), which contain critical residues in the active site of the enzyme.

putatively contain critical residues found in the active site; several regions of possible N-glycosylation and phosphorylation sites; a serine/threonine-rich region; either of two cysteine-rich regions; and a carboxyl-terminal region. This exon organization is similar, at least in part, to arrangements found in chitinase genes from a yeast, *Saccharomyces cerevisiae*, and a nematode, *B. malayi* (Kuranda and Robbins, 1991; Fuhrman, 1995). Other than the chitinase gene of *M. sexta*, the intron-exon organization of no other insect chitinase gene is known. cDNA clones coding for other chitinolytic

enzymes from insect sources have been isolated and characterized only recently (Krishnan *et al.*, 1994; Nagamatsu *et al.*, 1995; Zen *et al.*, 1996).

The amino acid sequence of *M. sexta* chitinase has relatively little similarity with sequences of classes I and II plant chitinases, but does show limited similarity to sequences of plant chitinases of classes III and V and microbial chitinases (Choi *et al.*, 1997). In particular, two highly conserved regions, including one proposed to be a part of the active site of the enzyme, are present in *M. sexta* and other invertebrate chitinases. The hornworm





bit an affinity for carbohydrate compounds (Kirkpatrick *et al.*, 1995; Malette *et al.*, 1995). Rather surprising is the similarity between the *Manduca* enzyme and a chitotriosidase from human serum (33% identity), which also exhibits chitinase activity and is probably an infection-response protein (Boot *et al.*, 1995; Renkema *et al.*, 1995).

Fig. 2 shows an alignment of six chitinase amino acid sequences from representative organisms, including insect, nematode, crustacean, mammalian, and bacterial sources. The alignment indicates that most of the sequence conservation is in the central domain, which includes the two regions implicated in catalytic activity. In addition, the alignment highlights the multidomain nature of the chitinases from different sources. Individual chitinases possess different combinations of these domains in addition to the catalytic domain. For example, a cysteine-rich carboxyl domain is present in the insect, nematode, and human chitinases but not in the other enzymes. A proline/glutamate/serine/threonine-rich (PEST) domain that precedes this carboxyl domain and is postulated to increase the susceptibility to proteolysis by a calcium-dependent protease (Rogers *et al.*, 1986) is absent in the *Serratia* and prawn chitinases. The bacterial enzyme has a unique N-terminal region that is missing in the other enzymes.

The *M. sexta* chitinase gene is not active during the larval feeding period. The enzyme is expressed only during narrow time frames just before larval-larval, larval-pupal and pupal-adult molting. The activity of this gene is regulated positively by ecdysteroid, and affected negatively by juvenile hormone (Kramer *et al.*, 1993). The tight developmental and hormonal regulation of chitinase expression suggests that the presence or absence of this enzyme might be detrimental to insect growth if expressed at an inappropriate time or if not expressed at an appropriate time. Similarly, green tissues of plants do not normally express chitinases but exhibit inducible chitinase expression when exposed to pathogens, insects, or other types of stress (Lin *et al.*, 1995). Plants and microorganisms constitutively expressing an insect or plant chitinase might thus be resistant to insects and fungi, because exposure to this enzyme might digest chitin in the gut lining of the herbivores and cell walls of plant pathogenic fungi. Even vertebrates contain inducible chitinolytic enzymes. Human macrophages secrete large amounts of a chitotriosidase upon activation, and the plasma of patients suffering from Gaucher's disease has high levels of this enzyme (Hollak *et al.*, 1994). These types of chitinolytic enzymes may play a defensive role against fungi and nematodes. Nematode-infected mammalian hosts often develop antibodies against nematode chitinases (Fuhrman *et al.*, 1992; Adam *et al.*, 1996).

### CHITINASE AS A BIOPESTICIDE

Considerable interest in the chitinolytic enzymes has been stimulated by their possible involvement as defens-

ive agents against chitin-containing pestiferous and pathogenic organisms, such as insects, nematodes, and fungi (Carr and Klessig, 1989; Linthorst, 1991; Sahai and Manocha, 1993). Resistance to undesired organisms can be imparted by degradation of their vital structures, such as the peritrophic membrane or cuticle of insects, egg shells or sheaths of nematodes, and the cell wall of fungal pathogens, or by liberation of substances that subsequently elicit other types of defensive responses by the host (Boller, 1987).

The peritrophic membrane and exoskeleton of insects act as physicochemical barriers to environmental hazards and predators. Both are composite materials made up primarily of chitin and protein, with the latter also containing some lipids, catecholamine metabolites, minerals, and other minor components. However, some entomopathogenic fungi such as *Metarhizium anisopliae*, *Beauveria bassiana*, *Nomuraea rileyi*, and *Aspergillus flavus* have overcome these kinds of barriers by producing multiple extracellular degradative enzymes, including chitinolytic and proteolytic enzymes that help the pathogens to penetrate the barriers and expedite infection (St Leger *et al.*, 1986, 1991a, b; El-Sayed *et al.*, 1989). Some types of insect venoms also contain hydrolytic enzymes, which might serve to facilitate the entry of components of the venom into chitin/protein-protected prey (Krishnan *et al.*, 1994). Similarly, the nematode, *B. malayi*, utilizes a chitinase to break down a protective chitinous extracellular sheath and/or the peritrophic membrane of the host mosquito to gain entry (Fuhrman *et al.*, 1992). Allosamidin, an inhibitor of chitinase, blocked the egress of malarial parasites trapped in the peritrophic membrane, which forms around the blood meal (Shahabuddin *et al.*, 1993). This inhibition could be overcome by the addition of an exogenous chitinase to the blood meal. Baculoviruses also contain genes for chitinases, which are related structurally to bacterial chitinases, but the precise role of those enzymes in infection of their hosts is unclear (Ayles *et al.*, 1994). A role in putrefaction of the host has been proposed (Hawtin *et al.*, 1995). Chitinolytic enzymes used by insects, nematodes, fungi, viruses, and other organisms for molting or penetration of structural barriers are potentially useful in pest management as targets for inhibitors. Alternatively, the pathogens or pests can be exposed to chitinases at inappropriate levels or developmental stages to increase their vulnerability to host defenses.

In the late 1970s, chitinases were shown to degrade insect gut peritrophic membranes *in vitro*. Brandt *et al.* (1978) proposed that chitinases cause perforations in the membranes, thus facilitating entry of the pathogens into the tissues of susceptible insects. However, not until many years later was evidence obtained that demonstrated such a physical effect *in vivo*. The addition of exogenous chitinase from *Streptomyces griseus* to the blood meal of the mosquito, *Anopheles freeborni*, prevented the peritrophic membrane from forming (Shahabuddin *et al.*, 1993). *Escherichia coli*-produced

recombinant endochitinase *ChiAII* encoded by *S. marcescens* was found by scanning electron microscopy to perforate *Spodoptera* larval midgut peritrophic membranes after *in vitro* incubations at concentrations of 0.1–10  $\mu\text{g/ml}$  (Regev *et al.*, 1996). Moreover, perforation of peritrophic membranes also occurred *in vivo* after fifth-instar larvae were fed a diet containing recombinant *ChiAII*.

Chitinases also facilitate the penetration of the host cuticle by entomopathogenic fungi (Coudron *et al.*, 1989; St Leger *et al.*, 1991a, b). Chitinases and  $\beta$ -N-acetylglucosaminidases are secreted when the entomopathogens, *M. anisopliae*, *B. bassiana*, *Verticillium lecanii*, and *N. rileyi*, are grown on insect cuticles (St Leger *et al.*, 1986; Coudron *et al.*, 1989). At the time of cuticle penetration, virulent isolates of *N. rileyi* exhibit substantially higher levels of chitinase activity than avirulent strains (El-Sayed *et al.*, 1989). Chitinase gene expression in entomopathogenic fungi is believed to be controlled by a repressor-inducer system in which chitin or some oligomeric products of degradation serve as inducers (St Leger *et al.*, 1986). The addition of carbohydrates, lipid, or proteins to chitin-grown cultures represses chitinase production.

Bacterial chitinases are generally ineffective in assays when some types of insects are fed diets containing the enzymes. No mortality of the nymphal stages of the rice brown plant hopper, *Nilaparvata lugens*, occurred when 0.09% (w/v) *S. griseus* chitinase was added to an artificial diet (Powell *et al.*, 1993). Similarly, *Serratia* and *Streptomyces* chitinases at 1–2% levels in the diet of the merchant grain beetle, *Oryzaephilus mercator*, caused no mortality (Kramer *et al.*, 1997). The inactivity of many of these chitinase preparations can be explained by the presence of primarily exo- instead of endo-cleaving enzymes, the former being substantially less effective than the latter in degrading chitin.

#### TRANSGENIC PLANTS EXPRESSING CHITINASES CONSTITUTIVELY

The role of plant chitinases in disease resistance is well documented (see Graham and Sticklen (1994) for a comprehensive review). Numerous plant and microbial chitinase cDNAs have been cloned. Some of these have been introduced into plants under the control of constitutive promoters, resulting in an enhancement of resistance of the host plant to fungal pathogens (Brogie *et al.*, 1991, 1993; Vierheilig *et al.*, 1993; Lin *et al.*, 1995). However, the role of endogenous chitinases from various sources in mediating plant resistance to insects is less well understood.

Chimeric gene constructs for tobacco hornworm chitinase were prepared by inserting the 1.8 kb *EcoRI* fragment from a cDNA clone containing the entire coding region (Kramer *et al.*, 1993) into binary vectors between single or double CaMV 35S promoters and either the *nos* or *pinII* polyadenylation signal sequences (Ding, 1995;

Ding *et al.*, 1997). These constructs were introduced into tobacco plants (*Nicotiana tabacum* var. *xanthi*) using an *Agrobacterium tumefaciens*-mediated transfer. Primary transgenic T<sub>0</sub> plants were shown by immunoblot analysis to express insect chitinase. The mass of the recombinant enzyme was only 46 kDa instead of 60 kDa or 85 kDa, which are the expected masses of the mature protein without and with glycosylation, respectively. Nonetheless, the small 46 kDa recombinant protein was enzymatically active (Wang *et al.*, 1996). N-Terminal sequences of both native (85 kDa) and plant-recombinant (46 kDa) proteins were identical, which demonstrated that the latter was truncated at the C-terminal end, whereas the former was not (Wang *et al.*, 1996). A similar situation in regard to truncated recombinant products occurred in the case of a chitinase from the nematode, *B. malayi*, which has a native molecular mass of 62 kDa (Venegas *et al.*, 1996). When a truncated cDNA of the *Brugia* chitinase gene was expressed in *E. coli*, a protein of only 43 kDa containing the N-terminal catalytic domain was produced. This protein was enzymatically active and had the same K<sub>m</sub> as the 62 kDa enzyme for oligo GlcNAc substrates but had a reduced affinity for high molecular weight chitin. Apparently, the C-terminal domain of the *Brugia* and *Manduca* chitinases plays a role in targeting and in enhancing the binding of the enzyme to chitin. Another chitinolytic enzyme that exhibits similar processing into distinct forms is human macrophage chitotriosidase (Renkema *et al.*, 1997). It is synthesized as a 50 kDa protein, but some of the protein is processed into a 40 kDa form. The spleen contains both 40 and 50 kDa enzymes (Renkema *et al.*, 1995). Like the recombinant *Manduca* chitinase from tobacco plants, the smaller human enzyme is C-terminally truncated. The C-terminal portion of the chitotriosidase contains O-linked glycans, as is probably the case for the *Manduca* chitinase.

Leaves excised from chitinase-positive and chitinase-negative transgenic tobacco plants expressing the *M. sexta* chitinase were fed to first instar larvae of the tobacco budworm, *Heliothis virescens* (Ding *et al.*, 1997). After 3 weeks, the total mass of larvae recovered from chitinase-negative leaves was nearly sixfold higher than the mass of larvae surviving on chitinase-positive leaves. Reductions in larval biomass and feeding damage also were observed using intact transgenic first generation progeny T<sub>1</sub> plants for bioassays. Larvae reared on plants lacking the *Manduca* chitinase gene consumed substantially more leaf tissue than did larvae feeding on plants expressing the gene. Overall, mortality was greater for larvae grown on chitinase-positive plants. Constitutive expression of the truncated insect chitinase in tobacco thus protected the plants from extensive feeding damage by the budworm.

In order to determine whether the *Manduca* chitinase from transgenic tobacco and several chitinases from other sources were directly toxic to insects, a beetle feeding study was conducted using the insect enzyme as well as



chitinases from two bacterial and one plant species. A relatively small insect species, the merchant grain beetle, *O. mercator*, was utilized in this bioassay because not enough recombinant enzyme from the tobacco plants could be purified for use in bioassays requiring larger insects. Recombinant *Manduca* chitinase from transgenic tobacco and chitinases from *Serratia* (bacterium), *Streptomyces* (Actinomycete), and *Hordeum* (plant) species were fed to neonate beetle larvae at a 1–2% level in the diet. The growth and survival of larvae consuming the microbial and plant chitinases were the same as those of larvae consuming the untreated diet, indicating that these chitinases have no adverse effect on beetle growth. On the other hand, all of the larvae consuming the recombinant insect chitinase-supplemented diet were dead within a few days after egg hatch (Wang *et al.*, 1996; Ding *et al.*, 1997). Therefore bioassay data obtained using both transgenic plants and a semi-artificial diet indicated that insect chitinase is more potent as a biopesticide than are several chitinases from other sources.

We know of few reports of the successful use of a plant chitinase in combating insect pests. Cereal grains do contain substantial levels of chitinases (10–100 µg/g are typical; Molano *et al.*, 1977; Wadsworth and Zikakis, 1984; Leah *et al.*, 1987). However, stored grains are susceptible to insect attack, suggesting that stored product insects have adapted to overcome the effects of plant chitinases. In further support of this conclusion, we found that transgenic rice plants expressing relatively high levels of a rice chitinase (0.05% total protein) had no detrimental effects on the growth of the fall armyworm, *S. frugiperda* (unpublished data). Nonetheless, enhanced resistance of plants to several fungal pathogens was obtained after the introduction of plant chitinase transgenes (Broglie *et al.*, 1991; Vierheilig *et al.*, 1993; Zhu *et al.*, 1994; Jach *et al.*, 1995; Lin *et al.*, 1995). Also, hen's egg white lysozyme, a vertebrate enzyme with both chitinase and muramidase activities, was found to inhibit the growth of several bacterial and fungal plant pathogens (Trudel *et al.*, 1995). However, enhancement of plant resistance to insects by these plant and animal enzymes was not reported. Another attempt was the use of a bean class I endochitinase gene for transformation of potato plants to enhance resistance to the tomato moth, *Lacanobia oleracea* (Gatehouse *et al.*, 1997). Although the bean chitinase accumulated at levels up to 2% of total soluble protein, it provided no protective effect against tomato moth larvae. Gatehouse *et al.* (1996) observed small but statistically significant decreases in the growth and fecundity of the peach-potato aphid, *Myzus persicae*, on transgenic potato plants expressing multiple genes that encode not only bean chitinase but a second potential biopesticide, snowdrop lectin. The results were interpreted to indicate that bean chitinase slightly reduced the total fecundity but had little overall effect on population build-up. Snowdrop lectin, on the other hand, appeared to delay aphid reproduction as well as population increase.

### SYNERGISM BETWEEN CHITINASES AND *BACILLUS THURINGIENSIS* (Bt)

Microbial chitinases have been used in mixing experiments to increase the potency of entomopathogenic microorganisms. Synergistic effects among chitinolytic enzymes and microbial insecticides have been known to occur since the early 1970s. Bacterial chitinolytic enzymes were used to enhance the activity of the bacterium, Bt, and a baculovirus, AcMNPV. Larvae of the spruce budworm, *Choristoneura fumiferana*, died more rapidly when exposed to chitinase-Bt mixtures than when exposed to the enzyme or bacterium alone (Smirnoff, 1973, 1974; Lysenko, 1976; Morris, 1976). The mortality of gypsy moth (*Lymantria dispar*) larvae was enhanced when chitinase was mixed with Bt relative to a treatment with Bt alone, in laboratory experiments (Dubois, 1977; Daoust, 1978). The toxic effect was correlated positively with enzyme levels (Gunner *et al.*, 1985). The larvicidal activity of a nuclear polyhedrovirus towards gypsy moth larvae was increased about fivefold when it was coadministered with a bacterial chitinase (Shapiro *et al.*, 1987).

Inducible chitinolytic enzymes from bacteria may cause mortality under certain conditions. These enzymes may compromise the structural integrity of the peritrophic membrane barrier and facilitate Bt toxin synergism by enhancing contact of the toxin molecules with their epithelial membrane receptors. For example, five chitinolytic bacterial strains isolated from the midguts of *Spodoptera littoralis* induced a synergistic increase in larval mortality when combined with *B. thuringiensis* spore-crystal suspensions relative to either an individual bacterial strain or a Bt suspension alone (Sneh *et al.*, 1983). An enhanced toxic effect towards *S. littoralis* also resulted when a combination of low concentrations of a truncated recombinant Bt toxin and a bacterial endochitinase were incorporated into a semisynthetic insect diet (Regev *et al.*, 1996). Crude chitinase preparations from *B. circulans* enhanced the toxicity of Bt *kurstaki* towards diamondback moth larvae (Wiwat *et al.*, 1996). These same researchers transformed Bt *israelensis* with chitinase cDNAs from two other bacteria, *Aeromonas hydrophila* and *Pseudomonas maltophilia*. Although the stability of the plasmids in the transformed Bt strain was demonstrated, the expression level of chitinase was very low, and no insect bioassay data were reported.

We recently demonstrated a synergistic interaction between insect chitinase and Bt using the tobacco hornworm as the test insect (Ding *et al.*, 1997). In contrast to results obtained with the tobacco budworm, studies with the hornworm revealed no consistent differences in larval growth or foliar damage when they were reared on first generation T<sub>1</sub> transgenic chitinase-positive plants as compared with chitinase-negative control plants. The possibility that some marginal detrimental effect on the structural integrity of the *M. sexta* peritrophic membrane was occurring, but not to the extent that affects

hornworm growth rate, was evaluated by measuring changes in the growth of insects reared on plants treated with sublethal doses of a Bt toxin. At levels where no growth inhibition was caused by either the transgenic insect chitinase or the toxin individually, combinations of both resulted in significantly less foliar damage and lower larval biomass production. The results indicated that the insect chitinase transgene can potentiate the effect of sublethal doses of Bt toxin and *vice versa*. Synergistic effects on Bt toxicity caused by other types of additives have been noted previously, i.e., synthetic pyrethroids (Saleem *et al.*, 1995).

To test for a potential synergism between Bt toxin and chitinase in another system, a bacterial chitinase gene, *chiAII*, from *S. marcescens*, was introduced by *Agrobacterium*-mediated transformation into alfalfa and tobacco, together with a synthetic Bt *cryIC* gene (Strizhov *et al.*, 1996). Unpublished data had indicated that chitinases might enhance the insecticidal activity of Bt toxins by destroying the chitinous peritrophic membrane of the insect midgut. Because the toxin levels attained in the chimeric plants alone were, however, sufficient to cause 100% mortality of beet armyworm, *Spodoptera exigua*, and Egyptian cotton leafworm, *S. littoralis*, testing for a synergistic interaction between chitinase *AII* and the *CryIC* toxin was not possible.

As a point of information, Guzzo and Martins (1996) recently reported that the application of a commercial formulation of Bt to coffee leaves caused local and systemic inductions of both chitinase and  $\beta$ -1,3-glucanase. Two- to threefold increases in enzyme activities were observed. Apparently, recent anthropic attempts to acquire synergistic effects involving Bt toxins and chitinases were not the first times this combinatorial strategy had been tried for pest control. This strategy may already be utilized rather frequently in nature.

#### BACULOVIRUS EXPRESSING INSECT CHITINASE HAS ENHANCED LARVICIDAL ACTIVITY

*M. sexta* chitinase has been found to increase the insecticidal activity of a recombinant baculovirus (Gopalakrishnan *et al.*, 1995). A recombinant non-occluded *Autographa californica* multiply embedded nuclear polyhedrovirus, which contained the 1.8 kb DNA fragment from *M. sexta* chitinase cDNA (Kramer *et al.*, 1993) under the control of the polyhedrin gene promoter, expressed a glycosylated chitinase with a mass of 85 kDa, which was indistinguishable in size and composition from the native enzyme in molting fluid. The recombinant chitinase was secreted into the growth medium when insect cell lines were infected with this virus. In addition, when the recombinant virus was injected into *M. sexta* larvae, chitinase was found in the hemolymph, where it does not occur normally. The recombinant baculovirus killed larvae of the fall armyworm (*S. frugiperda*) in approximately three-quarters of the time required for the wild-type virus to kill the larvae

( $LT_{50}$  = 65 h vs. 88 h, respectively; Gopalakrishnan *et al.*, 1995). The results indicated that insect chitinase, by increasing the killing rate of insect pathogens, may be useful as an enhancing factor for biological control agents.

#### FUTURE WORK AND CONCLUDING REMARKS

Insect chitinases appear to possess properties that make them uniquely useful for insect control, including efficacy towards species that are not susceptible to bacterial or plant chitinases. The cloning of the genes for several chitinolytic enzymes from insects has opened the possibility of obtaining transgenic crop plants with greater resistance to targeted pests. One of the difficulties in the generation of transgenic plants expressing insect genes appears to be the low level of translation of insect mRNA with codon usage quite different from that of plant mRNAs. Synthetic versions of the insect chitinase genes with plant-preferred codons should help to alleviate this problem. The accumulation of truncated forms in transgenic plants also may arise from the susceptibility of the foreign chitinases to host proteolytic enzymes. The short half-life of the insect chitinase, which is found briefly in the molting fluid only during the molting period, may be a necessary property for insect survival. However, the half-life of the enzyme in transgenic plants might be increased by deleting regions of the insect chitinase molecule that render the protein susceptible to proteolysis. We suspect that the serine/threonine-rich domains of the chitinases, which are also rich in proline and glutamic acid, may resemble the "PEST" region that has been proposed to be the target of a protease that facilitates protein turnover (Rogers *et al.*, 1986). Additional modifications of the chitinase genes by site-directed mutagenesis and/or deletions or swapping of specific functional domains may help to target the chitinase or enhance its activity and stability in insect pests.

We are currently developing occluded baculoviruses expressing an insect chitinase gene for possible direct use in insect control. Large-scale isolation of insect chitinases from culture media of baculovirus-infected cell lines or insects can lead to their use as an additive to microbial pesticide formulations, in an attempt to reduce  $LD_{50}$  levels of the pathogen. Such pyramiding strategies may also delay the development of insect resistance to insecticides such as Bt toxins. In addition, by weakening the peritrophic membrane, dietary chitinase may increase the susceptibility of insects to pathogenic bacteria, viruses, or bioactive compounds normally present in plants.

We are continuing to develop insect chitinase as a biocide for insects and plant pathogens, by introducing its gene into crop plants such as rice, wheat, corn, and sorghum. We are also making baculovirus vectors expressing various deletions and mutant forms of chitinase to identify regions that influence activity, stability, and affinity for the chitin matrix. These experiments may help researchers to design enzymes with improved insect-

ticidal properties. To target a wide range of pest species, additional research needs to be conducted into improving the expression levels of the enzyme and on using combinations of the chitinase gene and other insecticidal genes in transgenic plants and microbial biocontrol agents.

Chitin metabolism has been considered a relatively unique target for insect control agents because higher animals do not use chitin as a structural element. As in the case of plants, vertebrates may also utilize chitinases for defense purposes. Several reports now document the existence of chitinolytic enzymes and families of related proteins in higher animals (Hakala *et al.*, 1993; Malette *et al.*, 1995; Renkema *et al.*, 1995, 1997; Choi *et al.*, 1997). A role for mammalian chitinases as a defense mechanism against chitin-containing pathogens has recently been proposed, and some evidence for antifungal activity in a host response using guinea pigs has been obtained (Overdijk *et al.*, 1996). A number of mammalian proteins are structurally similar to family 18 chitinases, but are devoid of glycosidase activity, because an active-site catalytic residue has been replaced by non-catalytic amino acid residues (DeSouza and Murray, 1995). Apparently, structural elements typical of chitinases are utilized for binding of specific carbohydrates on surfaces of target cells by appropriate receptors. Many of these proteins are expressed in situations where tissue remodelling occurs, but their precise physiological roles remain unknown. Prior to the use of any chitinase as a pest control agent, relationships between the structure and function of that chitinase and vertebrate homologues should be determined, as well as any potential side-effects on non-target organisms. To the best of our knowledge, no investigations into the safety of food derived from transgenic plants with modified chitinase content have been published (Franck-Oberaspach and Keller, 1997).

Many host plant resistance strategies are based on multiple genes encoding a diverse set of resistance factors. A polygenic resistance system probably helps to prevent plant-feeding pest species from overcoming resistance by the host plant (Pimental, 1991). The addition of chitinolytic enzyme genes, whose encoded proteins adversely affect insect pests and microbial pathogens, to the repertoire of other defense genes in plants and entomopathogens, such as pest-specific toxins, protease inhibitors, antigens,  $\alpha$ -amylase inhibitors, hormone agonists and antagonists, and lectins, should enhance the effectiveness of this type of biotechnological control strategy. A more thorough understanding of the chemistry and regulation of chitin metabolism and other vital metabolic pathways in insects will help to facilitate the development of novel ways to control insect pests.

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