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Review

Protease Interactions With *Bacillus thuringiensis* Insecticidal Toxins

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The microbe *Bacillus thuringiensis* (Bt) produces crystals that contain insecticidal crystal proteins (ICPs) used to control many major pests. ICPs are degraded by proteases from a variety of sources, including those endogenous to the bacterium, those purified from animals and plants, or those found in insects. Proteases in the bacterium function in protein metabolism during sporulation; in some cases they hydrolyze ICPs. Insect proteases are implicated in Bt toxin specificity, mode of action and insect adaptation to Bt. This review describes the current knowledge of protease interactions with ICPs with special emphasis on the role of proteases in insect resistance to Bt toxins. Arch. Insect Biochem. Physiol. 42:1–12, 1999.

Key words: *Bacillus thuringiensis*; insecticidal toxins; proteases; resistance; review

INTRODUCTION

Bacillus thuringiensis (Bt) is a ubiquitous bacterium that forms crystals containing insecticidal proteins (ICPs) which are used to control lepidopteran, dipteran, and coleopteran insects (Schnepf et al., 1998). For many years, Bt has been used in spray applications to reduce damage by agricultural and forest insect pests. Spray applications have limited efficacy because ICPs degrade rapidly in ultraviolet light. This problem is circumvented in crops transformed with genes encoding ICPs. However, the consistent expression of Bt toxins in transgenic plants will increase the selection pressure in insects. Survival and propagation of resistant pests is a major concern.

ICPs, or Cry proteins, are in the form of protoxins in the crystal. ICPs are solubilized and processed to toxic peptides by gut proteases in susceptible insects. Because proteases are important to toxicity, research into interactions of proteases with Bt proteins may lead to improved toxin efficacy.

Proteases are defined as peptide hydrolases and include all enzymes that hydrolyze peptide bonds (Beynon and Bond, 1993). Proteinases refer to a specific class of proteases and are synonymous with the term endopeptidases, which cleave internal bonds in a peptide. Most of the proteases that degrade Bt ICPs are proteinases.

Proteases endogenous to the bacterium have been described. Bt proteases with physiological functions within the bacterium can degrade ICPs and affect insect toxicity. Insect gut

Abbreviations used: Bt, *Bacillus thuringiensis*; ICP, insecticidal crystal protein.

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proteases are involved in crystal dissolution and protoxin activation and contribute to toxin specificity. In some cases, insect proteases can further degrade activated ICPs; they may also be involved in receptor-toxin interactions and post-binding events. More important, insect proteases are implicated in some cases of pest adaptation to Bt toxins.

This overview of protease interactions with Bt insecticidal proteins presents a summary of data, together with discussion of the importance of research in this area with respect to the specificity of Bt toxins and resistance development. It is not intended as a comprehensive review of insect proteases or Bt insecticidal toxins. Several current reviews in these areas are available (Schnepf et al., 1998; Reeck et al., 1999; McGaughey and Oppert, 1999). On a final note, Bt crystal proteins have recently been renamed under a new nomenclature (Crickmore et al., 1998), which will be used in this review.

Endogenous Proteases in Bt

During the early sporulation phase, an increase in intracellular protease activity occurs in Bt cultures. Proteases endogenous to Bt have been described from the cysteine, metallo, and serine families of enzymes (Li and Yousten, 1975; Bulla et al., 1977; Chestukhina et al., 1980; Nickerson and Swanson, 1981; Stepanov et al., 1981; Bibilos and Andrews, 1988; Dalhammar and Steiner, 1984; Pfannenstiel et al., 1984; Andrews et al. 1985; Kumar and Venkateswerlu, 1997; Reddy and Venkateswerlu, 1997; Reddy et al. 1998). Major proteases in most Bt species are thermostable and many are metalloproteases, with some exceptions. Bt subsps. *tenebrionis*, which has ICPs with selectivity for coleopteran insects, and *kurstaki*, with selectivity for lepidopteran insects, have proteases from the metalloprotease class. However, serine proteinases constituted approximately 50% of the total activity in subsp. *israelensis*, which has ICPs selective for dipteran insects (Reddy et al., 1998).

The endogenous proteolytic activities in Bt may hydrolyze crystal proteins (Chestukhina et al., 1980; Bulla et al., 1981; Andrews et al., 1985; Haider et al., 1986; Bibilos and Andrews, 1988; Carroll et al., 1989; Kunitate et al., 1989; Dai and Gill, 1993; Donovan et al., 1997; Kumar and Venkateswerlu, 1997). A reduction in the size of Bt subsp. *tenebrionis* ICPs occurred during sporulation, and proteolysis was prevented by the addition of protease inhibitors (Carroll et al., 1989). Mosquitocidal ICPs were also degraded in the crystal (Dai and Gill, 1993). Genetic engineering to delete neutral protease A from Bt resulted in higher concentration of full-length ICPs (Donovan et al., 1997).

ICPs from Bt subsp. *kurstaki* crystals, incubated in denaturing and reducing conditions, were hydrolyzed by metalloproteases in the crystal (Kumar and Venkateswerlu, 1997). Interestingly, the toxin produced under these conditions was highly active against the cotton leafworm, *Spodoptera littoralis*, a species insensitive to native *kurstaki* crystals or toxins generated by exogenous proteases (Kumar and Venkateswerlu, 1998a). Although the crystal contained multiple Cry proteins, the toxin was homogenous, as demonstrated by two-dimensional polyacrylamide gel electrophoresis, and lacked the first 29 amino acids of the protoxin N-terminus (Kumar and Venkateswerlu, 1998b). This differed from a trypsin-generated toxin, which lacked an N-terminal peptide of 28 amino acids (Bietlot et al., 1989). Therefore, different polypeptides were obtained by incubation with enzymes from different sources, resulting in a difference in toxicity.

ICP Processing

The mode of action of Bt in the gut of a susceptible insect is complex, involving many steps in the conversion of ICPs to toxins (Gill et al., 1992). In the crystal, ICPs interact through hydrogen bonding, disulfide linkages, and hydrophobic interactions. In lepidopteran insects, ICPs are released in the alkaline gut and are hydrolyzed to toxins by proteases. Toxins bind to brush border membrane cells in the midgut; receptor/toxin aggregation leads to pore formation, ionic imbalance, cell lysis, and septicemia.

In early studies on the conversion of Bt protoxin to toxin, urea denaturation of ICPs was reported to be necessary for protoxin processing by plant or animal proteases (Lecadet and Dedonder, 1967). However, gut proteases from the cabbage butterfly, *Pieris brassicae,* were able to degrade protoxins under nondenaturing conditions, although the alkaline buffer may have aided

in dissolution of the crystals. With midgut fluid from the silkworm, *Bombyx mori*, alkaline dissolution of the crystals was demonstrated as a prerequisite to proteolysis (Faust et al., 1967). The authors suggested that crystal dissolution in vivo always precedes proteolytic activation. From in vitro experiments, it was determined that the alkaline conditions of the gut initiated swelling of Bt crystals, and proteases were involved in the subsequent dissolution and release of crystal protoxins (Tojo and Aizawa, 1983).

In Lepidoptera, gut proteases process ICPs from 130–140 kDa protoxins to toxic proteins of approximately 60–70 kDa. The processing of a 130 kDa lepidopteran-specific protoxin by mammalian serine proteinases proceeded via seven distinct sequential cleavages every 80–90 amino acids from the C-terminus (Chestukhina et al., 1982; Choma et al., 1990). Ten kilodalton fragments were rapidly hydrolyzed to smaller peptides and could not be detected electrophoretically. Proteolysis was discontinued with the production of a 67 kDa proteolytically-resistant core, which was the toxin.

Processing of the protoxin to toxin has also been reported to proceed by removal of both N- and C-terminal peptides. As with ICPs hydrolyzed in the crystal, processing of the N-terminus was determined by the type of protease. Processing of protoxins from Bt subsp. *kurstaki* strains HD-1 and HD-73 by midgut fluids from the smaller tea tortrix, *Adoxophyes* sp, *B. mori*, common cutworm, *Spodoptera litura,* diamondback moth, *Plutella xylostella*, housefly, *Musca domestica* resulted in polypeptides with different N-termini (Ogiwara et al., 1992). While most hydrolysates contained peptides beginning with arginine-28, those incubated with *P. xylostella* and *S. litura* gut proteases contained peptides initiating with leucine-46 and glycine-66 at the N- terminus, respectively. However, no correlation between N-terminal processing of protoxin and subsequent toxicity was found, as toxins obtained by all protease sources were toxic to *P. xylostella*.

Serine proteinases from different sources are involved in protoxin activation. In addition to serine proteinases in the bacterium, mammalian trypsin and chymotrypsin can degrade the protoxin (Cooksey, 1968; Chestukhina et al., 1982). Insect proteinases with trypsin- or chymotrypsin-like specificities can also hydrolyze Bt protoxins (Lecadet and Dedonder, 1966; Tojo et al., 1986; Milne and Kaplan, 1993; Dai and Gill, 1993; Oppert et al., 1996; Martínez- Ramírez and Real, 1996; Carroll et al., 1997).

Coleopteran insects have guts that are more neutral to acidic and coleopteran-specific ICPs are similar to the size of lepidopteran-specific toxins. Therefore, the processing of coleopteran-specific ICPs was considered unnecessary for toxicity. However, researchers have demonstrated that coleopteran-ICPs undergo dissolution. ICPs from Bt subsp. *san diego* (*tenebrionis*) dissolved only at pH values above 10 and below 4 (Koller et al., 1992). Furthermore, the soluble toxin was more toxic than crystals to the cottonwood leaf beetle, *Chrysomela scripta,*, suggesting that protoxin solubility and/or activation may be limiting in some beetles.

Recent data demonstrated that coleopteran-ICPs are solubilized and hydrolyzed to smaller toxic polypeptides. Hydrolysis of Bt subsp. *tenebrionis* ICPs, which contain Cry3 protoxins, by mammalian trypsin or gut extracts from the yellow mealworm, *Tenebrio molitor*, resulted in conversion from a 73 kDa protoxin to a 55 kDa toxin lacking the N-terminal 158 amino acid peptide (Carroll et al., 1989). Gut proteases from the Colorado potato beetle, *Leptinotarsa decemlineata,* hydrolyzed Cry3A to a 42 kDa protein that bound proteins in the midgut (Martínez-Ramírez and Real, 1996). Chymotrypsin-processing of Cry3A was necessary to obtain a toxic product that was soluble at a neutral pH, which is close to the physiological pH of 5–6 in susceptible beetles (Carroll et al., 1997). Furthermore, binding of the toxin to *L. decemlineata* gut membranes could only be demonstrated with chymotrypsin-treated or gut-protease processed Cry3A toxin (Martínez-Ramírez and Real, 1996; Carroll et al., 1997). Thus, coleopteran-active toxins are obtained via proteolytic processing, and this processing is necessary for toxicity.

The importance of beetle-specific ICP solubilization and activation is further emphasized in a study of Cry3C protoxins. Native crystals containing Cry3C were not toxic to *L. decemlineata*, whereas toxicity was enhanced significantly by

in vitro solubilization of the crystals in alkaline/reducing buffer and activation by either trypsin or *L. decemlineata* midgut fluid (Lambert et al., 1992). This "unmasking of silent activity" was also reported in Lepidoptera with crystals from Bt subsp. *dendrolimus* (Lecadet and Martouret, 1987).

The mosquitocidal protoxin Cry11Aa1 was hydrolyzed by bacterial proteases, mammalian trypsin and chymotrypsin, or mosquito gut proteases (Ibarra and Federici, 1986; Pfannenstiel et al., 1986; Dai and Gill, 1993). Cry11Aa1 was partially processed from 72 to 32–40 kDa proteins within the crystal by endogenous Bt proteases (Ibarra and Federici, 1986). Similar processing occurred with nonbacterial proteases. Based on N-terminal sequencing of proteolytic fragments, it was reported that trypsin-, chymotrypsin-, and thermolysin-like proteases from the southern house mosquito, *Culex quinquefasciatus*, were responsible for toxin processing (Dai and Gill, 1993).

Protoxin and Toxin Stability

Studies of proteases and Bt proteins have provided information about functional stability. Harsh denaturation and hydrolysis of Cry1 toxins by serine proteinases resulted in two smaller protease-resistant fragments (Chestukhina et al., 1990; Convents et al., 1991). Sequencing revealed that these regions are subdomains of the toxin, corresponding to the conserved, toxic region that participates in pore formation, and the variable, host recognition region that binds to midgut receptors. This same subdomain organization was found in Cry3 toxins (Ort et al., 1995).

DNA was reported to copurify with ICPs and apparently maintains the structural integrity of the protoxin necessary for proteolytic digestion (Bietlot et al., 1993; Clairmont et al., 1998). The protoxin was associated with a 20 kilobase DNA fragment, which was resistant to digestion by DNase. However, removal of the fragment from the protoxin by extensive DNase digestion prevented proteolysis and subsequent activation of the protoxin by trypsin. Therefore, it was proposed that the association with DNA provided the complex with a tertiary structure amenable to proteolysis, and removal of the DNA altered the protoxin structure such that the proteolytic sites were protected.

Research with mutant toxins indicated that toxin stability and/or expression levels are affected by the N-terminal amino acid sequence. Trypsin-hydrolysis of protoxin Cry9Ca1 produced a toxin with an N-terminus of arginine-44 (Lambert et al., 1996). The toxin was further degraded to smaller nontoxic peptides after extensive incubation with trypsin. Elimination of trypsin cleavage sites in mutants resulted in a toxin resistant to further degradation, but toxicity was not enhanced.

A similar report described the alteration of a gene encoding Cry1C to increase expression levels. Purified Cry1C is effective against the *Spodoptera* sp. armyworms, yet transgenic plants containing Cry1C toxins have not provided protection from these insects, presumably due to low toxin expression. The trypsin-resistant core of the toxic fragment of Cry1C ranged from isoleucine-28 to arginine-627 (Strizhov et al., 1996). However, bacteria expressing toxins with truncated N-termini did not grow, suggesting that the N-terminus is important to cell viability and toxin stability and/or expression.

Bt Toxins and Proteases

While much is known about the initial interactions of proteases and ICPs, relatively little is understood about the involvement of proteases in toxicity once the toxin is formed. In vitro, the toxin can be degraded further with papain, elastase, or trypsin after boiling under denaturing conditions (Choma et al., 1990). Some insensitive insects were found with either higher proteolytic activity or a relatively higher concentration of proteases in the gut, which resulted in hydrolysis of the core polypeptide (Ogiwara et al., 1992). This provided evidence that proteases may be involved in toxin specificity and resistance development, as will be discussed in subsequent sections.

Proteases have also been reported as membrane receptors for Bt toxins. Aminopeptidase N, an exopeptidase in the brush border membrane, has been documented as a Cry1Ac binding protein in the tobacco hornworm, *Manduca sexta*, tobacco budworm, *Heliothis virescens, P. xylostella*, the gypsy moth, *Lymantria dispar* (Knight et al., 1994; Sangadala et al., 1994; Gill et al., 1995; Valaitis et al., 1995; Lee et al., 1996; Luo et al.,

1997). However, there is no evidence that the aminopeptidase hydrolyzes toxin. Toxin binding did not affect aminopeptidase activity, unlike alkaline phosphatase in *H. virescens*, which was inhibited by Cry1Ac (English and Readdy, 1989).

Another Cry1A binding protein in *M. sexta*, a 210 kDa cadherin-like protein, is associated with proteases (Vadlamudi et al., 1993; Francis and Bulla, 1997). A trypsin-like protein copurified with the cadherin protein, and the activity of the trypsin-like protein was unaffected by toxin binding. Decreased binding of Cry1Ab was observed when membrane vesicle preparations were held at room temperature, indicating that the receptor was degraded by intrinsic proteases.

Few data are available on the involvement of proteases with the later stages of toxicity, such as protease interactions with toxin-receptor complexes or the membrane pore formation process. However, the ability of toxins to form channels in lipid bilayers was dependent upon the enzyme source used for protoxin activation, suggesting that proteases may influence pore formation (Smedley et al., 1997).

Role of Proteases in Toxin Selectivity

Because proteases are involved in the solubilization and activation of Bt protoxins, they are thought to control the degree of toxicity at an early step in the mode of action. Haider et al. (1986) provided data suggesting that insect proteases could determine Bt specificity. This study examined ICPs from Bt subsp. *aizawai* (formerly *colmeri*), which are toxic to both lepidopteran and dipteran insects. Protoxins activated by gut extracts from the mosquito, *Aedes aegypti*, were toxic only to dipteran cell lines and larvae, whereas those activated by extracts from *P. brassicae* were toxic only to lepidopteran cells and larvae. Incubation of the protoxin with lepidopteran proteases or mammalian trypsin resulted in conversion to a 55 kDa protein, whereas hydrolysis with dipteran proteases resulted in a 52 kDa protein. Further processing of the lepidopteran-specific toxin by dipteran gut proteases yielded the smaller, dipteran-specific form. Haider and Ellar (1987) also found that trypsin activation of *aizawai* protoxin yielded toxins that bound specifically to lepidopteran but not dipteran cells. However, when these trypsin-activated toxins were further hydrolyzed with gut proteases from *A. aegypti*, a protein was obtained that bound to *A. aegypti* but not lepidopteran cells.

The types and/or activity levels of gut proteases may influence toxin sensitivity. In research on *P. brassicae, Mamestra brassicae, S. littoralis*, a direct correlation was found between the toxicity of Bt subsp. *thuringiensis,* gut protein concentration, and protease activity (Bai et al., 1990). Toxin degradation was proposed as the mechanism of toxin insensitivity in the cotton bollworm, *Heliothis armigera* (Shao et al., 1998). Bt subsp. *kurstaki* ICPs, toxic to *B. mori* but not to *H. armigera*, were hydrolyzed to toxin when incubated with *B. mori* gut proteases. Relatively lower amounts of toxin were produced with *H. armigera* midgut fluid because of excessive toxin degradation by chymotrypsin-like proteases. In vivo, combinations of protoxin and serine protease inhibitors resulted in a synergism of activity in *H. armigera* larvae. These results support the hypothesis that serine proteinases were responsible for the lack of toxicity in *H. armigera* because of a reduction in toxin titer.

Excessive toxin degradation was also implicated in the insensitivity of the Eastern spruce budworm, *Choristoneura fumiferana,* to Bt (Pang and Gringorten, 1998). Low amounts (1%) of *C. fumiferana* midgut fluid incubated with Bt subsps. *kurstaki* and *sotto* yielded large amounts of toxin and relatively higher toxicity to *B. mori* than incubations containing 50 times more midgut fluid. Increasing volumes of midgut fluid from *B. mori,* which is more sensitive to *kurstaki,* resulted in correspondingly more toxin and enhanced toxicity. Therefore, it was predicted that *C. fumiferana* had a proteinase-mediated mechanism to eliminate toxins, rendering it less susceptible to Bt toxins than *B. mori*, which lacks such an adaptive mechanism.

Another possible mechanism for the reduction of Bt toxicity is removal of the activated protein by sequestration. Toxin sequestration by gut proteases has been reported in *C. fumiferana.* Precipitation of Bt subsp. *sotto* toxins by a gut proteinase of *C. fumiferana* led to limited proteolysis of the toxin and a loss of larval toxicity (Milne et al., 1995). The precipitating proteinase had

elastase-like activity and interacted with the C-terminal region of Cry1Aa toxin. Combined, these results suggested that some insensitive insects may degrade, precipitate, or eliminate the toxin faster than susceptible strains.

A suspension of crystalline Cry3A was toxic to the potato aphid, *Macrosiphum euphorbiae*, yet Cry3A which was solubilized and filtered to remove spores or crystalline toxin lacked activity (Walters and English, 1995). It was suggested that the toxin may have been more potent as a suspension due to the need for slow solubilization in the aphid midgut. These results indicated that activation/solubilization processes may occur in insects other than Lepidoptera and Coleoptera and affect Bt toxicity.

Larvae often undergo a decrease in sensitivity to Bt toxins as they age (McGaughey, 1978; Sneh et al., 1981; Bai et al., 1993; Johnson and McGaughey, 1996) and this may also be related to proteinase activity. In *S. littoralis,* an increase in toxin degradation in fifth instar larvae was associated with an increase in the specific activity of gut proteases, which was proposed to account for the loss of Cry1C sensitivity in older larvae (Keller et al., 1996).

Other in vivo studies of gut proteases and the specificity of activated toxins have been inconclusive. Toxins were obtained by incubating different strains of Bt with proteases from *S. littoralis* and *P. brassicae* (Lecadet and Martouret 1987). Toxins from either protease source were as active as native crystals in *S. littoralis* larvae. However, *P. brassicae* larvae were much more sensitive to native crystals than to proteolytically activated toxins.

The toxicities of 14 different Bt strains were determined for *P. brassicae, H. virescens, S. littoralis* (Jacquet et al., 1987). The relative toxicities varied greatly, depending on whether insects were fed crystals, solubilized crystals, or in vitro-activated toxins. Similarly, solubility of crystals was a factor for toxicity in some lepidopteran larvae but not others (Aronson et al., 1991). Variable toxicities were reported in lepidopteran forest pests to HD-1 activated by *B. mori* midgut fluid, although no comparisons were made to crystal toxicity (van Frankenhuyzen et al., 1991).

Insect Proteases in the Development of Resistance to Bt

Successful insect control with Bt transgenic plants has resulted in the expanded use of Bt crops. Extensive planting of Bt transgenic crops will increase toxin exposure to insects, leading to additional selection pressure for resistance. Insects have been selected for resistance to Bt toxins in the laboratory(reviewed in McGaughey and Oppert, 1999). Field resistance has also been reported in *P. xylostella* collected in fields where Bt sprays were used (Kirsch and Schmutterer, 1988; Tabashnik et al., 1990; Hama et al., 1992; Shelton et al., 1993; Perez and Shelton, 1997). Assessment of the frequency of resistance alleles in field populations of *H. virescens* indicated that resistance in nontarget species could develop in 3 to 4 years (Gould et al., 1997). Because proteases are important at different stages in the mode of action of Bt, resistance management may be improved by studying protease interactions with Bt toxins in insects that survive on Bt transgenic plants.

Much of the data on physiological adaptations in resistant insects suggest that changes occur in toxin receptors (reviewed in McGaughey and Oppert, 1999). Receptor-mediated mechanisms may include a loss of toxin binding sites, increase in nonspecific binding unrelated to toxicity, and reduction in toxin/receptor aggregation that is associated with pore formation. However, resistance is also caused by changes in ICP solubilization and/or activation reactions that are mediated by proteases. These resistance mechanisms may include changes in gut pH, or modifications in gut proteases that could lead to changes in solubility, differences in the degree of protoxin activation, enhanced toxin or receptor-toxin degradation.

Laboratory studies with the Indianmeal moth, *Plodia interpunctella*, found no differences in midgut proteinase activity from susceptible and Bt *kurstaki*-resistant strains (Johnson et al., 1990). However, another strain of resistant *P. interpunctella* insects, selected with Bt subsp. *entomocidus,* had significantly lower soluble gut proteinase activities (Oppert et al., 1994, 1996). Proteinases in gut extracts from the *entomocidus*-resistant insects processed Bt protoxin less efficiently than those from the susceptible parent

strain or a strain resistant to Bt subsp. *kurstaki*. Comparison of the phenotypic expression of gut proteinases in *P. interpunctella* strains revealed the absence of a major serine proteinase activity in *entomocidus*- and *aizawai*-resistant strains (Oppert et al., 1997). This proteinase was shown to hydrolyze Bt protoxin; the loss of the proteinase was genetically linked to Bt resistance. Furthermore, the absence of this proteinase was not transient. When insects lacking the serine proteinase were placed on untreated diets, the proteinase activity was never recovered, indicating that this genotype was stable (unpublished data). Because serine proteinases are involved in the activation of Bt protoxin in *P. interpunctella* (Oppert et al., 1996), loss of enzymes involved in protoxin activation could contribute to toxin resistance.

Slower Cry11A1 protoxin processing was also reported in Bt-resistant strains of *C. quinquefasciatus* (Dai and Gill, 1993). Similarly, differences in Cry1Ab protoxin processing were described in Bt-resistant *H. virescens* (Forcada et al., 1996). However, not only did proteases from an HD-73-resistant strain hydrolyze Cry1Ac protoxin more slowly than those from a susceptible strain, but a subsequent degradation of the activated toxin was also faster with resistant strain proteases. Increased toxin degradation results in lower toxicity, but other toxin-eliminating mechanisms, such as toxin sequestration, and precipitation, have not yet been reported in resistant insect populations. Any toxin-eliminating resistance mechanism would have serious implications for transgenic plants expressing the activated forms of Bt toxins.

Transgenic Plants With Bt Toxin and Protease Inhibitor Genes

Transgenic plants have been constructed with a combination of Bt toxin genes and protease inhibitor genes in an effort to increase insecticidal activity and reduce the potential of resistance development. Trypsin and chymotrypsin inhibitors potentiated the insecticidal activity of Bt toxins both in the diet and in tobacco plants with transgenes from Bt (Cry1Ab) and squash (*Curcurbita maxima*, trypsin protease inhibitor) (MacIntosh et al., 1990). With *P. xylostella*, however, soybean trypsin inhibitors had no significant effect on Bt toxicity or resistance to Bt (Tabashnik et al., 1992). The conflicting results may be due to the choice of inhibitors, difference in bioassay procedures, or a compensation by the insect for the presence of inhibitor, as has been reported in other insects (reviewed in Oppert, 1999).

Several other transgenic plants have been described with the coexpression of genes for Bt toxins and protease inhibitors. Individual *Arabidopsis thaliana* plants were transformed with a gene encoding Cry1C or the trypsin inhibitor from cowpea, *Vigna unguiculta*, and were bred for the coexpression of both genes (Santos et al., 1997). Protection from insect predation with plants expressing both genes was less than transgenic Cry1Ac-expressing plants but better than transgenic cowpea trypsin inhibitor-expressing plants. However, tobacco plants homozygous for Bt toxin and cowpea trypsin inhibitor genes were highly resistant to damage by *H. armigera*, achieving almost 100% mortality (Zhao et al., 1996). These results correlated to those of Shao et al. (1998), in which a synergism of activity in *H. armigera* was observed with both Bt protoxin and serine proteinase inhibitors.

Bt-transgenic tobacco was compared to cowpea trypsin inhibitor-transgenic tobacco in field trials (Hoffmann et al., 1992). Each transgenic was protected from damage by the corn earworm, *Helicoverpa zea*. However, Bt-transgenics exhibited better control with less variation than inhibitor-transgenics. In addition to insect control, Bt toxins and proteinase inhibitor genes are being coexpressed in cotton to delay pest adaptation to both groups of insecticidal control proteins (Pannetier et al., 1997).

The use of protease inhibitors in combination with Bt toxins presents an interesting paradox. Interference with proteases that activate Bt protoxins would decrease the production of toxins; this may partially explain the negative results obtained thus far with Bt toxins and protease inhibitors. This hypothesis is also supported by protease-mediated resistance in insects that survive when fed Bt-treated diets because they lack Bt-activating proteases. However, an increase in the expression of and/or activity levels of toxin-degrading proteases could

also result in toxin insensitivity in some insects. It was suggested that the increase in Bt toxicity observed with protease inhibitors may be due to a reduction in the degradation of toxin in insects with an appropriate adaptive mechanism, such as those with proteases capable of hydrolyzing toxin (Pang and Gringorten, 1998). The introduction of proteinase inhibitors may increase the activity spectrum to insects that degrade toxin, either by a species-specific trait or an adaptive mechanism. Regardless, information is lacking on the complexity and regulation of insect digestive proteases. Until we gain more knowledge in this area, responses to the ingestion of proteins that interact with digestive proteases cannot be predicted.

Concluding Remarks

Many factors are involved in Bt toxicity to insects, including the type of toxin, physicochemical conditions of the lumen, proteases that influence solubility, and/or processing, and target receptors in the insect. There are major differences in the hydrolytic pattern of Bt protoxins, depending on the source of ICP or protease. Differences in toxicity result from protoxin processing with proteases from different sources.

The complement and/or relative activity of proteases in the insect can also determine an insect's physiological response to different Bt toxins. Toxin-insensitive insects may not process the protoxin efficiently, or they may hydrolyze the toxin excessively. Adaptations in insects that promote either of these factors will lead to decreased sensitivity.

Understanding protease differences in insects that are susceptible or tolerant to Bt is useful from several aspects. Clearly, toxin receptors provide toxin selectivity in susceptible insects and are involved in some resistance events. However, insect proteases are also involved in toxin selectivity and in resistance development to Bt, the characterization of these proteases will provide critical information needed to develop effective resistance management of Bt toxins.

If insects are insensitive to Bt or have adapted through a change in protease expression by changing either the type(s) of proteases expressed or the level of expression, the efficacy of Bt toxins can be enhanced in several ways. Research has demonstrated that protease inhibitors can be used to increase Bt toxicity in insects that eliminate the toxin via degradation. Bt toxin genes used to transform plants for insect resistance may also be modified so that proteolytic recognition sites are eliminated. Alternatively, in cases where the protoxin is not hydrolyzed efficiently, protease-recognition sites may be engineered into the toxin to enhance toxicity. Continued research on insect proteases will identify improvements for enhanced efficacy of Bt insecticidal toxins.

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