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CRY PROTEINS FROM *BACILLUS THURINGIENSIS* BERLINER: FROM LABORATORY TO FIELD APPLICATION

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ABSTRACT

Conventional application of Bt-based microbial insecticides has resulted to varying degrees of success. The use of Cry proteins from *Bacillus thuringiensis* Berliner for insect pest management has been resurrected through the application of modern biotechnology. Highly significant advances in the area of Cry protein biochemistry and molecular mode of action are also presented.

Key words: *Bacillus thuringiensis*, Cry proteins, Bt transgenics, genetic engineering, host plant resistance, biotechnology, IPM

INTRODUCTION

It was in a silkworm farm in Japan that Ishiwata first took notice of a diseased silkworm (*Bombyx mori* L.) larvae. Since then, *Bacillus thuringiensis* Berliner (Bt) has made a name for itself as it is known today. Several natural insecticides have been discovered but none has generated so much interest from the academe, government policy makers and the corporate world than Cry proteins from Bt. Cry proteins are effective insecticides when delivered properly and are safe to non-targets including humans. The success in using Cry proteins for insect pest management is well known and its use has contributed to the significant reduction in the use of synthetic chemical insecticides. It is of interest to review the development of Cry proteins as a microbial insecticide in light of its modern application for improving our agricultural output.

BIOLOGY, CRY PROTEIN STRUCTURE AND MODE OF ACTION

Bt is a Gram-positive spore-forming bacterium. It is closely related to *Bacillus cereus* except for the production of proteinaceous inclusion bodies during sporulation. The inclusion body is made up of insecticidal Cry proteins which account for up to 25% of the dry weight of sporulated cells (Agaisse & Lereclus, 1995). This much accumulation of protein in the inclusion body is made possible by expressing the corresponding gene from a strong promoter in a non-dividing cell in order to circumvent protein dilution by cell division (Agaisse & Lereclus, 1995). Bt also produces other less known toxins. The α-exotoxin is proteinaceous and thermolabile (Krieg, 1971) but it is also toxic to vertebrates (Krieg, 1971). The β-

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exotoxin was first detected in Bt cultures by McConnell & Richards (1959). It is heat-stable and kills insects when injected into the hoemocoel. Phospholipase C is a thermolabile enzyme believed to act on cellular contents after penetrating the cell membrane (Heimpel, 1967). Recently, a lepidoptericidal vegetative insecticidal protein (vip) with no homology with the Cry proteins was discovered by Estruch *et al.* (1996). The protein is secreted by Bt into the medium during both vegetative and sporulation stages of growth.

The *Bacillus thuringiensis* insecticidal crystal protein is composed of about 130 kDa protoxins. Upon solubilization of the crystal protein into protoxin, further proteolytic action in the insect gut produces the 65 kDa activated Cry protein (Bietlot *et al.*, 1989; Choma *et al.*, 1990; Hofte *et al.*, 1986). The 3-D structures of four Cry proteins (Cry1Aa, Cry2A, Cry3A and Cry3B) have been determined. The overall structure is similar among the four Cry proteins. For example, Cry3A tertiary structure (Li *et al.*, 1991) clearly showed that the two folding domains hypothesized from previous proteolysis and unfolding studies (Choma *et al.*, 1990; Convents *et al.*, 1990) correspond to the α-helical domain I and the other half as domains II and III combined as a unit (Knowles, 1994). The 3-D structure of Cry1Aa toxin is similar to Cry3A (Grochulski *et al.*, 1995). Cry1Aa also has a 3-domain structure composed of seven α-helical bundle domain I, the loop region of domain II and the mainly β-sheet domain III.

After activation in the alkaline midgut lumen of susceptible lepidopteran larvae, the Cry protein binds to midgut receptors. Cry protein binding to midgut receptors was first hypothesized by Van Rie *et al.* (1989) to be irreversible. Liang *et al.* (1995) demonstrated the irreversibility of Cry1A toxin binding to the gypsy moth *Lymantria dispar* midgut. In Cry3A, loop 3 in domain II is found to affect irreversible binding in *Tenebrio molitor* (Wu & Dean, 1996). Domain III, loop 2 residues in Cry1Ab were involved in the irreversible binding process in *Manduca sexta* (Rajamohan *et al.*, 1995; 1996). Chen *et al.* (1995) have also discovered that certain residues in domain I affect irreversible binding to *M. sexta* midgut receptors. The role of domain III in binding was explored by Lee *et al.* (1995). They found out from domain III exchanges between Cry1Aa and Cry1Ac that residues 451-623 from Cry1Ac had strong binding to aminopeptidase N. Dean *et al.* (1996) proposed that domain I and III insert into the membrane and possibly comprise one of the units in a functional ion channel.

The receptors involved in the interaction with Bt toxin were recently identified and cloned. The first insect receptor to be cloned was a 210 kDa cadherin type Cry1Ab receptor from *M. sexta* (Vadlamudi *et al.*, 1995). In the same year, Knight *et al.* (1995) reported a *M. sexta* 120 kDa aminopeptidase N receptor (APN) for Cry1Ac. This receptor has a glycosyl-phophatidylinositol (GPI) membrane anchor that can be cleaved from the rest of the APN producing a 115 kDa molecule by phosphatidylinositol-specific phospholipase C (PIPLC) (Lu & Adang, 1996).

Following the binding of toxin to receptors and irreversible insertion of the Cry protein to the apical membrane of the insect midgut, ion channels are believed to be formed by the toxin. Helix 4 (Masson *et al.*, 1999) and Helix 7 (Alcantara *et al.*, 2001) of domain I were found to be involved in ion channel activity of the Cry protein. At the tissue level, the effect of ion channel formation is manifested as the collapse of transepithelial potential in open-circuited conditions and the inhibition of short-circuit current measured in voltage clamped midguts. Once ion

channels are formed by the Cry protein across the midgut apical membrane, the entry of extraneous material into the cell cytoplasm results in classic histopathological changes (Bravo *et al.*, 1992) that eventually leads to the death of the larva.

BT FERMENTATION

When formulating a fermentation medium, factors such as cellular stoichiometry and the desired amount of biomass to be produced must be considered. The basic concept is simply a material balance: small organic and inorganic molecules are converted into biomass during the course of cellular growth (Bailey & Ollis, 1986). Nutrients must be supplied in sufficient amounts and proper proportions in order to synthesize the specified amount of biomass. The provision of necessary minerals is also important in medium formulation.

In semi-solid fermentation, Bt is grown in a liquid medium which has been absorbed on the surface of small particles of a carrier. The result is a large liquid-gas interface due to high ratio of surface area to volume. Possible sources of carrier are wheat bran, ground corn, peanut meal and rice hulls. It is also possible to use inorganic carriers such as diatomaceous earth, vermiculite and expanded volcanic glass. To maximize aeration and reduce clumping, a mixture of bran and inorganic carrier is often used. Organic carriers provide nutrients to Bt during fermentation and also serve as absorption base (Dulmage & Rhodes, 1971).

Submerged fermentation starts with providing seed culture produced in increasing volume of medium in shake flasks and small fermenters. Aseptic transfers are timed when Bt is in a vigorous stage of growth. The production fermenter is then inoculated with 0.1% up to 10% by volume of the seed. Temperature, aeration, pH and foaming are carefully controlled during fermentation. Batch fermentation of Bt usually takes about 3 days. Fermentation and recovery costs are higher than in semi-solid fermentation but yields are usually greater in submerged fermentation and the product can be concentrated (Dulmage & Rhodes, 1971).

FORMULATION

The products of Bt fermentation (crystals, spores) are usually mixed with inert carrier to preserve potency and to facilitate delivery to the target insect pest. Commercial liquid formulations of Bt are usually in the form of concentrated emulsion which is directly prepared from the fermentation product (thick paste) (Angus & Luthy, 1971). On the other hand, Bt produced from semi-solid fermentation is usually formulated as dust. Additives are usually added to the formulation to maximize delivery of Bt spores and crystals to target insect pest. For example, spreaders or wetting agents are added to water diluent to ensure wetting of surface to be sprayed. Spreaders stabilize the liquid/solid interface. Stickers are also included to create a weather-resistant film on surface of foliage. Protectants minimize the effects of prolonged exposure to sunlight. For example, Cry protein encapsulated in transformed and heat-killed *Pseudomonas* cells performed better than conventional Bt isolates in the field (Gelernter, 1990).

BIOTECHNOLOGY

It was Gonzalez *et al.* (1981) who confirmed that Cry protein production is associated with plasmid DNA. In the same year, Schnepf & Whiteley cloned one of the subsp. *kurstaki* Cry genes in *Escherichia coli*. As a result, novel approaches were made to improve delivery of Cry proteins to target insect pests. Cry genes were transferred to endophytic bacteria which serve as delivery agent (Dimock *et al.*, 1988). Spectral activity was also altered leading to improved insecticidal activity to more than one target insect pest (Carlton, 1988).

The successful expression of Cry genes in plants offered new possibilities for insect pest management. Reports of production of Bt Cry proteins in tobacco (Barton *et al.*, 1987; Vaeck *et al.*, 1987), tomato (Fischhoff *et al.*, 1987), cotton (Perlak *et al.* 1990), corn (Koziel *et al.* 1993) and rice (Fujimoto *et al.* 1993) soon followed. Eventually, Bt cotton, Bt potato and Bt corn were already available in the U.S. market in 1995-1996 (James, 1997).

SUMMARY AND CONCLUSION

The availability of three-dimensional structures of Cry1Aa, Cry2A, Cry3A and Cry3B has permitted mutational analyses that led to the understanding of the complex mode of action of Cry proteins. It has now been established that Cry proteins are activated by proteolytic enzymes in the midgut of susceptible insects followed by binding to midgut receptors, irreversible insertion into the midgut apical membrane and lethal pore formation. Since Cry proteins are stomach poisons, proper delivery to target insects is critical for effective insecticidal action. Application of Cry proteins in various formulated products has been done with varying degrees of success due to very limited field persistence. A more efficient approach in using Cry proteins for insect pest management is through genetic engineering. It is now possible to produce Cry proteins *in planta* (Bt crops) such that commercial production of several high value Bt crops is now a reality.

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