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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

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New tool for spreading proteins to the environment: Cry1Ab toxin immobilized to bioplastics

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Abstract A new tool to provide an environmentally friendly way to deliver active proteins to the environment has been developed, based on the use of polyhydroxyalkanoate (PHA, bioplastic) granules. To illustrate this novel approach, a derived Cry1Ab insect-specific toxin protein was in vivo immobilized into PHA granules through the polypeptide tag BioF. The new toxin, named Fk-Bt1, was shown to be active against *Sesamia nonagrioides* (Lepidoptera: Noctuidae). The dose–mortality responses of the new toxin granule formulation (PFk-Bt1) and purified Cry1Ab have been compared, demonstrating the effectiveness of PFk-Bt1 and suggesting a common mode of action.

Introduction

The development of novel tools and concepts for environmental applications of recombinant technologies that could overcome public concerns about the environmental release of genetically modified organisms is a challenge in terms of ecology and industrial sustainability (de Lorenzo 2001).

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Polyhydroxyalkanoates (PHAs, bioplastics) are produced by certain bacteria and accumulated as reserve granules in the cytoplasm when the culture conditions are not optimal for growing (Steinbüchel et al. 1995; Madison and Huisman 1999). The PHA granules contain phospholipid-coated polyesters and granule-associated proteins (GAPs) at the surface (Steinbüchel and Hein 2001). Phasins are the main components of GAPs (Liebergesell et al. 1992; Pieper-Fürst et al. 1995; Schembri et al. 1995; Wieczorek et al. 1996; McCool and Cannon 1999; Prieto et al. 1999) that are used to stabilize the PHA granules by generating a hydrophilic interphase between the cytoplasm and the hydrophobic core of polymer (Pötter and Steinbüchel 2005). Taking advantage of the physiological role of these proteins, we have recently developed a novel protein fusion tag system (named BioF tag) that allows the construction of different chimeric fully functional reporter proteins immobilized in vivo into the PHA granules. Besides, these fusion proteins coprecipitate with the granules in a simple centrifugation process (Moldes et al. 2004). The BioF tag was constructed using the N-terminal region of phasin PhaF, contained in the medium-chain-length PHA granules of *Pseudomonas putida* GPo1, which behaves as a functional domain able to bind PHA granules (Prieto et al. 1999; Moldes et al. 2004).

On the other hand, growing interest in biorational pest control has placed the Cry toxins from *Bacillus thuringiensis* (Bt) at the leading biopesticides. Bt spore–crystal proteins are a useful alternative to synthetic chemical pesticide application in commercial agriculture, forest management, and mosquito control (Schnepf et al. 1998). *B. thuringiensis* has also been a key source of *cry* genes for transgenic expression in plants to render crops resistant to insect pests (Sharma et al. 2004). They have no toxicity to humans and most beneficial insects, making Cry proteins crucial components of integrated pest management strategies aimed at preserving natural enemies of insect pests (Glare and O'Callaghan 2000). Selection of environmentally safe and cost-effective formulations to extend the insect host range and to increase the residual activity of commercial Bt products is one of the major

needs for widening their use in pest control programs (Navon 2000).

In this report, a BioF insect-specific toxin immobilized to bioplastics based on Cry1Ab protein has been generated, suggesting that the BioF tag could be exploited as a new tool for spreading active polypeptides to the environment.

Materials and methods

Strains, plasmids, and DNA manipulations

The strains and plasmids used in this study are listed in Table 1. To construct pGNF2, the BioF tag encoding DNA fragment was PCR-amplified by using the previously described NF1 and CF1 oligonucleotides (Moldes et al. 2004) and cloned directly in pGEM-T (Table 1; Fig. 1). The DNA fragment coding for the 653 amino acids of the wild-type Cry1Ab protein with known insecticidal activity (Fig. 1) (Schnepf et al. 1998) was isolated by PCR using as template the total DNA of the strain *B. thuringiensis* spp. *kurstaki* CECT4454. The primers applied were as follows: 5BT, 5'-CCGTTGACATAGAAACTGGTTACACCCC-3' 3BTL, 5'-AACTGCAGTTACTCAACTAAATTGGATAC TTGATC-3'. For PCR amplifications, we used 2 U of *AmpliTaq* DNA polymerase (Perkin-Elmer Applied Biosystems, Norwalk, CT), 1 μg of template DNA, 1 μg of each deoxynucleoside triphosphate, and 2.5 mM of $MgCl_2$ in the buffer recommended by the manufacturer. Conditions for amplification were chosen according to the G + C content of the corresponding oligonucleotides. The PCR product was cloned directly in pGEM-T (Table 1; Fig. 1), producing the plasmid pBTLT. General procedures for DNA manipulations were as indicated elsewhere (Sambrook and Russell 2001).

Table 1 Bacterial strains and plasmids with relevant genotype and phenotype

Strain or plasmid	Relevant genotype or phenotype	Reference
Strains		
Pseudomonas putida		
GPG-Tc6	GPo1 derivative, Tc^r, Km^r, P_{C_1}::*lacZ*, *phaF*::miniTn*5*Tc	Prieto et al. 1999
Escherichia coli		
DH5α	Host for *E. coli* plasmids	Sambrook and Russell 2001
CC118(λ-*pir*)	Host for pVLT35-derived plasmids	Herrero et al. 1990
Bacillus thuringiensis ssp. *kurstaki* HD-1	*cry1Ab*, ATCC 33679	Johnson et al. 1980
Plasmids		
pUC18	Ap^r, cloning vector	Sambrook and Russell 2001
pVLT35	Sm^r, $lacI^q$, shuttle expression vector	de Lorenzo et al. 1993
pGEM-T	Ap^r, cloning vector	Mezei and Storts 1994
pGNF2	pGEM-T derivative, *bioF*	This study
pBTLT	pGEM-T derivative, *k-bt1*	This study
pUCBT	pUC18 derivative, *k-bt1*	This study
pNFBT1	pGEM-T derivative, *fk-bt1*	This study
pNFBT2	pVLT35 derivative, *fk-bt1*	This study

Growth conditions and granule isolation

Escherichia coli and *Pseudomonas* strains were cultivated in Luria-Bertani medium (Sambrook and Russell 2001), with aeration at 37 and 30°C, respectively. For PHA production, *Pseudomonas* strains were grown as described previously (Moldes et al. 2004), using the 0.1 N M63 medium, which is a nitrogen-limited minimal medium [13.6 g/l KH_2PO_4, 0.2 g/l $(NH_4)_2SO_4$, 0.5 mg/l $FeSO_4{\cdot}7H_2O$ adjusted at pH 7 with KOH], plus 15 mM octanoic acid (Huisman et al. 1992). Growth was monitored with a Spectrophotometer Shimadzu UV-260 at 600 nm. Cells were broken by a fourfold French press passage (1,000 psi). Antibiotics were added to growth media to the following final concentrations: ampicillin (100 μg/ml), tetracycline (12.5 μg/ml), kanamycin (50 μg/ml), and streptomycin (200 μg/ml). Transformation of *E. coli* cells was carried out by using the RbCl method or by electroporation (Gene Pulser, Bio-Rad; Sambrook and Russell 2001). Plasmid transfer to the target *Pseudomonas* strains was done with the filter-mating technique (Herrero et al. 1990). Granule isolation was carried out by a simple centrifugation (4,000×*g*, 30 min) of a total crude extract prepared by French press cell breakage as described for the BioF system (Moldes et al. 2004).

Analytical procedures

A Western blot analysis of Bt products was carried out as described (Sambrook and Russell 2001) using a polyclonal anti-Cry1Ab serum at a dilution of 1:1,000.

A densitometer (Molecular Dynamics) was used to quantify the protein content as described before (Kraak et al. 1997). For PHA content determination, lyophilized cells were analyzed according to a described method (Lageveen et al. 1988). A CP-Sil 5CB column (Chrompack) was applied to identify the methanolyzed PHA monomers by gas chromatography.

Bioassay

Insecticidal activity of Bt products has been tested, evaluating its toxicity against larvae of the *Sesamia nonagrioides* (González-Núñez et al. 2000). Probit analysis using the POLO-PC computer package (LeOra Software 1987) was used to assay the mortality caused by PFk-Bt1 granules compared with that caused by Cry1Ab crystals

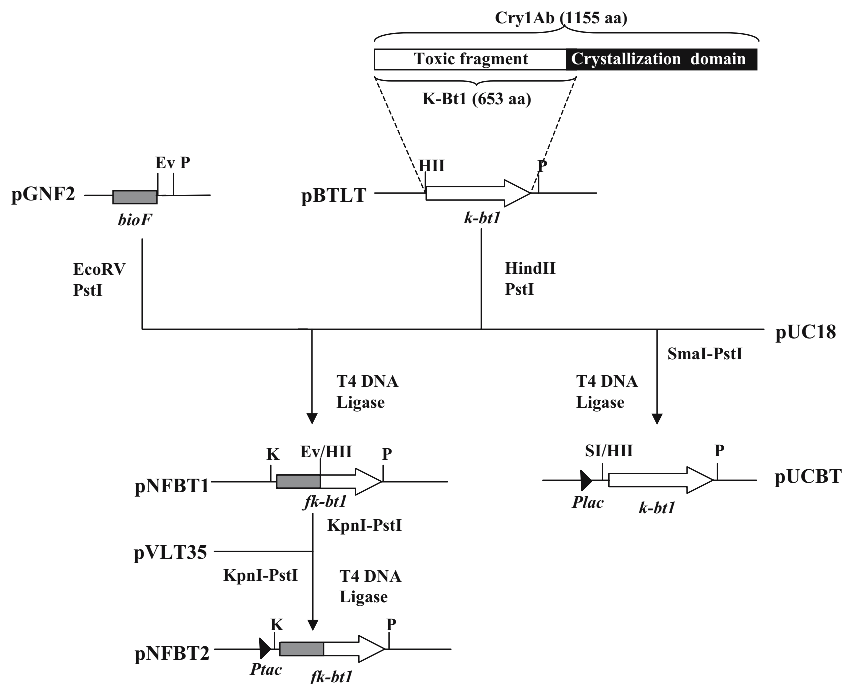

Fig. 1 Cry1Ab toxin and outline of the plasmids pUCBT and pNFBT2. The schematic representation of the domain organization of Cry1Ab toxin with the K-Bt1 encoded fragment cloned and the construction of the *Escherichia coli* expression vector pUCBT and the shuttle plasmid pNFBT2 are shown. *White arrows* represent the DNA fragment coding for the truncated K-Bt1 protein. *Gray boxes* represent the DNA coding for the BioF tag. *Ev*, *Eco*RV, *HII*, *Hin*dII, *K*, *Kpn*I, *P*, *Pst*I, *SI*, *Sma*I, *Ptac*, *tac* promoter of pVLT35, *Plac*, *lac* promoter of pUC18

(81% purity) from *B. thuringiensis* ssp. *kurstaki* HD1-9 provided by Syngenta. Bioassays were conducted as described (González-Núñez et al. 2000). Briefly, toxin crystals were resuspended in 0.1% (v/v) Triton X-100, and PFk-Bt1 granules were diluted with 15 mM Tris–HCl, pH 8. Then, 50 µl of the corresponding solutions at seven different concentrations were applied on the surface of 1 ml of the meridic diet used to rear *S. nonagrioides*, dispensed in the cells of plastic trays (Bio-Ba-128, Color-Dec Italy, Capezzano Pianore, Italy). The concentration of the toxin associated to the PHA granules was 10 µg Fk-Bt1/mg of PHA, calculated by Western blot using an antibody against the BioF tag by using the ECL Western Blotting Detection Kit (Amersham). Fifty microliters of toxin-free PHA granules obtained from *P. putida* GPG-Tc6 at the same concentration of the highest dose tested or 50 µl of distilled water were used as control. One neonate larva (<24 h old) were placed in each cell and confined with a cover. Each dose was replicated three times, and each replicate included 16 observations (48 insects per dose). Trays were incubated in a growth chamber at 25±0.3°C, 70±5% relative humidity, and constant dark. Mortality was assessed after 7 days.

Results

Production of granules carrying a BioF fusion protein with a truncated version of a Cry toxin

We have constructed a BioF fusion protein with a truncated version of a Cry toxin from *B. thuringiensis* ssp. *kurstaki* HD-1 (CECT4454) consisting of the first 653 amino acids of the wild-type Cry1Ab native protein (named K-Bt1 for killing-Bt-Cry1Ab toxin) (Fig. 1) with known insecticidal activity against Lepidoptera (Schnepf et al. 1998). The truncated *cry1Ab* gene was PCR-amplified with appropriate oligonucleotides and cloned into plasmid pGEM-T to obtain pBTLT. Using this plasmid and the plasmid pGNF2, which encodes the BioF tag (Table 1), we constructed the plasmid pNFBT2 (Table 1; Fig. 1), derived from the shuttle expression vector pVLT35 (Table 1), which carries the hybrid gene *fk-bt1* (*bioF-killing-Bt-cry1Ab*) gene. The recombinant plasmid pNFBT2 was transferred by conjugation to *P. putida* GPG-Tc6 (Table 1) for producing PHA granules carrying the Fk-Bt1 fusion protein (these granules are named PFk-Bt1 hereafter) when cultured under nitrogen limitation in minimal medium plus octanoic acid.

The toxin content of the isolated granules was qualitatively tested by Western blot using an antibody against Cry1Ab (Fig. 2) by comparing the PFk-Bt1 granule fraction isolated from *P. putida* GPG-Tc6 (pNFBT2) with that

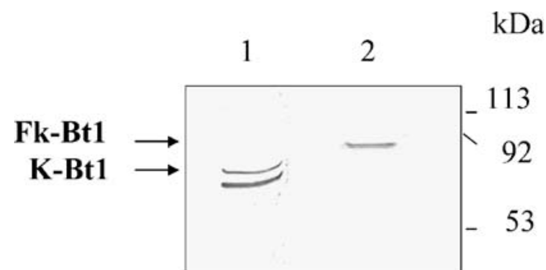

Fig. 2 Identification of CryAb fusion products. Western blot analysis of different cellular fractions using a polyclonal anti-Cry1Ab serum at a dilution of 1:1,000. *Lane 1* Fifteen micrograms of crude extract of a recombinant *E. coli* DH5α strain (Sambrook and Russell 2001) harboring the plasmid pUCBT and producing the K-Bt1 truncated protein, *lane 2 P. putida* GPG-Tc6 (pNFBT2) granule fraction (PFk-Bt1) containing 0.8 µg of total protein. Positions of CryAb products are indicated by *arrows*. The molecular mass (in kilodaltons) of the standard marker proteins is indicated

Table 2 Susceptibility of *Sesamia nonagrioides* larvae to PFk-Bt1 granules and Cry1Ab toxin crystals

	Slope±SE[1]	LC_{50} (95% CI)[2]	Relative potency (95% CI)[3]
Cry1Ab	1.78±0.21a	23 (16–30)a	7.2 (4.8–10.6)a
PFk-Bt1	1.08±0.32a	130 (40–215)b	1b

Mortality data were analyzed by probit analysis using the computer program POLO-PC (LeOra Software 1987)

[1]Slope of the dose–response curve. Values followed by the same letter indicate that the slopes are parallel (likelihood ratio test of parallelism, $P<0.05$)

[2]Concentration that produces 50% mortality (LC_{50}) and 95% confidence intervals (CI), expressed as nanograms of toxin per square centimeter of treated diet surface area. Values followed by different letters indicate significant differences based on overlapping confidence intervals ($P<0.05$)

[3]Relative potency of Cry1Ab toxin with respect to PFk-Bt1. Values followed by different letters indicate significant differences ($P<0.05$)

of *E. coli* DH5α (pUCBT) (Table 1; Fig. 1) which produces the truncated K-Bt1 toxin. This analysis showed a prominent band in the granule fraction corresponding to the expected molecular mass of the fusion protein Fk-Bt1 (88.6 kDa) (Fig. 2, lane 2). It is worth mentioning that the K-Bt1 protein produced in *E. coli* DH5α (pUCBT) appears as a double band in this Western analysis, due to an alternative start codon or, most probably, due to the action of intracellular proteases, as observed for other Cry1Ab producer strains (Fig. 2, lane 1) (Almond and Dean 1994). In contrast, the Fk-Bt1 protein immobilized in the PHA granules showed a single band, suggesting that this new formulation hinders its proteolytic degradation.

Insecticidal activity of PFk-Bt1 granules

To evaluate the insecticidal activity of PFk-Bt1 granules, we have tested its toxicity against larvae of the Mediterranean corn borer, *S. nonagrioides*, a Cry1Ab highly susceptible lepidopteran pest (González-Núñez et al. 2000). Mortality caused by PFk-Bt1 granules has been determined by probit analysis and compared with that caused by purified Cry1Ab crystals. Toxin-free PHA granules at the same concentration of the highest dose tested or distilled water was used as control. Bioassays were conducted as described in "Materials and methods." Table 2 shows that PFk-Bt1 granules caused mortality in *S. nonagrioides* larvae. The concentrations of PFk-Bt1 and Cry1Ab causing 50% of mortality (LC_{50}) were 130 and 23 ng toxin/cm^2, respectively. No toxicity was associated with the PHA granules obtained from the parental strain *P. putida* GPG-Tc6, which was comparable to that found with larvae fed on distilled water control diet. The slopes of dose–mortality responses were parallel, allowing the calculation of a relative potency of Cry1Ab with respect to the PFk-Bt1 toxin. Although PFk-Bt1 was 7.2-fold less active against *S. nonagriodes* than purified Cry1Ab crystals (Table 2), the data reported here show that the susceptibility of *S. nonagrioides* larvae to PFk-Bt1 and the standard Cry1Ab was in the range of the lethal concentrations of Cry1Ab obtained with field populations of this species (González-Núñez et al. 2000).

Discussion

Over the past few years, a major global trend of agricultural and industrial sectors is implementing biotechnology applications that, when coupled with other technologies, could offer unique opportunities for developing environmentally friendly, energy-efficient, and sustainable processes. Bioprocessing is experiencing a great impetus to improve and adapt modern biotechnology to classical fermentation technologies. This success has been promoted by the design of different heterologous gene expression systems and, particularly, by the creation of alternative fusion protein methodologies to facilitate downstream processing of proteins after fermentation (Sassenfeld 1990; Uhlén et al. 1992; La Vallie and McCoy 1995). In this context, the BioF system represents one of the most innovative and versatile fusion protein methodologies recently exploited (Moldes et al. 2004). In this work, we propose to use the BioF system as a new tool to provide an environmentally friendly way to deliver active proteins to the environment using pest control as one example of an environmental problem that could be challenged by this novel approach. In addition, our study illustrates a novel and noticeable advantage of the BioF system, i.e., the possibility of immobilizing insecticidal proteins in a biodegradable support.

The data demonstrate that the toxin linked to the granules retained most of its insecticidal activity. Since dose–mortality responses were parallel for both toxins, the decrease in PFk-Bt1 toxicity with respect to purified Cry1Ab may be attributed to a slightly limited availability of the toxin incorporated to the bioplastic, but not to a variation in the mechanism of action. The advantages of this novel toxin formulation (see below) are far above the minor limitation that causes toxin availability, which in fact can be optimized by modifying some specific parameters of the plastic formulation, e.g., by reducing granule size, by increasing the content of PFk-Bt1 per granule (the crystal provides a highly concentrated toxin), or by changing the PFk-Bt1 design to improve toxin release, which will require further investigation.

The BioF system can be successfully applied for the synthesis of a biodegradable plastic carrying Fk-Bt1, a truncated form of Cry1Ab toxin, opening a new avenue for environmental release of recombinant polypeptides, here exemplified by a pest control application. Benefits from the BioF system as a new tool for the formulation of active polypeptides (e.g., enzybiotics, fitohormones, catalyzers) would include the following:

(1) Low-cost processing, based on a competitive fermentation (Durner et al. 2001) (many raw materials can be used) and a simple downstream process (lysis and centrifugation)
(2) Biodegradable support, i.e., an environmentally friendly product that could be spread to the environment without generating contaminant wastes
(3) A sticky material, which may increase the protein persistence in the environment by reducing its water rain washing (Morales-Ramos et al. 1998)
(4) Protein/polypeptide composition designed by genetic engineering, which can be very useful to generate in vivo in a single organism tailor-made complex protein/polypeptide formulations.

In the case of biopesticides, this system can avoid current toxin complexity in conventional Bt formulations. Moreover, the production of modified toxins or combination of toxins with different target sites could also be implemented by BioF, which meets the growing needs to enlarge the host range of Bt products and to manage insect resistance. All these properties make the BioF system an innovative and alternative way to deliver active proteins/polypeptides over currently available technologies.

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