Toxicon 247 (2024) 107807

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Short communication

UV protection and insecticidal activity of microencapsulated Vip3Ag4 protein in *Bacillus megaterium*

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ARTICLE INFO

Handling editor: Ray Norton

Keywords:
Bacillus thuringiensis
Vip3 proteins
Bacillus megaterium
Priestia megaterium
Microencapsulation
Bioinsecticide formulations
Biological control
Insect pests

ABSTRACT

In this study, secretable Vip3Ag4 protein was encapsulated in *Bacillus megaterium* and used for quantitative bioassays, in order to determine the UV photoprotective capacity of the cell, for preventing inactivation of the insecticidal activity of the protein. The non-encapsulated and purified protein was exposed to the UV light showing a LC_{50} of 518 ng/cm^2 against *Spodoptera littoralis* larvae, whereas the exposed encapsulated protein exhibited 479 ng/cm^2. In addition to the capability to accumulate Vip3 proteins for the development of novel insecticidal formulates, the *B. megaterium* cell has demonstrated to provide moderate protection against the deleterious action of UV light.

Bacillus thuringiensis is a Gram-positive spore-forming bacterium able to produce a number of different proteins showing insecticidal activity. The crystal proteins including Cry and Cyt proteins (commonly known as δ-endotoxins) form spore-associated crystals and are both the most abundant and best studied insecticidal proteins from *B. thuringiensis* (Schnepf et al., 1998).

In addition, other insecticidal *B. thuringiensis* proteins are produced during the vegetative growth phase and referred generically as vegetative insecticidal proteins, which show insecticidal activity after ingestion and proteolytic activation by insect-midgut proteases (Chakroun et al., 2016). These proteins include binary Vpb1/Vpa2 (formerly known as Vip1/Vip2) proteins with insecticidal activity against Coleoptera (Warren et al., 1998), the Vip3 proteins with insecticidal activity against Lepidoptera (Estruch et al., 1996), the Vpb4 (formerly known as Vip4) with Vpb4Da2 protein toxic against *Diabrotica virgifera virgifera* (Coleoptera) (Yin et al., 2020) and Mpp5, (formerly known as Sip1A) showing insecticidal activity against *Diabrotica undecimpunctata howardi* and *D. virgifera virgifera* (Donovan et al., 2006).

Some genes encoding Vip3A proteins have been used in the construction of transgenic plants (also known as Bt crops) showing insect resistance: in example, cotton Cot102XCot67b expresses both Cry1Ab and Vip3Aa19 proteins to protect cotton plants from *Helicoverpa zea* and *Heliothis virescens* (Lepidoptera) (Thompson and Schwab). However, these proteins have not been used as active ingredients of formulated bioinsecticides because they encode a signal peptide sequence at the N-terminus that drives their secretion into the culture medium, despite this signal peptide is not typically N-terminally processed after secretion (Chakroun et al., 2016; Estruch et al., 1996). The significative loss of Vip3 proteins during secretion have prevented their production as optimized (dosable) sprayable Vip3-based biopesticides where the unique active ingredient is constituted by pure Vip3, necessary for manufacturing commercially available products. In addition, a main disadvantage of biopesticides is their loss of activity upon the deleterious action of abiotic factors in the field including the sunlight. The decrease in stability with loss of effectiveness of *B. thuringiensis*-based biopesticides after exposure to the UV radiation from sunlight has been

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https://doi.org/10.1016/j.toxicon.2024.107807
Received 29 February 2024; Received in revised form 6 June 2024; Accepted 12 June 2024
Available online 17 June 2024

already reported (Jalali et al., 2020; Pozsgay et al., 1987; Sansinenea et al., 2015).

Therefore, the aim of this work was to test the photoprotective capacity of *Bacillus megaterium* on Vip3Ag4 protein, expressed and encapsulated in this bacterium in a previous work (Palma et al., 2023) by performing quantitative insect bioassays, in order to assess the UV light protection capacity of the cell for preventing inactivation of the insecticidal activity due to deleterious action of UV light. As discussed below, several efforts have been made to achieve this goal using Gram-negative bacteria, particularly *Pseudomonas fluorescens* (Grace and Ewart1, 1996; Hernández-Rodríguez et al., 2013; Thompson and Schwab, 1996). However, the presence of cell-wall endotoxins and the necessity of employing expensive inducers of protein expression, such as isopropyl β-D-1-thiogalactopyranoside (IPTG), have hindered further advancements in the agribusiness sector, relegating the use of secretable Bt proteins to transgenic insect-resistant plants only. Therefore, the development of low-cost, effective inducers should help in overcoming these challenges.

B. megaterium(currently *Priestia megaterium*) is anaerobic, spore-forming and non-pathogenic bacterium that is able to grow on a wide range of substrates; it does not contain any endotoxin and is commonly found also colonizing the leaves of cotton (Bora et al., 1994). In addition, *B. megaterium* has been described by the US Food and Drug Administration as a GRAS organism (Generally Recognized as Safe) (Mohammed et al., 2014). Currently, there are several expression vectors available for transforming *B. megaterium* (Biedendieck et al., 2007) allowing intracellular expression of proteins using (D)-xylose as a low cost-effective inducer of gene expression. Moreover, the *B. megaterium* expression system used here for encapsulation of Vip3Ag4 protein has also been used successfully, in industrial production of a wide range of proteins with different purposes (Korneli et al., 2013; Vary et al., 2007).

The Vip3Ag4 protein was expressed, encapsulated and purified in *B. megaterium* as previously described by Palma et al. (2023). Briefly, the *vip3Ag4* gene was cloned into pN-His-TEV1622 plasmid (shuttle vector) in *Escherichia coli*, and transformed into asporogenic *B. megaterium* WH320 protoplasts (Mobitec). Protein expression was induced at 37 °C in 2× TY medium supplemented with tetracycline (10 μg/mL), when culture reached an OD_{600} of 0.3 with a final concentration of 0.5 % (w/w) xylose (Palma et al., 2023). Purification of the 6 × His tagged Vip3Ag4 protein was performed using Protino Ni-TED 2000 Packed Columns according to the manufacturer's instructions (Macherey–Nagel) and induced cells killed with lugol as previously described (Palma et al., 2023).

The toxicity of Vip3Ag4 protein was evaluated in larvae of the lepidopteran *Spodoptera littoralis* following the method of surface contamination of the diet. For this, a general diet of lepidoptera (Greene et al., 1976) was used, which was distributed in 24-well plates (Corning). Once the diet had solidified, each well surface was impregnated with 35 μl of purified soluble protein. For each treatment, five concentrations with a constant dilution factor were used. In order to study the photoprotective capacity of microencapsulation, the purified protein (1 mg/mL) and the encapsulated protein (22.2 ng of encapsulated Vip3Ag4 protein per μl of resuspended cells) were subjected to a fixed pulse of UV radiation (40 kJ/cm^2). This radiation level represents the maximum energy received per surface unit at parallel 42° (Pamplona, Spain) during two sunny days.

To carry out photo exposure tests, the toxin or the suspensions of cells were dispensed in 12-well plates that were allowed to dry at 28 °C overnight. The dry protein or cell suspension were exposed to UV radiation in a Stratalinker 1800 crosslinker (Agilent Technologies), previously set up for automatically delivering a UV pulse of 40 kJ/cm2 in about 4 s. The sample was later rehydrated and dispensed onto solidified diet as described above. In the case of using purified Vip3Ag4 protein, water was used as negative control, and in the case of the protein encapsulated in *B. megaterium*, non-induced *B. megaterium* cells were used as negative control for bioassay. Bioassays should be conducted with healthy individuals and control populations should exhibit no more than 10 % mortality (Ibarra and Del Rincón-Castro, 2001). In our bioassays, the control groups showed neither insect mortality nor signs of impaired growth, supporting the fact that mortality was only caused by the action of the purified or encapsulated Vip3Ag4 protein. Dehydration controls were carried out in order to study the effect of dehydration both on the purified protein and on the encapsulated one. Once the protein dilution was dispensed on the diet, the boxes were allowed to dry at room temperature in a laminar flow hood until the water was eliminated by evaporation. Next, a neonate larva of *S. littoralis* was placed in each well and the plate was closed by placing three layers of absorbent paper to avoid excess moisture under the plate lid. The bioassay was kept under controlled conditions for 16:8 h (light:darkness), 25 ± 1 °C and 70 ± 5 % relative humidity. Three repetitions of each treatment were carried out with 24 larvae per concentration. Mortality was evaluated at 7 days and the dose-mortality data were analyzed using a Probit analysis with the POLO-PC software (LeOra Software Company) through which the Lethal Concentration producing 50 % mortality (LC_{50}) was determined for each treatment.

The insecticidal activity results showed that the Vip3Ag4 protein produced in *B. megaterium* is highly toxic against *S. littoralis* larvae. Column-purified Vip3Ag4 protein from *B. megaterium* lysate had an LC_{50} of ~28 ng/cm^2 (Table 1).

The toxicity of purified Vip3Ag4 protein was similar to that obtained in a previous work by Palma et al. (2013), in which the protein was produced in *E. coli* BL21 (DE3), and had an LC_{50} of ~35 ng/cm^2 (Palma et al., 2013). In the case of the protein produced and encapsulated in *B. megaterium*, it was also observed that it was toxic against *S. littoralis* larvae, with an LC_{50} of ~260 ng/cm^2 (Table 1). This shows that the LC_{50} of the encapsulated protein (eVip3Ag4) is about 10-fold higher than that of the purified protein. This difference may be due to the fact that the protein inside *B. megaterium* cells is surrounded by the cell wall of the microorganism, which could have led both to a reduced bioavailability and an inefficient proteolytic activation process. In the case of the purified Vip3Ag4 protein exposed to UV radiation (Vip3Ag4+UV), the LC_{50} value rose to 518 ng/cm^2 causing a reduction by about 20-fold compared to the unexposed purified Vip3Ag4 protein. On the other hand, the encapsulated Vip3Ag4 protein exposed to UV radiation (eVip3Ag4+UV) showed a LC_{50} value of 479 ng/cm^2, while the non-exposed encapsulated protein (eVip3Ag4) had a LC_{50} of 258 ng/cm^2. This indicates only a 1.66-fold reduction (non-statistically significant) in LC_{50}, rather than the expected 19.8-fold reduction. The relative potency values between purified and encapsulated proteins, both exposed and unexposed, reveal that the cell wall of the killed bacterium provides a photoprotective effect against the deleterious effects of UV exposure.

B. thuringiensis is able to produce a wide range of different pesticidal proteins demonstrating its potential and safety as biocontrol agent for at least the last four decades (Bravo et al., 2011; Ibrahim et al., 2010). These pesticidal proteins include crystal and secretable proteins highly toxic against invertebrate species from different orders (de Maagd, 2015; Schnepf et al., 1998; van Frankenhuyzen, 2009). This allowed that natural *B. thuringiensis* strains have been successfully incorporated in the production of sprayable products (biopesticides) wherein the active material is the spore-crystal mixture. The genes encoding such insecticidal proteins have also been used in innovative formulated insecticides and for the construction of insect resistant transgenic crops (Sanchis, 2011). However, the vegetative insecticidal proteins Vip3 are produced during the vegetative growth phase and mainly secreted and diluted into the culture medium, although recently, a report showed that traces of Vip3Aa remain associated with spores in *B. thuringiensis* HD-1 strain boosting its insecticidal activity against *S. exigua* (Donovan et al., 2006; Estruch et al., 1996).

The significative loss of Vip3 proteins during secretion has limited the production of optimized sprayable Vip3-based biopesticides were the unique active ingredient is constituted by pure Vip3 proteins, relegating their exploitation exclusively to transgenic crops, suggesting that

Table 1
Median lethal concentration (LC_{50}) of purified Vip3Ag4 and encapsulated protein in killed cells (eVip3Ag4), treated with 40 kJ/cm2 UV light (+UV) against neonate larvae of *S. littoralis*. Relative potency indicates how many times more active a protein is than the comparator (e.g., wild type Vip3Ag4 is 19.8 times more active than treated Vip3Ag4+UV). Differences between LC_{50} values between purified Vip3Ag4 and purified Vip3Ag4+UV are significant. The difference between eVip3Ag4 exposed or not exposed to UV is non-significant.

Treatment	Regression lines		LC_{50} (ng/cm^2)	Goodness of fit		Relative Potency	Fiducial limits (95%)
	Slope ± SE	Intercept ± SE		χ^2	DF		
Vip3Ag4	1.8 ± 0.2	2.5 ± 0.3	27.5	2.1	3	19.8	(13.3–29.7)
Vip3Ag4+UV	1.4 ± 0.2	1.2 ± 0.5	518.0	2.4	3	1	–
eVip3Ag4	1.8 ± 0.2	0.7 ± 0.4	258.5	2.0	3	1.66	(1.0–2.5)
eVip3Ag4+UV	3.0 ± 0.3	3.1 ± 0.8	479.0	2.1	3	1	–

innovative methods need to be used in order to develop Vip3-based sprayable formulations suitable for the biocontrol of susceptible insect pests (Qin et al., 2010).

Nowadays, we can find in the scientific literature several examples of systems using *E. coli* for the recombinant production of different insecticidal proteins from *B. thuringiensis*. In addition, we can also find other studies that used non-pathogenic strains of the Gram-negative bacterium *P. fluorescens* for the microencapsulation of secretable Cry1Ia and Vip3 proteins (Hernández-Rodríguez et al., 2013). This system was used by Mycogen Corporation for the production of MVP™ and M-TRAK™ formulations for control of some lepidopterans and beetles, respectively (Panetta, 1993). After the *P. fluorescens* cells were induced for protein expression, the cells containing Cry proteins were fixed (killed) by application of physical or chemical methods (e.g., chemical fixation using formaldehyde or lugol). This process showed improved effectiveness of the insecticidal proteins and increased their persistence in the field by protecting them against the inactivation produced by the solar ultraviolet light (Gaertner et al., 1993). However, the presence of natural endotoxins (also known as lipid A) in the membranes of Gram-negative bacteria is a potential drawback. Along with the high cost of effective inducers such as IPTG, this has hindered the development of further biopesticide formulations.

In a previous work, the expression and encapsulation of active insecticidal Vip3Ag4 protein in *B. megaterium* was achieved (Palma et al., 2023), however, its potential photoprotective capacity concerning the insecticidal activity after UV light exposure was not determined.

In this work, we report for the first time, data of toxicity and UV protection of encapsulated Vip3Ag4 protein in *B. megaterium.* The LC_{50} value of the encapsulated protein was about 10-fold higher than that of the purified protein. This difference may be due to the fact that the protein inside the *B. megaterium* cell is surrounded by the cell wall of the microorganism, which could have led both to a reduced bioavailability and an inefficient proteolytic activation process in the insect midgut. This behavior was also described in Hernández-Rodríguez et al. (2013), where two proteins, Cry1Ia and Vip3Aa, purified from and encapsulated in *P. fluorescens*, were tested against two lepidopteran species. In both cases, it was seen that the LC_{50} of the encapsulated proteins was slightly higher than that of the purified proteins (Table 1), but showed no significant differences between the two LC_{50} values (Hernández-Rodríguez et al., 2013). When non-encapsulated Vip3Ag4 protein was exposed to UV radiation, the LC_{50} value increased by about 20-fold compared to the unexposed protein, whereas encapsulated Vip3Ag4 showed an increase of $<$ 2-fold, revealing that *B. megaterium* confers a moderate protection of Vip3g4 protein against UV radiation.

The significance of this study is that encapsulation of Vip3 proteins into *B. megaterium* cells, can enhance the stability and efficacy of biopesticide formulations by protecting insecticidal proteins from UV degradation. However, further experiments are needed to fully understand and optimize these protective effects.

In addition to the main findings presented in this study, further research should be developed to unveil other potential advantages of utilizing *B. megaterium* as encapsulation host for insecticidal proteins. Beyond its moderate UV protective properties, future investigations should explore additional protective attributes against other abiotic factors, thereby enhancing the stability and efficacy of biopesticide formulations both in the field and the storage. Also, exploring the adaptability of *B. megaterium* to different culture media could provide crucial information for scalable production methods. Large-scale production and field application studies are essential steps towards translating laboratory findings into practical solutions for pest management in agriculture, in order to decide if *B. megaterium* would stand as 'host of choice' for the production of efficient Vip3-based formulations and the development of a new generation of 'killed' microbial pesticides.

Ethical Statement

Hereby, I Leopoldo Palma consciously assure that for the manuscript **"UV protection and insecticidal activity of microencapsulated Vip3Ag4 protein in *Bacillus megaterium*"** the following is fulfilled.

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in the context of prior and existing research.
6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

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I agree with the above statements and declare that this submission follows the policies of Solid State Ionics as outlined in the Guide for Authors and in the Ethical Statement.

CRediT authorship contribution statement

Leopoldo Palma: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Iñigo Ruiz de Escudero:** Formal analysis, Data curation, Conceptualization. **Francisco Mañeru-Oria:** Investigation. **Colin Berry:** Writing – review & editing, Formal analysis, Conceptualization. **Primitivo Caballero:** Writing – review & editing, Validation, Resources, Project administration, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequence of this study is available at GenBank (Acc. Num. HQ414237). Other data including bioassays with insects is available at https://academica-e.unavarra.es/handle/2454/12228?show=full

Acknowledgments

This research was funded by the Spanish Ministry of Science and Innovation grant number AGL2009-13340-C02 and by the Universidad Pública de Navarra. We acknowledge the technical support of N. Gorría and I. Ibañez for their help in insect rearing. Leopoldo Palma would like to express gratitude to the Spanish Government-Universities Ministry, the NextGenerationEU and Recovery, Transformation, and Resilience plans for funding his awarded María Zambrano contract (ref. ZA21-003).

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