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Improved Insecticidal Toxicity by Fusing Cry1Ac of *Bacillus thuringiensis* with Av3 of *Anemonia viridis*

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Abstract Av3, a neurotoxin of *Anemonia viridis*, is toxic to crustaceans and cockroaches but inactive in mammals. In the present study, Av3 was expressed in *Escherichia coli* Origami B (DE3) and purified by reversed-phase liquid chromatography. The purified Av3 was injected into the hemocoel of *Helicoverpa armigera*, rendering the worm paralyzed. Then, Av3 was expressed alone or fusion expressed with the Cry1Ac in acrystalliferous strain Cry$^-$B of *Bacillus thuringiensis*. The shape of Cry1Ac was changed by fusion with Av3. The expressed fusion protein, Cry1AcAv3, formed irregular rhombus- or crescent-shaped crystalline inclusions, which is quite different from the shape of original Cry1Ac crystals. The toxicity of Cry1Ac was improved by fused expression. Compared with original Cry1Ac expressed in Cry$^-$B, the oral toxicity of Cry1AcAv3 to *H. armigera* was elevated about 2.6-fold. No toxicity was detected when Av3 was expressed in Cry$^-$B alone. The present study confirmed that marine toxins could be used in bio-control and implied that fused expression with other insecticidal proteins could be an efficient way for their application.

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Introduction

Bacillus thuringiensis is a Gram-positive spore-forming bacterium that shows high-insecticidal activity and produces crystalline protein inclusions during sporulation. To date, over 100 crystal proteins have been identified from different strains of *B. thuringiensis*. The majority of these proteins are Cry proteins, which are especially toxic to lepidopterans, dipterans, coleopterans, hymenoptera, and even nematodes [11, 16]. Cry1Ac is expressed as a 130 kDa rhombic crystalline protoxin which has potent toxicity against lepidopteran insects while not animals. The protoxin was processed into a 60 kDa toxin by midgut proteases after ingestion by insects. The activated toxin binds to specific receptors on the brush border membrane of gut epithelial cells and generates pores by inserting into the membrane, leading to cell death by osmotic shock and, eventually, insect mortality [3].

Several means to enhance the toxicity of Cry1Ac, such as site-directed mutagenesis, application of synergetic substances [4, 14], co-expression, or fused expression with other anti-insect proteins [5, 10], have been investigated. Previous report showed that the toxicity of Cry1Ac to the larvae of *Spodoptera exigua* was elevated about fivefold after fusion with spider neurotoxin ω-ACTX-Hv1a [6]. Compared with wild Cry1Ac, fused expression of Cry1Ac with the neurotoxin huwentoxin-I from the venom of the Chinese bird spider (*Selenocosmia huwena*) showed higher toxicity against the third instar larvae of *Plutella xylostella* [17].

The venoms of sea anemones contain many peptide toxins acting on ion channels. Among these toxins, site-3 voltage-gated Na^+ channel peptide toxins have been extensively studied. To date, more than 50 Na^+ channel peptide toxins have been identified [12]. Av3 (previously

named ATX-III) is one of the three short peptide toxins first isolated from the venom of sea anemone *Anemonia viridis* (previously named *Anemonia sulcata*) [1]. It is a type III toxin composed of 27 amino acids with three disulfide bonds and has shown to be active on crustaceans, blowfly larvae, and cockroaches but inactive in mammals. It inhibits the inactivation process of the voltage-gated sodium channel by binding to receptor site-3 [7, 8].

As the toxicity of Av3 on agricultural pests has not been reported before, it was expressed in *E. coli* Origami B (DE3) and purified by HPLC in this study, and then analyzed on *Helicoverpa armigera* by hemocoel injection. In order to introduce Av3 in bio-control and elevate the toxicity of Cry1Ac, the two proteins were fusion expressed in *B. thuringiensis* acrystalliferous strain Cry$^-$B, and the toxicity assay was performed on *H. armigera* orally.

Materials and Methods

Strains and Plasmids

The bacterial strains, plasmids, and primers used in this study are listed in supplementary table (Online Resource 1). *Escherichia coli* Origami B (DE3) was purchased from Biomeik Corporation (Tianjin, China) and used for Av3 expression. The *E. coli* GB2005 used for cloning and *E. coli* GBDir used for Red/ET recombination were provided by Youming Zhang. The pET32b vector was provided by Yehu Moran (Tel-Aviv University, Israel) and used to express Av3 in *E. coli* Origami B (DE3). The shuttle vector, pHT315, was used to express Av3 and Cry1AcAv3 fusion protein in *B. thuringiensis* Cry$^-$B.

Vector Construction

The *av3* gene constructed on pUC57 vector was synthesized chemically (Sangon) according to Av3 amino acid sequence (UniProt accession no. P01535) with an enterokinase recognition site in the 5′ term and designated as pUCAv57. The *av3* gene was amplified from pUCAv57 using the primers AcF-*Kpn*I and AcR-*BamH*I. After cleavage with *Kpn* I and *BamH* I, the *av3* DNA fragment was subcloned into the corresponding sites of the pET32b vector and fusion expressed with thioredoxin. For the Av3 expression vector pHT1AcPT-av3, the cry1Ac promoter was amplified from pH1AcPT-chi with primers AcpS-*Hind*III and AcpR, and *av3* was amplified from pUCAv57 using primers Av3S and Av3R-*Kpn*I. The *cry1Ac* promoter and *av3* were then ligated by overlap extension polymerase chain reaction (PCR), and the 1AcPT-chi fragment of pH1AcPT-chi was substituted by the *cry1Ac* promoter-*av3* fragment at *Hind* III/*Kpn* I sites.

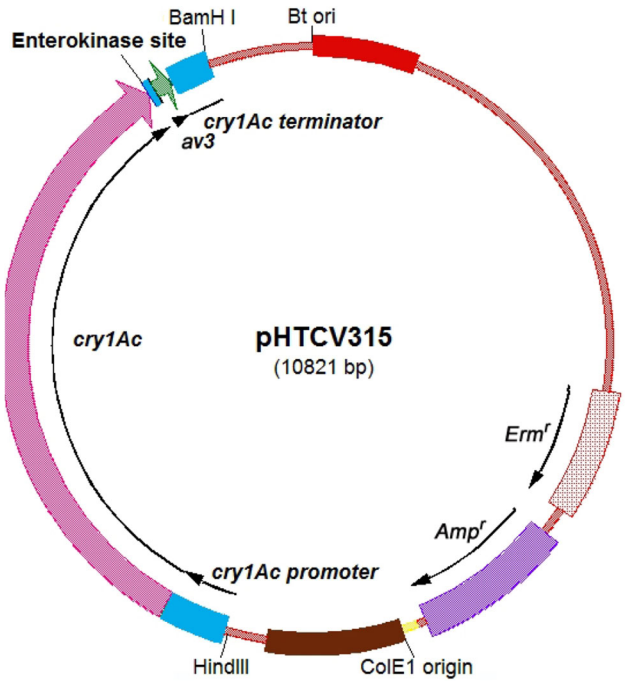

Fig. 1 Profile of fusion expression vector pHTCV315. *av3* was fused with *cry1Ac*, and an enterokinase site was inserted to link the two genes. The fusion gene was controlled by *cry1Ac* promoter and constructed on the shuttle vector pHT315

The Cry1Ac-Av3-fused expression vector pHTCV315 (Fig. 1) was constructed as follows. The *av3* gene was amplified from pUCAv57 using the primers AvF-*Sal*I and AvR-*EcoR*I. The terminator of *cry1Ac* was amplified from the plasmid pHAc35 using the primers ActF-*EcoR*I and ActR-*BamH*I. After ligation by overlap extension PCR, the *av3-1acter* fragment was inserted on pUC19 vector at *Sal* I and *BamH* I sites. The primers REDCVF (with a homolog of *cry1Ac* ORF and an enterokinase site) and REDCVR (with a homolog of the pHAc35 vector) were then used to amplify the *av3-1acter* fragment. Then, the *av3-1acter* fragment and linearized pHTAc35 were co-transformed into recombineering strain *E. coli* GBDir, and the final-fused expression vector pHTCV315 was obtained by Red/ET recombination.

Expression and Purification of Recombinant Av3

The Av3 expression vector pETAv32b was transformed into *E. coli* Origami B (DE3). The expression and purification of Av3 was performed as previously reported [8]. Overnight cultured cells were inoculated into LB fresh medium with 100 μg ml^{-1} ampicillin and grown at 37 °C. When OD_{600} of 0.6 was reached, IPTG was added to the mixture to a final concentration of 0.5 mM. The cells were allowed to grow for another 4 h at 30 °C. After collection by centrifugation at 7,000×*g* for 8 min, the pellet was suspended in the binding

buffer (PBS with 20 mM imidazole, pH 9.4) in the presence of 0.2 mM PMSF and heated for 10 min at 80 °C to denature thermally unstable cell proteins. After sonication, the cells were lysed and centrifuged at 14,000×*g* for 30 min. The supernatant was passed through 0.22 μm filters and loaded onto a HisTrap FF crude column (GE Healthcare) connected to an AKTA purifier. The column was washed by a 15-column volume of binding buffer and the TrxB–Av3 fusion protein was eluted with elution buffer (PBS with 500 mM imidazole, pH 9.4). After desalting by ultrafiltration (Vivaspin 2, MWCO 10 kDa, Saritoes) at 4,000 g, enterokinase was added to remove TrxB-tag. The protein mixture was incubated at 25 °C for 16 h. The recombinant Av3 was then purified by reversed-phase liquid chromatography (RPLC) using a linear gradient of 25–30 % acetonitrile with 0.1 % trifluoroacetic acid and a YMC 3 ml column. The recombinant Av3 was determined using LTQ-XL mass-spectrometry system.

Fused Expression and Atomic Force Microscopy Observation

The fusion expression vector pHTCV315 and the Av3 individually expression vector pHT1AcPT-av3 were transformed into *B. thuringiensis* acrystalliferous strain Cry$^-$B and designated as BHCV and BHAv3, respectively. BHCV was inoculated into LB broth and cultured overnight at 30 °C, 2 % (v/v) overnight culture was then inoculated into G-Tris medium and shaken at 30 °C for 60 h. Cry$^-$B and BHAc (Cry$^-$B with pHAc35) were set as control. The BHCV, BHAc, and Cry$^-$B bacteria pellets were suspended in 100 μl of reducing sample buffer and boiled for 10 min. After centrifugation at 12,000×*g* for 2 min, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed (with a 12 % polyacrylamide separating gel and a 5 % stacking gel) to detect the expression of fusion protein Cry1AcAv3, the fused protein was then in-gel digested and determined using mass spectrometry. Meanwhile, the pellet of BHCV was washed twice with PBS and ddH_2O, and the shape of Cry1AcAv3 was observed under atomic force microscopy (AFM).

Western Blot Analysis

1 mg of purified TrxB-Av3 was injected into the endothelium on the back of New Zealand rabbits (purchased from Hunan experimental animals Ltd., China) four times. TrxB-Av3 antibody was harvested from the rabbits after the fourth immunization, and the concentration was detected by means of dot blotting. After separation of proteins by SDS-PAGE, Western blot was performed to detect the expression of fusion protein.

Toxicity Bioassay

One microgram of purified Av3 was injected into the hemocoel of the fourth-instar *H. armigera*. 1 μg of BSA was injected as controls. Five insects were injected for each group. The movement and food intake of insects were monitored. The engineering bacteria BHCV, BHAv3, and BHAc were cultured in G-Tris broth for 60 h, and the spore-crystal mixtures were washed twice with 0.5 M NaCl and ddH_2O. Toxicity was detected on *H. armigera* by oral administration thrice as previously reported [13]. After 96 h, the mortality was recorded, and 50 % lethal concentrations (LC_{50}) were determined by probit analysis using SPSS software.

Results and Discussion

Construction of Av3 or Cry1AcAv3 Expressing Bacteria

The *av3* and the *cry1Ac-av3* genes constructed into the corresponding expression vectors were sequenced correctly. The final recombinant *E. coli* Origami B (DE3) with pETAv32b, *E. coli* GBDir with recombined pHTCV315, *B. thuringiensis* strain BHCV with pHTCV315, and *B. thuringiensis* BHAv3 with pHT1AcPT-av3 were tested by colony PCR (Online Resource 2). Red/ET recombination technology was used to fuse *cry1Ac* of *B. thuringiensis* and *av3* of *A. viridis*. It is a homolog recombination technique mediated by RecA, and does not rely on the presence of suitable restriction sites [9]. Most importantly, it avoided mutations of the *cry1Ac* gene, which might occur in PCR.

Protein Expression and Identification

TrxB-Av3 was abundantly expressed in *E. coli* Origami B (DE3) (Online Resource 3) and purified by the HisTrap column. After enterokinase cleavage of TrxB-Av3, Av3 was purified by RPLC and further confirmed by mass spectrometry (Fig. 2a). SDS-PAGE analysis indicated that a ~136 kDa Cry1AcAv3 fusion protein was highly expressed in the BHCV cells (Fig. 3a), and the fusion protein was detected by means of Western blot analysis using TrxB-Av3 antirabbit serum (Fig. 3b). There was no Av3 been detected in BHAC and Cry$^-$B. Meanwhile, Av3 peptide fragments were successfully detected in Cry1AcAv3 trypsin digestion mixtures by mass spectrometry (Fig. 2b).

AFM Scanning

After cultivation for 48 h in G-Tris broth, inclusion bodies formed in BHCV and most of the cells lysed. Under optical

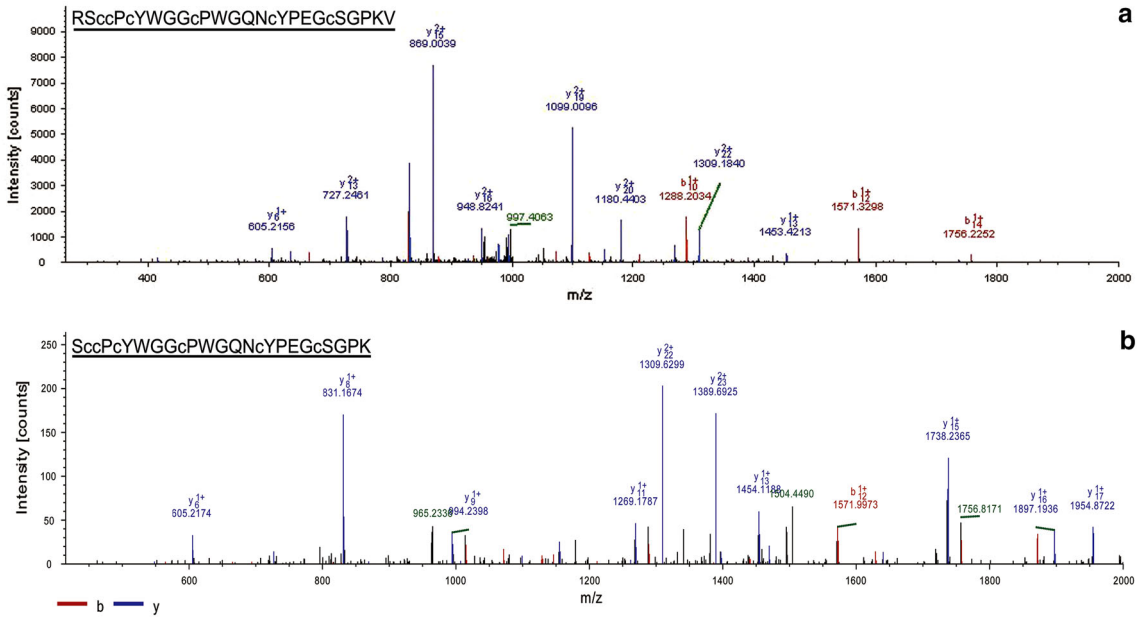

Fig. 2 Mass-spectrometry identification of **a** Av3 and **b** Cry1AcAv3. Av3 peptide purified from *E. coli* OrigamiB (DE3): pETAv32b was loaded on LC–MS directly. The ~136 kDa fused protein Cry1AcAv3 expressed in Cry^{-}B was in-gel digested by 2 % (w/v) trypsin and determined by means of mass spectrometry using an LTQ-XL system. Av3 peptide fragments were detected successfully (Color figure online)

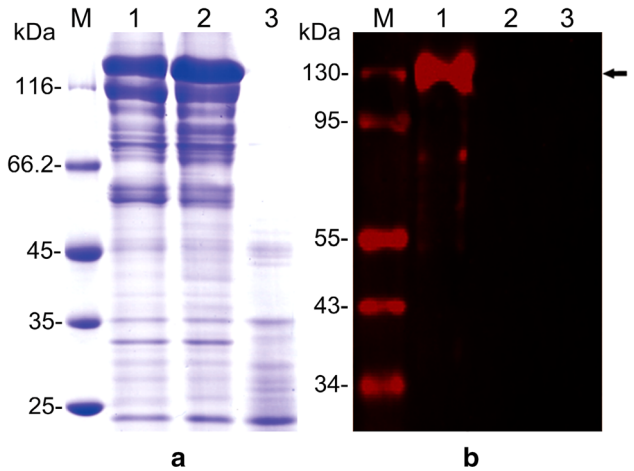

Fig. 3 SDS-PAGE and Western blot analysis of Cry1AcAv3 fusion protein expressed in Cry^{-}B. **a** Cell total proteins of BHCV, BHAC, and Cry^{-}B were separated on SDS-PAGE and stained with Coomassie *brilliant blue*. Cry1AcAv3 (136 kDa) was expressed in BHCV (*lane 1*) and found to be slightly heavier than the 133 kDa Cry1Ac expressed in BHAC (*lane 2*). **b** After SDS-PAGE and transmembrane, the membrane was incubated with TrxB-Av3 antirabbit serum (1:1,000) at 37 °C for 2 h. IRDye® 680 conjugated goat antirabbit IgG (LI-COR, America) was added to detect the TrxB-Av3 antibody. Then, the membrane was scanned using an Odyssey infrared imager (LI-COR, America). Cry1AcAv3 (indicated by *arrow*) was successfully detected. No Av3 band was detected in BHAC and Cry^{-}B. *M* protein molecular weight, *lane 1* BHCV, *lane 2* BHAC, and *lane 3* Cry^{-}B (Color figure online)

microscope, the shapes of inclusion bodies between BHCV and BHAc were not apparently different. However, when observed under AFM, the Cry1AcAv3 fusion protein exhibited irregular rhombus- or crescent-shaped crystalline inclusions (Online Resource 4), which was differing from the regular rhombic Cry1Ac crystals. The reason for the shape changes might be ascribed to the structural change of the C-terminal. The cysteine residues in the C-terminal region of the protoxin play an important role in forming a stable crystal structure by generating interchain or intrachain disulfide bridges [2]. The cysteine residues in the Av3 molecule probably affected the crystal formation process of Cry1Ac by disorganizing interchain or intrachain disulfide formation, resulting in different shapes of fusion proteins.

Bioassay

H. armigera revealed potent contraction paralysis 15 min after injection of the purified Av3. Over 5 days of experiment, all of the insects injected with Av3 failed to show normal movement and ingestion and instead showed spasms and eventual death (Online Resource 5). For the oral administration of the fusion protein Cry1AcAv3, the growth of the insects was obviously restricted at low concentrations. The summarized results of the bioassays are shown in Table 1. Cry1AcAv3 expressed in Cry^{-}B showed higher toxicity against *H. armigera* larvae than primitive Cry1Ac, with an LC_{50} value (11.9 μg ml^{-1}) decreased by about 61.2 % compared with the primitive Cry1Ac (30.7 μg ml^{-1}). Additional data are given in Online Resource 5. By contrast, Av3 expressed alone in Cry^{-}B showed inclusion bodies (data not shown) and very

Table 1 Insecticidal activity of engineering bacteria against *Helicoverpa armigera*

Bacteria	LC_{50} (μg ml^{-1}) (95 % FL)[a]
Cry-B (pHTCV315)	11.9 (7.9–17.1)
Cry-B (pHAc35)	30.7 (20.5–49.4)
Cry-B (pHT1AcPT-av3)	>10,000

Toxicity was detected from *H. armigera* after oral administration at six concentrations (i.e., 2, 4, 16, 32, 64, and 128 μg spore-crystal mixture ml^{-1}). Forty-eight first-instar larvae were detected for each concentration, and sterile ddH_2O was used as the negative control

[a] LC_{50} values (μg spore-crystal mixture ml^{-1}) and 95 % fiducial limits (FL) were determined from three independent assays

weak oral toxicity ($LC_{50} > 10{,}000$ μg ml^{-1}). A highly efficient *cry1Ac* promoter and the environment of *Bt.* cells probably lead to the failure of correct mating of disulfides in Av3 structure, thus limiting its toxicity. While fused expression with Cry1Ac was likely to induce the correct formation of disulfide bonds in Av3 molecule. This implies that fusion express with other proteins could be an efficient way to apply peptide toxins. In addition, according to the conservation of voltage-gated Na^+ channels between different species of insects, Av3 might be also active in other kind of insect even beneficial insects. Although Cry1Ac is active in relatively narrow range of agricultural pests, the pest control spectrum of the fusion protein Cry1AcAv3 might be broadened. Further study on this area is necessary.

Conclusion

The polypeptide neurotoxin Av3 was expressed in *E. coli* OrigamiB (DE3) and purified by HPLC, bioassay showed that it was toxic to agricultural pest *H. armigera* when performed by hemocoel injection. Then Av3 was fusion expressed with Cry1Ac in *B. thuringiensis* Cry^-B, the shape of Cry1Ac crystals was changed by fused expression. Bioassay results showed that Av3 lost its toxicity when expressed in Cry^-B alone but improved the toxicity of Cry1Ac by fused expression, which implies that polypeptide toxins could be applied by fused expression.

The co-expressions of Cry1Ac and *Pinellia ternata* agglutinin in the tetraploid *Isatis indigotica* Fort have broadened the pest control spectrum [18]. The expression of synthetic cry1Ac and cry2Ab genes in tobacco shows a broader anti-insect spectrum [15]. Our study verified the insecticidal capacity of sea anemone peptide toxins. However, its active range has yet to be studied.

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