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Article *in* Biochemical and Biophysical Research Communications · January 2001

DOI: 10.1006/bbrc.2000.3998 · Source: PubMed

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Recombinant Cry3Aa Has Insecticidal Activity against the Andean Potato Weevil, *Premnotrypes vorax*

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Received November 2, 2000

The Andean potato weevil, *Premnotrypes vorax*, an insect of the order Coleoptera, is a major cause of damage to potato crops in the Andean regions of South America. The insecticidal Cry proteins from *Bacillus thuringiensis* are useful biological pesticides, and some are toxic to Coleopteran insects. We overexpressed recombinant, histidine-tagged Cry3Aa protein in *Escherichia coli* host cells. The recombinant protein was solubilized at high pH with urea, purified using Ni^{2+}-nitrilo-triacetic acid affinity resin, and dialysed to lower pH and remove urea. Bioassays were performed with an insect media whose surface was spread with 70 μg/mL purified native or recombinant toxins. First instar larvae exposed to toxin treated media for 5 days exhibited mortalities from 57% (native Cry3Aa) to 52% (recombinant Cry3Aa). Purified native and recombinant Cry3Aa proteins appeared to be equally toxic to the Andean potato weevil.

Key Words: **Cry3Aa; Cry3A; *Premnotrypes vorax;* Andean potato weevil; *Bacillus thuringiensis.***

The Andean potato weevil, or white grub, *Premnotrypes,* an insect of the order Coleoptera, is a major cause of damage to potato crops in the Andean regions of South America (1). In Colombia, 10–30% of the potato harvest is lost due to this insect. *Bacillus thuringiensis,* and the insecticidal proteins it produces, have long been used as biological pesticides (2–4). Their advantages over chemical pesticides include high insect specificity, low toxicity to other organisms, and low environmental impact. Close to 30 different insecticidal proteins, of *B. thuringiensis* have been identified and many are cloned and sequenced (5). The Cry insecticidal proteins interact with specific receptors on insect midgut epithelial cells, insert into the membrane and form pores, causing osmotic shock and death of the insect (2). Cry3 proteins are toxic to Coleoptera (3). Below we report the cloning and recombinant expression of Cry3Aa from *B. thuringiensis* var. *san diego,* and demonstrate that Cry3Aa is toxic to the Andean potato weevil, *Premnotrypes vorax.*

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MATERIALS AND METHODS

Materials. Biochemical reagents were from Sigma Chemical Company. Oligonucleotides were from Corpogen, Bogotá, Colombia. The T-overhang vector, pCRII, was from Invitrogen, and the expression vector pPROEXHtb was from Life Technologies. *B. thuringiensis* var. *san diego* was a gift from Dr. S. Orduz, CORPOICA.

Construction of pPROEXHtb-Cry3Aa. *B. thuringiensis* var. *san diego* cells were grown in Luria-Bertani media for 3 days at 30°C, and plasmid DNA was isolated using an alkaline lysis method (6). PCR was performed using as a template the purified plasmid together with sense primer 5′CTGGATCC*ATG*AATCCGAACAATCGA3′, and antisense primer 5′TAGCGGCCGCTTAATTCACTGGAATAAA3′. Primers were based on the published Cry3Aa sequence (GenBank Y00420, 7) and contained restriction sites (underlined) for *Bam*H1 (sense) and *Not*1 (antisense) to facilitate cloning. The start codon in the sense primer is shown in italics. A single amplification product of the expected size (1934 bp) was obtained, and showed the correct pattern of DNA fragments upon restriction with *Eco*RI (data not shown). Because of apparent incomplete *Bam*H1 and *Not*1 digestion of the PCR product, which subsequently caused difficulties during subcloning, the PCR product was first ligated into pCRII. Purified pCRII-Cry3Aa was digested with *Bam*H1 and *Not*1, and the fragment containing Cry3Aa coding sequence was ligated into the expression vector pPROEXHtb.

Expression of pPROEXHtb-Cry3Aa recombinant protein in Escherichia coli. *E. coli* DH5α cells transformed with pPROEXHtb-Cry3Aa were grown in 2xYT media (6) in the presence of 100 μg/mL ampicilin. Expression was induced by addition of 0.5 mM isopropyl-beta-D-thiogalactopyranoside when cultures reached an optical density of 0.8. Cells were harvested 3 h postinduction.

Purification of pPROEXHtb-Cry3Aa recombinant protein. The cell pellet from 250 mL *E. coli* DH5α culture was resuspended in 10 mL phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) and sonicated on ice (5 × 3 min), with a Branson model 250 sonifier micro tip set at output control 4, 70% duty cycle. The sonicated cells were centrifuged at

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16000*g*, 5 min. The pellet was solubilized by incubation in 10 mL Solubilization Solution (5.0 M urea, 0.1 M sodium carbonate, pH 11.2) at 4°C for 1 h with 250 rpm agitation, and the solubilized protein was centrifuged 16,000*g*, 2 min. Two milliliters of Ni^{2+}-nitrilo-triacetic acid affinity resin (Ni-NTA, Life Technologies) equilibrated with Solubilization Solution + 20 mM imidazole were added to the supernatant of solubilized protein, and incubation was continued at 4°C for 1 h. The resin was poured into a column and washed with Solubilization Solution + 20 mM imidazole. Protein was eluted with Solubilization Solution + 200 mM imidazole. Column fractions were pooled and dialyzed at 4°C against Buffer A (20 mM Tris–HCl pH 8.0, 10% glycerol, 100 mM KCl, 5 mM β-mercaptoethanol) with three changes. Protein concentration was measured by the method of Bradford (8) using reagents from BioRad.

Purification of native Cry3Aa from B. thuringiensis. *B. thuringiensis* cells were grown as described above. Cells were centrifuged and the pellet was washed twice in cold distilled water. The pellet was resuspended in 0.01% Triton, and sonicated as described above. Cry3Aa was purified according to a published method (9), except that a discontinuous gradient of saccharose, not sucrose, was used.

Production of polyclonal antibodies against native Cry3Aa. Polyclonal antibodies were prepared based on a procedure described previously (10). Two New Zealand white rabbits were each inoculated with 1 mL of a 1:1 mixture containing Freund's complete adjuvent and 0.5 mg of purified, wild-type Cry3Aa. A second inoculation was made on day 32, using the mixture above in which Freund's complete adjuvent was replaced with Freund's incomplete adjuvent. Polyclonal antibodies were obtained from serum harvested on day 48.

Protein electrophoresis and electroblotting. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a Hoeffer model SE650 electrophoresis unit using 1.5 mm, 7.5–15% gradient gels with 5% stacking gels and the buffer system described by Laemmli (11). Protein was stained with Coomassie Brilliant Blue R.

Protein was transferred from SDS–polyacrylamide gels to nitrocellulose using a Hoeffer Transphor unit for ~12 h at 6 V/cm. Antibody-reacting protein bands on the membranes were visualized according to published procedures (12). The nitrocellulose membrane was incubated overnight at room temperature with the polyclonal antibodies raised against native pPROEXHtb-Cry3Aa at a dilution of 1/3000. The membrane was incubated for 1 h at room temperature with the secondary antibody, anti-rabbit peroxidase conjugate (Sigma), at a dilution of 1/1000.

Bioassays. Mature *Premnotrypes vorax* were collected near Chocontá, Cundinamarca, Colombia. Insects were maintained in a greenhouse in earth-filled pots covered with wheat or barley straw, and fed with potato foliage. Eggs were collected and incubated for approximately 30 days between paper towels in plastic boxes. First instar larvae were collected and then grown under conditions and on media developed by one of us (ACM). One liter of media was made by mixing 45.5 mL sterilized distilled water, 9.3 g agar agar, and 364 g cooked potatoes in a sterilized blender. After cooling, 200 mL distilled water containing 1 g methyl paraben and 2.4 g ascorbic acid were added. Media was poured into sterile plastic feeding cups (2.5 cm diameter, 3 cm height) to a depth of 0.5 cm, and allowed to solidify in a laminar flow hood for 2 h. Two hundred microliters of one of the following solutions was spread on the surface of media and was allowed to dry for 1 h. Buffer A, Buffer B (25 mM K_2HPO_4, pH 7.0), 70 μg/mL purified recombinant Cry3Aa in Buffer A, 70 μg/mL purified native Cry3Aa in Buffer B, or 700 μg/mL crude crystal/spore extracts of *B. thuringiensis* in Buffer B. Two to three larvae were placed in each cup and sealed with fine-meshed cloth held in place with rubber bands. Cups were incubated at 15 ± 10°C, 90 ± 10% humidity, with a photo period of 12 h. Larvae were scored for mortality after 5 days.

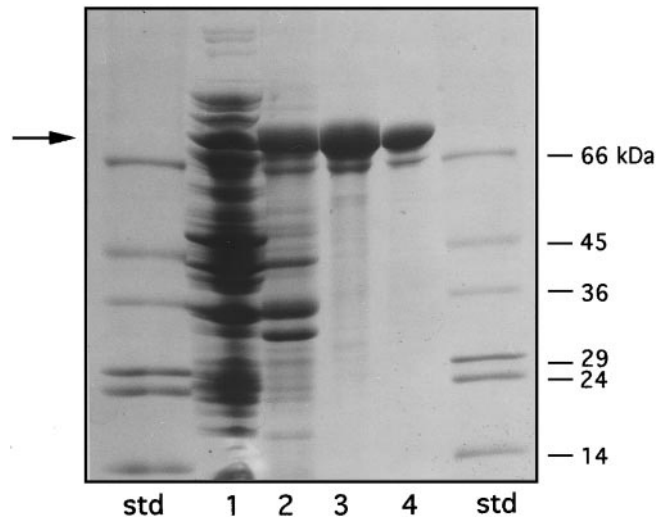

FIG. 1. Purification of recombinant histidine-tagged Cry3Aa protein. SDS–PAGE was performed to monitor purification of recombinant Cry3Aa protein. Lane 1, sonicated cell extract of *E. coli* DH5α expressing Cry3Aa; lane 2, extract pellet after solubilization with urea at high pH; lane 3, Cry3Aa-containing fraction eluted from Ni-NTA; lane 4, Cry3Aa-containing fraction after dialysis; std, molecular weight markers. Sizes of molecular weight markers are indicated on the right. The position of recombinant Cry3Aa is shown by the arrow.

RESULTS AND DISCUSSION

Perlak and coworkers (13) have shown that transgenic Russet Burbank potato plants expressing Cry3A are resistant to all stages of the Colorado potato beetle. Resistance to the sweet potato weevil is observed in transgenic sweet potato plants expressing Cry3Aa (14). We wanted to determine whether the *B. thuringiensis* Cry3Aa toxin was toxic to the Andean potato weevil, *P. vorax*, which is responsible for large crop losses in Colombia and other South American countries. To this end we produced and purified a recombinant Cry3A and tested its effect on first instar *P. vorax* larvae.

To simplify protein purification, we chose an expression system which added a tag of six histidine residues to the N-terminus of the recombinant protein. The Cry3Aa coding sequence was PCR amplified from plasmid isolated from *B. thuringiensis* var. *san diego.* The sense primer contained a *Bam*H I site 5′ to the Cry3Aa coding sequence, while the antisense primer contained a *Not* I site 5′ to the stop codon. The PCR product was ligated into the T-overhang vector, pCRII, to produce pCRII-Cry3Aa, and the Cry3Aa insert was obtained by digesting this construct with *Bam*H I and *Not* I. The insert was ligated into the expression vector pPROEXHtb, which had been treated with the same restriction enzymes, to produce the construct pPROEXHtb-Cry3Aa. Bacterial cells transformed with pPROEXHtb-Cry3Aa were grown under conditions inducing expression of the recombinant protein. The molecular mass of the recombinant

histidine-tagged toxin was calculated to be 73 kDa. A protein band near this size was observed when the sonicated cells were subjected to SDS–PAGE (Fig. 1, lane 1). The band was absent in cells transformed with parent vector (data not shown).

The recombinant protein was expressed in insoluble form. *B. thuringiensis* Cry insecticidal proteins have been solubilized using high pH alone (9, 15, 16) or high pH in combination with urea (17). We found the latter conditions to be most effective in solubilizing recombinant histidine-tagged Cry3Aa (Fig. 1, lane 2). The urea and high pH treatment solubilized a number of contaminating proteins. Cry3Aa was further purified by batch adsorption to Ni-NTA affinity resin. Elution of the protein from the resin resulted in a Cry3Aa preparation free from other contaminating proteins (Fig. 1, lane 3). The final step in purification was a dialysis to remove urea and to decrease the pH to 8 (Fig. 1, lane 4). Approximately 0.8 mg purified protein were obtained from 1 liter of cell culture.

To verify the identity of the recombinant protein, polyclonal antibodies were raised against native Cry3Aa purified from *B. thuringiensis.* Solubilized recombinant Cry3Aa and sonicated induced cells transformed with pROEXHtb-Cry3Aa were fractionated by SDS–PAGE and transferred to nitrocellulose. The recombinant Cry3Aa protein reacted with the polyclonal antibodies at a dilution of 1:3000, confirming the identity of the recombinant protein (Fig. 2). A small amount of protein migrating near 66 kDa was present in the purified Cry3Aa preparation and also reacted with the antibodies. This lower molecular mass fragment may have been the result of proteolysis by host cell proteases on the C-terminus of the recombinant protein.

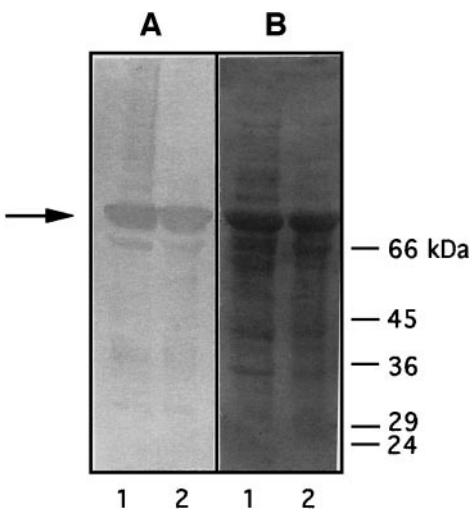

FIG. 2. Immunoblot showing recognition of recombinant Cry3Aa protein by polyclonal antibodies directed against purified native Cry3Aa. (A) nitrocellulose membrane stained with polyclonal antibodies diluted 1:3000; (B) the same membrane stained with Amido Black and India ink. Lane 1, sonicated cell extract of *E. coli* DH5α expressing Cry3Aa; lane 2, extract pellet after solubilization with urea at high pH. Sizes of molecular weight markers are indicated on the right. The position of recombinant Cry3Aa is shown by the arrow.

TABLE 1

Effect of Recombinant and Native Cry3Aa on First Instar Larvae of *Premotrypes vorax*

Solution applied to media	Number dead larvae	Number live larvae
1 None	12	69
2 Buffer A	12	69
3 Buffer B	12	69
4 Recombinant Cry3Aa in Buffer A	42	39
5 Native Cry3Aa in Buffer B	46	35
6 Crude *B. thuringiensis* Extract in Buffer B	44	37

Note. Buffer A was composed of 20 mM Tris–HCl pH 8.0, 10% glycerol, 100 mM KCl, 5 mM β-mercaptoethanol. Buffer B was composed of 25 mM K_2HPO_4, pH 7.0. The protein concentrations of purified recombinant and native Cry3Aa used were 70 μg/mL. The protein concentration of the *B. thuringiensis* extract was 700 μg/mL. Treatments 1–3 are not significantly different from each other, and treatments 4–6 are not significantly different from each other, according to Tukey's test.

To test the toxicity of Cry3Aa, first instar larvae of *P. vorax* were exposed for 5 days to media whose surface was spread with 70 μg/mL purified native or recombinant toxins (Table 1). A nonspecific mortality of ~15% was observed. Larvae exposed to toxin treated media exhibited mortalities from 57% (native Cry3Aa) to 52% (recombinant Cry3Aa). Under the conditions tested both purified native and recombinant Cry3Aa proteins appeared to be equally toxic to the Andean potato weevil.

ACKNOWLEDGMENTS

We would like to thank M. Pulido for helpful discussions. This work was supported in part by funds from Colciencias, Colombia.

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