Applied and Environmental Microbiology, Dec. 2008, p. 7145–7151 Vol. 74, No. 23
0099-2240/08/$08.00+0 doi:10.1128/AEM.00752-08

Solubilization, Activation, and Insecticidal Activity of *Bacillus thuringiensis* Serovar thompsoni HD542 Crystal Proteins▽

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Received 31 March 2008/Accepted 23 September 2008

Cry15Aa protein, produced by *Bacillus thuringiensis* serovar thompsoni HD542 in a crystal together with a 40-kDa accompanying protein, is one of a small group of nontypical, less well-studied members of the Cry family of insecticidal proteins and may provide an alternative for the more commonly used Cry proteins in insect pest management. In this paper, we describe the characterization of the Cry15Aa and 40-kDa protein's biochemical and insecticidal properties and the mode of action. Both proteins were solubilized above pH 10 in vitro. Incubation of solubilized crystal proteins with trypsin or insect midgut extracts rapidly processed the 40-kDa protein to fragments too small to be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas the Cry15 protein yielded a stable product of approximately 30 kDa. Protein N-terminal sequencing showed that Cry15 processing occurs exclusively at the C-terminal end. Cry15 protein showed in vitro hemolytic activity, which was greatly enhanced by preincubation with trypsin or insect gut extract. Larvae of the lepidopteran insects *Manduca sexta*, *Cydia pomonella*, and *Pieris rapae* were susceptible to crystals, and presolubilization of the crystals enhanced activity to *P. rapae*. Activity for all three species was enhanced by preincubation with trypsin. Larvae of *Helicoverpa armigera* and *Spodoptera exigua* were relatively insensitive to crystals, and activity against these insects was not enhanced by prior solubilization or trypsin treatment. The 40-kDa crystal protein showed no activity in the insects tested, nor did its addition or coexpression in *Escherichia coli* increase the activity of Cry15 in insecticidal and hemolytic assays.

Bacillus thuringiensis is a gram-positive bacterium, which during sporulation produces crystalline inclusions consisting of one or more insecticidal proteins known as delta-endotoxins. Based on sequence similarity, delta-endotoxins, mostly designated Cry proteins, may be divided into several broad classes (for a review, see reference 7). The vast majority of characterized Cry proteins show a number of conserved motifs, probably share similar structures, and therefore could tentatively be called three-domain Cry proteins. However, there exists a so-far small number of Cry proteins not related to the three-domain proteins, some of which can be arranged in small homology groups. These are the Cyt proteins, the Bin-like proteins, the Mtx2/3-like proteins, and the unique Cry6 and Cry22 proteins (9). These proteins may have very different modes of action compared to that of the 3-domain proteins and, therefore, are interesting subjects for further study.

Cry15Aa from *B. thuringiensis* serovar thompsoni (5) is a member of the Mtx2/3-like group, due to its similarity to the mosquitocidal Mtx2 and Mtx3 proteins from *Bacillus sphaericus*. Other members of this group, which are more similar to Cry15 than are Mtx2 and Mtx3, are the Cry23, -33, -38, and -45 proteins from *B. thuringiensis*. Their amino acid sequences show weak similarity to ß-barrel pore-forming, mammalian toxic proteins, such as aerolysin from *Aeromonas hydrophila*, cytotoxin from *Pseudomonas aeruginosa*, and epsilon toxin from *Clostridium perfringens* (9). Moreover, the three-dimensional structure of Cry23 with its binary partner Cry37 is similar to that of proaerolysin (22).

Cry15 protein, like its most similar putative homolog, Cry33 protein from *B. thuringiensis* serovar dakota (13), occurs naturally in a crystal together with an unrelated protein of approximately 40 kDa. The gene encoding Cry15's companion is arranged in an operon with *cry15* (5). Not much is known of the potential insect target spectrum of Cry15. It was originally described as toxic to tobacco hornworm (*Manduca sexta*) and, with less detail, to small white larvae (*Artogeia rapae*, now *Pieris rapae*) and as not toxic to tobacco budworm (*Heliothis virescens*) or cabbage looper (*Trichoplusia ni*) (5). For *M. sexta*, the 40-kDa protein was reported to be not toxic, and it did not increase the toxicity of Cry15Aa (5). This appeared to be different for codling moth larvae (*Cydia pomonella*), in which the presence of the 40-kDa protein-encoding gene in *B. thuringiensis* increased the activity of Cry15Aa (20). Still, less is known of the modes of action of the Cry15Aa and 40-kDa proteins, studies of which were hampered by the failure to in vitro solubilize Cry15Aa/40-kDa protein crystals in a way that maintained their biological activities (5). In this article, we present the results of the biochemical characterizations of Cry15Aa and the 40-kDa protein expressed in *B. thuringiensis*

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▽ Published ahead of print on 3 October 2008.

serovar thompsoni HD542 as well as in a heterologous system, *Escherichia coli*, and their insecticidal properties. Solubilization of Cry15Aa/40-kDa protein crystals while retaining their biological activities was achieved, enabling a more detailed analysis of the contribution of the individual proteins in insecticidal activity and the role of solubilization and proteolytic activation therein.

MATERIALS AND METHODS

Spore/crystal preparations of *B. thuringiensis* HD542. Crystal/spore preparations from *Bacillus thuringiensis* serovar thompsoni HD542 provided by the ARS (NRRL) Culture Collection, National Center for Agricultural Utilization Research (Peoria, IL), were prepared as described earlier (16). Progress of sporulation was monitored by phase-contrast light microscopy. When 75 to 80% of the cells had reached sporulation (i.e., released spores into the medium), cultures were harvested and further processed. For purification, crystals were applied to a discontinuous NaBr gradient as described before (8). The amount of crystal protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by densitometry, using a bovine serum albumin calibration curve. The protease inhibitor cocktail (complete protease inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany) was added prior to electrophoresis, according to the manufacturer's instructions.

Crystal solubilization. In order to test solubilization of the crystal/spore preparations, three different buffers were used as follows: for pH 6.0 and pH 7.0, phosphate-buffered saline (10 mM Na_2HPO_4/KH_2PO_4, 0.8% NaCl); for pH 8.0, pH 9.0, and pH 10.0, carbonate buffer (50 mM $NaHCO_3$, 100 NaCl); and for pH 11.0, CAPS (3-cyclohexylamino-1-propanesulfonic acid) buffer (50 mM CAPS, 100 mM NaCl). The buffer set described above was prepared in duplicate, with and without 10 mM dithiotreitol (DTT), added prior to use. Crystal/spore samples were incubated in buffer at 37°C for 2 h with regular shaking, followed by centrifugation at 39,000 × *g* for 20 min. Solubilization efficiency was checked by SDS-PAGE comparison of supernatants before and after centrifugation.

Cloning and expression of the Cry15Aa- and 40-kDa protein-encoding genes and the Cry15Aa/40-kDa protein-encoding operon in *E. coli*. For cloning of the Cry15Aa- and 40-kDa protein-encoding operon as well as of both genes individually, a PCR-based approach was used. For amplification of the open reading frames (ORFs) of Cry15Aa and 40-kDa protein, two sets of primers were designed: 34F (CATGCCATGGCAATTATGAATGATATTGC) and 34R (GGCGGATCCTATTCTTTATCATAATCGCGTTCATACTTC) for amplification of the *cry15Aa* gene, and 40F (CATGCCATGGATTTTAAACAATATCACAGG) and 40R (GGCGGATCCTAATCCATTACACCTATATTTCGTTG) for amplification of the 40-kDa protein-encoding ORF. The fragment encompassing the two consecutive ORFs was amplified using the 40F and 34R primers. Through these PCRs, an NcoI restriction site overlapping with the start codon was introduced, and a stop codon followed by a BamHI restriction site was introduced at the end of the coding sequence—at bp 1020 for *cry15Aa*, bp 930 for the 40-kDa protein-encoding ORF, and bp 1919 for the tandem combined Cry15/40-kDa protein-encoding ORFs. The PCR products were digested with NcoI and BamHI, run on a 0.8% agarose gel, excised and purified using a QIAEX II agarose gel extraction kit (Qiagen Benelux B.V., Venlo, The Netherlands), and subsequently used to replace the NcoI-BamHI-digested fragment (1,960 bp) of pMH10, described earlier (17), giving *E. coli* expression vectors pSN63 (*cry15Aa*), pSN64 (40-kDa protein-encoding gene), and pSN65 (Cry15Aa/40-kDa protein-encoding gene operon). Protein expression and partial purification from *E. coli* were performed as described earlier for three-domain Cry1 proteins (11).

Protease treatments. To test the effect of trypsin and insect midgut proteases on partially purified and solubilized crystals or on Cry15Aa protein expressed in *E. coli* XL1-Blue, the samples were diluted to a final concentration of 1 mg/ml, and the pH was adjusted to 9.0 using 1 M Tris-HCl, pH 8.0. Next, 10% (wt/wt) trypsin was added, and the samples were incubated at 37°C for 1 h. Insect midgut extract preparations were done as follows: 25 mg of insect midguts was ground in a microcentrifuge tube and suspended in 100 μl of sterile distilled water. After centrifugation at 15,000 × *g* for 30 min at 4°C, the protein concentration of the supernatant was determined. Midgut extract was added to each solubilized crystal or protein preparation at a 10% (wt/wt) protein extract, and samples were incubated at room temperature in time intervals from 15 min to 24 h at room temperature. To prevent further proteolytic processing after this incubation, samples were immediately heated to 96°C in SDS-PAGE sample buffer containing 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, and protease inhibitor cocktail ("Complete"; Boehringer Mannheim, Germany). The resulting products were analyzed on a 10% SDS-polyacrylamide gel and visualized using Coomassie brilliant blue R250 staining.

Proteomics. N-terminal sequencing by Erdman degradation was performed by Cambridge peptides (Birmingham, United Kingdom). Native or *C. pomonella* midgut protease, as well as trypsin-treated solubilized Cry15 proteins, was run on a 10% SDS-polyacrylamide gel. Subsequently, the proteins were blotted onto a polyvinylidene difluoride membrane for 1 h at 50 V in 10 mM CAPS buffer, pH 11, with 10% (vol/vol) methanol. Protein bands were visualized by Coomassie brilliant blue R250 staining and excised for sequencing.

Insect bioassays. To assess toxicity of preparations against *Pieris rapae*, leaves of *Arabidopsis thaliana* were used in a leaf dip bioassay by using the procedure described earlier for potato leaves (17). Briefly, single fresh *A. thaliana* ecotype Wassilewskija leaves were immersed in a test solution of Triton X-100 (0.05%) containing appropriate dilutions of proteins to be tested. After 10 s, these were taken out and allowed to dry on Whatman filter paper for 15 to 30 min at room temperature. Control leaves were immersed in test solution only. The leaves were then placed flat on water agar (0.6% agar in water) in individual plastic petri dishes. Five neonate larvae were placed on each leaf, and the experiment was replicated on different days. Mortality was scored after 4 days. Bioassays for *M. sexta*, *Spodoptera exigua*, or *Helicoverpa armigera* were performed with neonate larvae as described earlier (4). For *Cydia pomonella*, a diet surface contamination assay was used with an artificial diet, which has been described earlier (3). Fifty-percent lethal concentration (LC_{50}; concentration giving 50% mortality) and its 95% fiducial limits were estimated by Probit analysis of the results from three or more independent experiments, using the PoloPC computer program (LeOra Software, Berkeley, CA) (21). For synergy testing of different Cry15/40-kDa protein ratios on *C. pomonella* larvae, 100 neonate larvae per dose per experiment were used, and the experiment was performed three times on different days.

Hemolysis assays. Membrane pore formation by protein preparations was tested in a hemolysis assay using mouse erythrocytes. Mouse erythrocytes were pelleted from blood by centrifugation for 5 min at 1,000 × *g* and washed twice with Ringer solution (0.6% NaCl, 0.0075% KCl, 0.01% $CaCl_2$, and 0.01% $NaHCO_3$ [pH 7.5]). To each 100-μl erythrocyte suspension (4×10^8 cells per ml), different amounts of dialyzed protein samples were added, and the final volume was adjusted to 200 μl. Protein dialysis was done against 2 liters of solubilization buffer (50 mM $NaHCO_3$, 100 NaCl [pH 10]) without DTT for 18 h at 4°C. Ten-kilodalton cutoff dialysis tubing was used (Pharmacia LKB, Sweden). Subsequently, the samples were incubated at 37°C for 1 h and centrifuged at 1,000 × *g* for 10 min, and the absorption of the supernatant at 415 nm was measured using a Jenway 6405UV/VIS spectrophotometer. Negative and 100% hemolysis controls were prepared in the way described above with addition of only Ringer solution instead of protein or Triton X-100 to a final concentration of 1%, respectively.

RESULTS

Insecticidal activity of *B. thuringiensis* serovar thompsoni HD542 spore/crystal mixtures. Crystal/spore mixtures of *B. thuringiensis* strain HD542 were tested in bioassays against larvae of five different lepidopteran species, and where possible, the LC_{50} values were determined. Results of the bioassays are shown in Table 1. Unlike in other reports and in order to allow comparisons among further treatments and protein mixtures, we calculated the LC_{50} for the actual amount of Cry15 protein present in the sample as determined by gel densitometry, as opposed to using the dry weight of crystal/spore mixtures or *E. coli* inclusion bodies with undetermined Cry15 protein content. As was reported previously, we found activity against tobacco hornworm (*Manduca sexta*) larvae, codling moth (*Cydia pomonella*) larvae, and small white (*Pieris rapae*) larvae. No measurable activity was found for spore/crystal mixtures against the larvae of the noctuid beet armyworm (*Spodoptera exigua*) or cotton bollworm (*Helicoverpa armigera*).

Solubilization of HD542 crystals. *B. thuringiensis* serovar thompsoni HD542 crystals consist of two proteins, Cry15Aa and 40-kDa protein. In order to be able to study the modes of

TABLE 1. Insecticidal activities of *Bacillus thuringiensis* serovar thompsoni HD542 crystal and of Cry15 and 40-kDa proteins from recombinant *E. coli* strains

Toxin	LC_{50} (95% fiducial limit) for[a]:				
	M. sexta	*C. pomonella*	*P. rapae*	*S. exigua*	*H. armigera*
HD542 crystal	52 (36–65)	10.9 (6.8–15.1)	1,211 (641–1,584)	>4,000	>4,000
HD542 solubilized crystal	132 (74–182)	7.2 (4.5–9.9)	103 (42.3–190)	>4,000	>4,000
E. coli-produced Cry15Aa	404 (278–1,398)	19.3 (8.4–31.0)	122 (55.6–231)	NT	NT
E. coli-produced trypsin-treated Cry15Aa	186 (153–246)	5.4 (2.8–9.2)	24.0 (10.0–39.3)	>4,000	>4,000
cry15Aa operon	NT	18.9 (12.7–25.5)	NT	NT	NT
Cry15Aa plus 40-kDa protein	NT	18.4 (8.3–30.1)	NT	NT	NT

[a] NT, not tested.

action of the crystals and to better understand the role of the individual components in toxicity, a number of solubilization experiments at different buffer compositions and pHs were done. Several different buffers with a pH ranging from 6.0 up to 11.0, with or without the reducing agent DTT (10 mM), were tested, and total protein amount was compared with the amount of protein in the supernatant after solubilization. As shown in Fig. 1A and B, simultaneous solubilization of the two proteins from the crystals was achieved only at pH 10.0 and pH 11.0, in sodium hydrogen carbonate and CAPS buffer, respectively. The solubility of crystals in the same buffers, without DTT, was tested as well (Fig. 1C and D). Our data (compare Fig. 1A and C) show that the reduction of sulfur bridges by DTT slightly increased solubility at lower pHs; both proteins are readily solubilized at pH 10 in the presence of DTT but not without. Ethanolamine buffer (20 mM ethanolamine, 50 mM NaCl [pH 10.0 and pH 9.0]), Tris buffer (50 mM Tris-HCl, 100 mM NaCl [pH 9.0]), or borate-buffered saline (100 mM H_3BO_3, 50 mM NaCl [pH 9.0]) did not solubilize a significant amount of either protein, with or without addition of DTT (data not shown).

The supernatant of the solubilized spore/crystal mixture was tested in bioassays in a way similar to that for the unsolubilized preparations as discussed above. As shown in Table 1, solubilized crystals maintained activity against *M. sexta*, *C. pomonella*, and *P. rapae*. For *C. pomonella* larvae, there appeared to be no significant difference between toxicities before and after solubilization. Since this observation rules out straightforward inactivation of (a fraction of) the crystal proteins during solubilization, there is no clear explanation for why solubilized crystals show a slight decrease in activity against *M. sexta*. Solubilized crystals had distinctly increased activity compared to that of unsolubilized crystals in the *P. rapae* bioassay. Solubilization did not affect activity against *S. exigua* and *H. armigera*, which remained undetectable.

Expression of Cry15Aa and 40-kDa proteins in *E. coli*, and their insecticidal activity. Previous studies have indicated that detectable expression of Cry15 protein in *B. thuringiensis* requires the presence and expression of the 40-kDa protein-encoding gene (19), while in *E. coli*, both simultaneous expression of the two genes in one operon as well as expression of the two genes separately resulted in detectable proteins in inclusion bodies (5). In order to be able to separately study the roles of the two constituent proteins of the HD542 crystal in toxicities toward different insects, we expressed the *cry15*/40-kDa protein-encoding gene operon, as well as the *cry15* and 40-kDa protein-encoding genes separately, in *E. coli* strain XL1-Blue. The standard fermentation and inclusion body purification procedures used for other delta-endotoxins yielded inclusion bodies containing either Cry15Aa, the 40-kDa protein, or both in more or less equal amounts, which were all soluble at pH 10. SDS-PAGE showed that the *E. coli*-produced proteins have the same relative molecular masses as the original *B. thuringiensis*-produced and solubilized crystal proteins (Fig. 2).

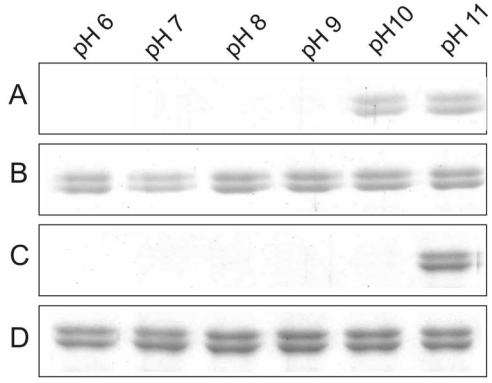

FIG. 1. Solubilization of *Bacillus thuringiensis* serovar thompsoni HD542 crystals. Solubilization at the following pHs in the presence (panels A and B) and in the absence (panels C and D) of 10 mM DTT: pH 6.0 (lanes 1), pH 7.0 (lanes 2), pH 8.0 (lanes 3), pH 9.0 (lanes 4), pH 10.0 (lanes 5), and pH 11.0 (lanes 6). Panels A and C show proteins in the soluble fraction, and panels B and D represent the total protein fraction.

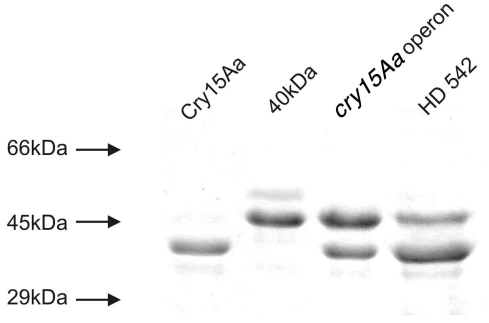

FIG. 2. Cry15Aa and 40-kDa protein produced by recombinant *E. coli* strain XL1-Blue and by *Bacillus thuringiensis*. Inclusion bodies or crystals solubilized, at pH 10, Cry15Aa from *E. coli* (lane 1), 40-kDa protein from *E. coli* (lane 2), Cry15Aa and 40-kDa proteins produced by expression of the *cry15Aa* operon in *E. coli* (lane 3), and crystals from *B. thuringiensis* serovar thompsoni HD542 (lane 4).

TABLE 2. Insecticidal activity of Cry15Aa at ½ LC_{50} (9.5 ng cm^{-2}) with the addition of different amounts of 40-kDa protein, produced from recombinant *E. coli* strains[a]

Cry15Aa/40-kDa protein ratio	Average mortality (%)		*P* value
	Expected	Observed	
1:0	20	22.0 ± 7.9	
1:1	20	18.0 ± 7.0	0.58
1:0.5	20	20.0 ± 6.9	0.75
1:0.25	20	14.6 ± 2.1	0.19
1:0.1	20	16.0 ± 5.2	0.33

[a] Observed mortality is the average of three experiments plus the standard deviation. *P* values were determined by analysis of variance.

Solubilized *E. coli*-produced preparations of Cry15 were tested in a bioassay against the insects previously shown to be sensitive to HD542 crystals. Compared to the activity of solubilized HD542 crystals, and based solely on the amount of Cry15 in each preparation, solubilized Cry15 produced in *E. coli* appeared slightly less active than *B. thuringiensis*-produced Cry15 for all three sensitive insects (Table 1). This difference might have been due to synergy with the presence of the 40-kDa protein or of residual *B. thuringiensis* spores in the spore/crystal preparations. In *E. coli* preparations, solubilized 40-kDa protein by itself had no activity against any of the tested insects (at 2,000 ng/cm^2 for *M. sexta*, *C. pomonella*, *S. exigua*, and *H. armigera* or at 5,000 ng/ml for *P. rapae*). As reported earlier (5), we found no increase in toxicity of Cry15 to *M. sexta* upon addition of equal amounts of solubilized 40-kDa protein. It was previously reported that the 40-kDa protein did increase the activity of *B. thuringiensis*-produced Cry15 against *C. pomonella* through synergistic action, although only when produced simultaneously in the same recombinant strain (20). Therefore, we studied the combined action of *E. coli*-produced Cry15 and 40-kDa proteins against this insect in more detail. Based on the amount of Cry15 protein only, the LC_{50}s of Cry15 alone and of Cry15 together with an equal amount of the 40-kDa protein, either produced in the same inclusion body or produced separately and then mixed, did not differ significantly (Table 1). In order to further study the potential Cry15Aa/40-kDa protein synergism in *C. pomonella* suggested by Rang et al. (20), 100 first-instar *C. pomonella* larvae were subjected to Cry15Aa at doses corresponding to 20% expected mortality, equivalent to ½ LC_{50} (9.5 ng cm^{-2}), and 40-kDa protein was added at five different Cry15/40-kDa protein ratios (1:0, 1:1, 1:0.5, 1:0.25, and 1:0.1). Comparison of the expected mortality with the observed mortality (Table 2) did not show a statistically significant (analysis of variance) increase of insecticidal activity of Cry15Aa by addition of 40-kDa protein.

In conclusion, we found no evidence of synergistic action of the 40-kDa protein on Cry15 activity against any of the tested insects, when using solubilized *E. coli*-produced proteins.

Proteolytic processing by insect gut proteases and bovine trypsin. Cry1 proteins require proteolytic processing in the gut of susceptible insects to produce the "activated" toxin, which has receptor-binding and pore-forming capacities. We investigated possible proteolytic processing of solubilized HD542 crystal proteins as well as of the *E. coli*-produced Cry15 and 40-kDa proteins. Aliquots of solubilized proteins were incubated with dilute gut extracts from all five tested insects for different time periods, after which proteolytic action was stopped by the addition of SDS-PAGE sample buffer with added protease inhibitors and heat. A typical time course is shown for one insect, *P. rapae*, in Fig. 3. As can be seen in Fig. 3, the 40-kDa protein in solubilized HD542 crystals is rapidly processed to smaller forms (Fig. 3, lanes 2 to 9, from 15 min to 24 h of treatment, respectively), whereas Cry15 is processed more slowly. With the gut extract dilution used in this experiment, after 8 h of incubation (Fig. 3, lane 8) a protein with an apparent molecular mass of approximately 28 kDa formed, and it appeared to remain stable for at least 24 h (Fig. 3, lane 9). Processing by gut proteases from the other four tested insects showed a similar pattern overall (not shown). All gut proteases produced a major protein product with the same apparent molecular mass (Fig. 4A) after prolonged incubation of solubilized crystals, with variable amounts of minor processing intermediate of slightly higher molecular mass (for example,

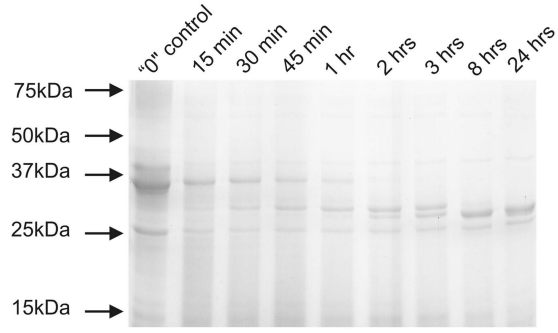

FIG. 3. Proteolytic processing of solubilized crystal proteins for 24 h by *P. rapae* gut extract. Solubilized HD542 crystals were incubated in diluted *P. rapae* gut juice and incubated at room temperature. At regular intervals, samples were removed and immediately boiled in SDS-PAGE sample buffer. Lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 45 min; lane 5, 1 h; lane 6, 2 h; lane 7, 3 h; lane 8, 8 h; lane 9, 24 h.

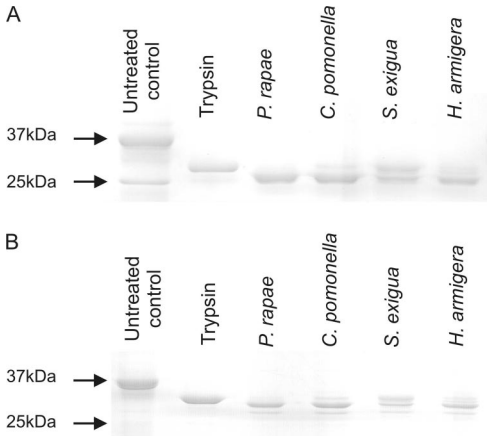

FIG. 4. Stable digestion products of Cry15Aa. Solubilized crystals produced by *B. thuringiensis* serovar thompsoni HD542 (A) or Cry15Aa produced by *E. coli* (B). Proteins were left untreated (lanes 1) or incubated with trypsin (lanes 2), *P. rapae* gut extract (lanes 3), *C. pomonella* gut extract (lanes 4), *S. exigua* gut extract (lanes 5), or *H. armigera* gut extract (lanes 6).

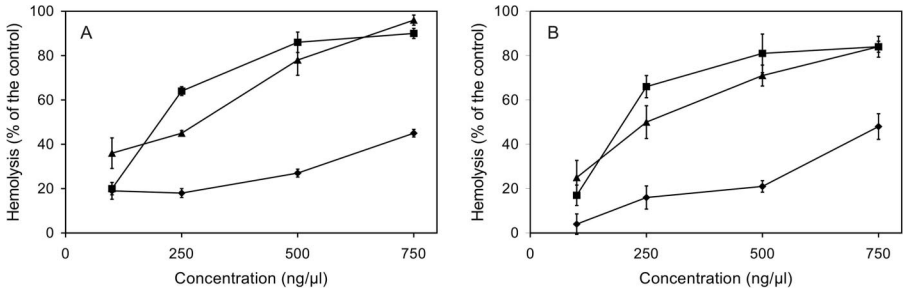

FIG. 5. In vitro hemolytic activities of Cry15Aa and 40-kDa protein. Hemolysis measured by absorption at 415 nm in mouse erythrocyte supernatant, expressed as percentage of absorption at complete hemolysis obtained with 1% Triton X-100. Concentrations are based on Cry15 content only, solubilized *E. coli*-produced Cry15Aa plus 40-kDa protein (*cry15Aa* operon; panel A), or Cry15Aa only (panel B). Protein was untreated (◆), pretreated with trypsin (■), or pretreated with *C. pomonella* gut juice (▲).

Fig. 4A, lane 4). Incubation of *E. coli*-produced, solubilized Cry15 protein resulted in apparently identical protein bands (Fig. 4B), suggesting that the visible proteolytic cleavage products from HD542 crystals consist of processed Cry15Aa only. This is supported by the observation that incubation of *E. coli*-produced, solubilized 40-kDa protein leaves no peptides with a molecular mass that may be detected by SDS-PAGE (not shown). Activation of Cry1 proteins by gut proteases can be mimicked by in vitro treatment with bovine trypsin (1). Similarly, treatment of solubilized HD542 crystals or solubilized Cry15Aa produced in *E. coli* with bovine trypsin yielded a protein with a slightly higher apparent molecular mass, as after treatment with gut proteases (Fig. 4A and B, lane 2).

To assess the effect of proteolytic processing of Cry15Aa on insecticidal activity, we tested in vitro trypsin-activated toxin in bioassays on all insects. For all three sensitive insects, trypsin pretreatment markedly increased activity, lowering LC_{50}s by two- to fourfold for *M. sexta* and *C. pomonella*, respectively, and by approximately fivefold for *P. rapae*. Trypsin-treated Cry15Aa had no detectable activity against *S. exigua* and *H. armigera*, indicating that the lack of activity of Cry15 against these insects is not likely to be caused by defective proteolytic processing in their guts (Table 1).

We also tested the activity of the *C. pomonella* gut extract processing product of Cry15Aa, as shown in Fig. 4B (lane 4), against first-instar *C. pomonella* larvae. This product lacked any measurable activity, suggesting that the active form of Cry15Aa is a processing intermediate larger in size than the protease-resistant final product, produced after 24 h of treatment (Fig. 3, lane 9). Such intermediate forms, which may be more similar to the trypsin-processed Cry15Aa, can be seen to be formed during gut extract treatments of between 15 min and 8 h (Fig. 3, lanes 2 to 8).

Structural characterization of gut extract and trypsin-digested Cry15. In order to understand the relation between protein primary structure and function as influenced by proteolytic processing, we characterized intact Cry15Aa and the major products from it by trypsin or *C. pomonella* gut protease action (Fig. 4B, lanes 2 and 4, respectively) in more detail. N-terminal sequencing revealed the sequence Met-Ala-Ile-Met-Asn for all three proteins. Since this sequence is identical to the predicted N-terminal sequence of intact Cry15, we conclude that neither trypsin nor *C. pomonella* gut proteases process Cry15Aa at its N terminus.

Nonspecific pore-forming activity of Cry15Aa and 40-kDa proteins in vitro. To further characterize the mode of action of Cry15Aa and the role of the 40-kDa protein therein, we have tested the nonspecific pore-forming activity of Cry15Aa in its different forms in a hemolysis assay on mouse erythrocytes. As is shown in Fig. 5, both Cry15Aa and a combination of Cry15Aa and the 40-kDa protein demonstrated hemolytic activity. A strong increase in hemolytic activity was observed after trypsin and *C. pomonella* midgut extract treatment of the same samples. The lack of significant difference in the hemolytic activities of Cry15Aa and the 40-kDa protein produced together by expression of the full operon in *E. coli* and in that of Cry15Aa alone produced by expression of the *cry15Aa* gene (with equal amounts of Cry15 tested) (Fig. 5A and B) showed that the presence of the 40-kDa protein did not change the hemolytic activity of Cry15Aa. Both trypsin preincubation as well as gut extract treatment increased hemolytic activity, in contrast to in vivo activity assays, in which complete gut extract processing resulted in loss of insecticidal activity.

DISCUSSION

Cry15 belongs to a small homology group of *B. thuringiensis* toxins (Fig. 6), which are likely to have a structure and mode of action different from the main group of *B. thuringiensis* toxins, the so-called three-domain toxins (9). They have different, yet relatively unexplored, host specificities ranging from *Lepidoptera* for Cry15 to *Coleoptera* for Cry23A (10) and Cry38A (M. J. Rupar, W. P. Donovan, C.-R. Chu, E. Pease, Y. Tan, A. C. Slaney, T. M. Malvar, and J. A. Baum, 2000, PCT international application WO0066742) and in vitro cytotoxicity against human cell lines for the so-far noninsecticidal Cry45Aa (14). Although these proteins have low overall sequence similarity, they share conserved sequence motifs consisting of an amphipathic loop region flanked by two Ser/Thr-rich regions (Fig. 6), with each other as well as with a large and diverse group of protein toxins from a variety of organisms (23). The three-dimensional structures of aerolysin (18), epsilon toxin (6), and *Laetiporus sulphureus* (a mushroom) hemolytic pore-forming lectin (23) suggest that these motifs represent beta-sheets and an amphipathic loop region, which in alpha-toxin has been shown to insert into the target membrane (15). These structural analogies suggest that Cry15 and the other toxins dis-

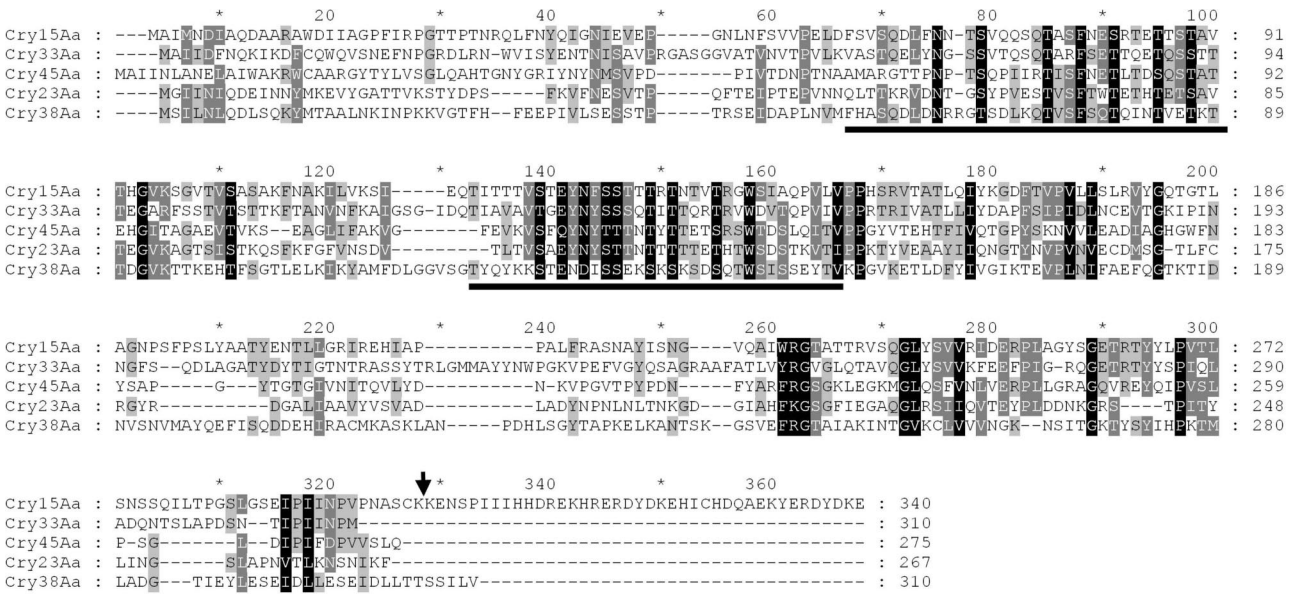

FIG. 6. Protein sequence alignment of the *Bacillus thuringiensis* Mtx2/3-like proteins. The S/T-rich regions flanking the putative pore-lining loop are underlined. A putative trypsin-processing site used by trypsin in vitro is indicated by an arrow.

cussed here are part of the β-pore-forming toxins, although in most cases this awaits experimental confirmation.

In this paper, we have investigated some of the biochemical and insecticidal properties of Cry15Aa and the 40-kDa protein, encoded by the *cry15Aa* operon. In contrast to earlier reports by Brown and Whiteley (5) and Rang et al. (20), we have managed to solubilize both proteins in carbonate buffer at pH 10, which gave us an opportunity to conduct more-accurate insect bioassays with respect to the amount of active protein and to the role of the two components of the crystal. We tested each protein separately, as well as together, in their natural Cry15Aa/40-kDa protein ratio and in equimolar combinations of both proteins produced separately. In contrast to an earlier study that suggested synergistic activity of the 40-kDa protein for insecticidal activity of Cry15 against *C. pomonella* (20), we did not observe such a role of the 40-kDa protein in our experiments. The apparent contradiction between these two sets of results could be explained by an earlier observation regarding the role of the 40-kDa protein in crystal formation in its natural environment, sporulating *B. thuringiensis* (19). Disruption of either of the two genes resulted in a lack of crystal formation and a concurrent decrease in production of the other protein. Expression of only *cry15* in *B. thuringiensis* resulted in low protein production and low activity against *C. pomonella*, which was raised 10-fold by introduction of the 40-kDa protein-encoding gene. In this case, spore/crystal preparations of *B. thuringiensis* were used, and the effect of the 40-kDa protein on Cry15 production was not considered (20). We therefore conclude that the 40-kDa protein protein does not truly synergize Cry15Aa action in the insects tested in our study, including in *C. pomonella*, although a possible active role in other insects cannot be excluded. The major role of the 40-kDa protein in its natural setting could be solely for crystal formation and for achieving higher Cry15Aa levels. Possibly, the 40-kDa protein is an essential partner for Cry15Aa in crystal formation, and in its absence, Cry15Aa is rapidly degraded.

Our preliminary structural and functional characterizations of the trypsin- and insect gut protease-treated Cry15Aa strongly suggest that (limited) C-terminal processing is necessary for insecticidal activity. Trypsin treatment of Cry15Aa resulted in a protein with an N-terminal sequence identical to that of unprocessed Cry15Aa, indicative of exclusively C-terminal processing. Preliminary quadrupole-time of flight analysis of in-gel trypsin-treated intact or trypsin-preincubated Cry15Aa suggests that the processing occurs at lysine residue 300 or 301, resulting in a protein of 32.45 or 32.59 kDa, respectively (results not shown). This would be in good agreement with the estimated molecular mass of 31 kDa observed during gel electrophoresis. This processing removes a 40-amino-acid-residue-long C-terminal extension of Cry15Aa compared to the other proteins of its similarity group (Fig. 6). This extension is rich in arginines and lysines, which make it particularly susceptible to trypsin degradation. Curiously, in this extension, positively and negatively charged amino acids alternate, which could result in opposite charges on the sides of the protein chain or β-sheet. Trypsin treatment also resulted in increased hemolytic activity. Together, these results show that Cry15Aa is activated by C-terminal processing similar to what was shown earlier for alpha-toxin (2) and aerolysin (12). Whereas an intermediary product with a size comparable to that of trypsin-treated Cry15Aa was observed during insect gut protease treatment, the final processing product was smaller, indicating that, with no N-terminal processing, gut proteases could process more extensively at the C-terminal side of Cry15Aa, possibly through the action of chymotrypsin-like proteases. Whereas *Cydia* gut protease-treated Cry15Aa retained hemolytic activity, insecticidal activity was lost. This suggests that for some aspects of insect-specific activity, such as possible

receptor binding, the C-terminal protein sequence around or just in front of residue 300 is essential.

In conclusion, we have characterized the first steps in the mode of action of Cry15Aa, a relatively less-known member of the extended and diverse Cry protein (or Bt toxin) family. Based on the biochemical properties of similar microbial proteins like alpha-toxin and aerolysin, we can speculate that further steps include receptor binding, oligomerization, and pore formation, but this awaits further experimentation.

ACKNOWLEDGMENTS

We gratefully acknowledge the help of a number of people in supplying us with insect eggs: Els Roode and Salvador Herrero (Department of Virology, Wageningen University) for *S. exigua*, Joop van Loon and Qingbo Tang (Department of Entomology, Wageningen University, Wageningen, The Netherlands) for *P. rapae* and *H. armigera*, and S. Reynolds and A. Meredith (University of Bath, Bath, United Kingdom) for *M. sexta*.

S.N. was supported by a fellowship from The Netherlands Organization for Scientific Research (NWO) International Fellowship Program for Romanian and Bulgarian postdoctorates. R.B. was partially supported by a short-term scientific mission fellowship from the EU program COST862, "Bacterial Toxins for Insect Control."

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