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# Binding of the CryIVD Toxin of *Bacillus thuringiensis* subsp. *israelensis* to Larval Dipteran Midgut Proteins

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**Ligand-blotting experiments on dipteran brush border membrane vesicles (BBMVs) showed binding of CryIVD toxin of *Bacillus thuringiensis* subsp. *israelensis* to proteins of 148 kDa in *Anopheles stephensi* and of 78 kDa in *Tipula oleracea*, both species being susceptible to CryIVD. Binding of CryIVD with BBMVs of *A. stephensi* resulted in a stronger signal than with BBMVs of *T. oleracea*. Likewise, larvae of *A. stephensi* are 10,000-fold more susceptible to the CryIVD toxin than are larvae of *T. oleracea*. Binding was also found with six proteins ranging in size from 48 to 110 kDa in BBMVs from the lepidopteran species *Manduca sexta*, but CryIVD was not toxic for *M. sexta* larvae. No binding of trypsinated CryIVD to BBMV proteins was observed. With the lepidopteran-specific toxin CryIA(b), no binding to dipteran BBMVs was found. Binding of CryIA(b) to nine different BBMV proteins ranging in size from 71 to 240 kDa was observed in *M. sexta*. The major binding signal was observed with a protein of 240 kDa for CryIA(b).**

Worldwide environmental problems and insect resistance to chemical pesticides have prompted the use of environmentally safe biopesticides. *Bacillus thuringiensis* represents the most promising microbial biocontrol agent, as well as a source of toxin genes for transgenic plants.

During the sporulation phase of *B. thuringiensis*, a parasporal proteinaceous crystal composed of one or more different insecticidal proteins is produced (17). The crystal is solubilized under the alkaline conditions of the insect midgut (18) and converted by midgut proteases from a protoxin into a toxin with specific binding properties to different receptor molecules situated on the midgut epithelium (9, 35). The presence and affinity of such receptors for particular toxins generally correlate with the susceptibility of the insect to these toxins (16, 34, 35), but the opposite may occur (40).

*B. thuringiensis* subsp. *kurstaki* has been widely applied in agriculture and forestry for the control of lepidopteran pests, whereas *B. thuringiensis* subsp. *israelensis* has been used successfully in the control of some dipteran pests. In addition to a 27-kDa cytolysin with general toxicity, *B. thuringiensis* subsp. *israelensis* produces at least three other proteins with molecular masses of 125 kDa (CryIVA), 130 kDa (CryIVB), and 68 kDa (CryIVD) (8, 10, 17), which are toxic exclusively for dipteran species belonging to the suborder Nematocera. Among the members of this suborder are insect vectors of important tropical diseases such as malaria, as well as some insect species of agricultural importance (*Tipula* sp. and *Sciarida* sp.). Tipulid larvae cause significant damage in grasslands and in horticultural crops. Field control of these soilborne larvae is still dependent on the environmentally unsafe organophosphate insecticides. The development of safe alternatives is urgently needed. Moreover, *Anopheles stephensi*, an important vector of malaria, is resistant to a large group of inexpensive conventional insecticides. *B. thuringiensis* subsp. *israelensis* is a good alternative larvicide for anopheline species. Many other dipteran agricultural pests, such as root, cabbage, stable, and onion flies, are insensitive to *B. thuringiensis* subsp. *israelensis* endotoxins. Studies on toxin binding and midgut physiology may elucidate the molecular basis of this specificity.

*B. thuringiensis*-resistant pest insects are likely to emerge as a consequence of frequent and large-scale application, especially of single-gene-based *B. thuringiensis* pesticides (22), along with the introduction into agriculture of *B. thuringiensis* transgenic plants (1, 29, 33). Resistance to certain *B. thuringiensis* toxins was found to be associated with a reduction of specific binding of the toxin to receptor molecules in the midgut (12, 36), but exceptions to this mechanism have also been observed (14).

Ravoahangimalala et al. (26) showed in vivo binding of CryIVD to the apical brush border of the gastric ceca and posterior stomach in histological sections of intoxicated *Anopheles gambiae* larvae fed with complete *B. thuringiensis* subsp. *israelensis* crystals. From their experiments, however, it was not clear whether binding was with the 65-kDa polypeptide protein or with its proteolytic cleavage products. Because in vivo processing of the CryIVD toxin is likely (5, 7), we studied the binding of both CryIVD and of trypsin-processed CryIVD, since trypsin is the major proteolytic enzyme in the mosquito larval midgut (3, 42). The larvae of the highly susceptible *A. stephensi*, the less susceptible *Tipula oleracea* (38), and the nonsusceptible *Manduca sexta* were used as target insects in our work. The binding studies with the CryIA(b) toxin, to which larvae of both dipteran species are nonsusceptible while larvae of the lepidopteran species *M. sexta* are highly susceptible, have been used as a control.

## MATERIALS AND METHODS

**Rearing of insects.** The origin of the mosquito strain and the rearing of the mosquito larvae were described previously (11). Adults were fed with blood through Parafilm stretched over a glass feeder (85 mm in diameter, 2 mm deep) (24) that was filled with bovine blood and kept at 37°C. *T. oleracea* larvae were reared as described previously (39), and second-instar larvae used in the bioassays were kindly supplied by G. Wiegers. Eggs of *M. sexta* (from Carolina Biological Supply Co., Burlington, N.C.) were placed at 25°C, and larvae were reared to the second instar on a semisynthetic diet.

**Bacterial strains, plasmids, and cloning strategy.** *B. thuringiensis* subsp. *israelensis* IPS-82, corresponding to strain 1884 from H. de Barjac, was used for the cloning of the *cryIVD* gene. Plasmid pUC18 and the shuttle vector pHV33 (25) were used in general cloning techniques (27). Transformation of *Escherichia coli* MC 1061 (Bio-Rad) and the plasmidless *B. thuringiensis* subsp. *israelensis* recip-

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ient strain 4Q7 (Bacillus Genetic Stock Center, Columbus, Ohio) was done by electroporation.

The gene coding for the CryIVD crystal protein was cloned previously (37). A 5-kb *Hin*dIII-*Eco*RV fragment encompassing the *cryIVD* gene and its regulatory region was subcloned into pUC18 cut with *Hin*dIII and *Hin*dII. To ensure replication of the construct in *B. thuringiensis*, the pC194 part of shuttle vector pHV33 was excised with *Hin*dIII and inserted at the *Hin*dIII site of the above-mentioned plasmid. This construct was electroporated into the nontoxic and acrystalliferous *B. thuringiensis* subsp. *israelensis* 4Q7, and selection on Luria-Bertani agar with chloramphenicol (7.5 μg/ml) resulted in *B. thuringiensis* subsp. *israelensis* recombinant 759.

**Purification and processing of crystal proteins.** A 10-ml portion of an overnight culture of *B. thuringiensis* subsp. *israelensis* 759, expressing the *cryIVD* gene, was diluted 400-fold in minimal medium (31) supplemented with chloramphenicol (10 μg/ml) and was incubated at 30°C for 5 days. At that time, the bacterial cells had sporulated, and the spore-crystal mixture was pelleted for 10 min at 6,000 × *g* and 4°C. The pellet was washed three times with distilled water and finally resuspended in 30 ml of 10 mM NaCl–1 mM EDTA (pH 7.5). After homogenization in a glass tissue grinder for 1 min at 0°C, crystals were separated from spores and cells by discontinuous sucrose gradient centrifugation (30). The gradient consisted of 12-ml layers of 79, 72, and 67% sucrose in 50 mM Tris-HCl (pH 7.5)–10 mM KCl. The gradient was overlaid with 1 ml of the homogenate and centrifuged for 18 h at 80,000 × *g* and 4°C. Three bands (one within the 67% sucrose layer, one on top of the 79% sucrose layer, and one within the 79% sucrose layer) and the pellet were collected and washed three times with 50 mM Tris-HCl (pH 7.5)–5 mM EDTA. The final pellet obtained from the four fractions was resuspended in 50 mM Tris-HCl (pH 7.5). The band located at the interphase between the 72 and 79% sucrose layers was sharp and intensely white and consisted predominantly of rectangular protein crystals, corresponding to the description given by Ibarra and Federici (19). A bioassay with the different sucrose gradient fractions after washing and with equal protein concentrations on second-instar *A. stephensi* larvae showed that the middle fraction was indeed the most toxic (data not shown).

Solubilized CryIVD toxin was obtained as follows. Crystals were pelleted in an Eppendorf centrifuge (14,000 × *g*), and the pellet was resuspended in an equal volume of 50 mM $Na_2CO_3$–50 mM dithiothreitol (pH 12.5). Solubilization was achieved by incubation for 1 h at 55°C. Undissolved crystals were removed by a 5-min centrifugation at 14,000 × *g*, and the supernatant was neutralized by addition of 1/20 volume of 12% citric acid.

Proteolytic processing was performed by adding trypsin or chymotrypsin to solubilized CryIVD (toxin/protease ratio, 8:1 [wt/wt]) at 37°C for 1 h. The digestion was stopped by the addition of phenylmethylsulfonyl fluoride at a final concentration of 100 μg/ml at 37°C for 30 min.

Fast protein liquid chromatography (FPLC)-purified and proteolytically activated CryIA(b) toxin was kindly provided by the *B. thuringiensis* research group of Centre for Plant Breeding and Reproduction Research (CPRO-DLO), Wageningen, The Netherlands (courtesy of D. Bosch).

**Ligand blotting.** Brush border membrane vesicles (BBMVs) were obtained by the $Mg^{2+}$ precipitation method of Wolfersberger et al. (41) as modified by Oddou et al. (23), who included protease inhibitors during dissection and all purification steps. This procedure has been successfully applied previously to obtain BBMVs from the dipteran insect *Aedes aegypti* (32).

Fourth-instar larvae of *A. stephensi* and *T. oleracea* were placed on an ice-cooled microscope platform in MET buffer [0.3 M mannitol, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 17 mM Tris-HCl (pH 7.5)]. By pulling the thorax and last abdominal segment in opposite directions with dissection needles, the alimentary tract was isolated. The midgut, i.e., the part between the cecum and the hindgut, without Malpighian tubules, was isolated in MET buffer and stored at −20°C until use. Midguts of 100 larvae in 500 μl of MET buffer were sonicated in three bursts of 30 s at 5% output power (ultrasonic microtip probe, 20 kHz; model UP-400; Sonicor Instrument Corp.) with a precooled probe and with ice cooling of the vessel (1.5-ml Eppendorf tube) for 1 min between the bursts. After differential centrifugation, the BBMV pellet was resuspended by sonication in 100 μl of MET–$MgCl_2$ (0.5× MET buffer, 12 mM $MgCl_2$) and homogenized by being passed five times through a 27-gauge syringe needle. The protein content of a BBMV suspension was determined by the Bio-Rad microassay with bovine serum albumin as a standard. *M. sexta* BBMV proteins were prepared without the use of proteinase inhibitors and were obtained from the *B. thuringiensis* research group of CPRO-DLO, Wageningen, The Netherlands (courtesy of D. Bosch).

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (20), BBMVs (10 μg of protein per lane) were heated at 95°C in 50 mM Tris-HCl (pH 6.8)–1% SDS–40 mM dithiothreitol before being loaded onto a 6% gel. Electroblotting to nylon-supported nitrocellulose (Hybond-ECL; Amersham) was done at 110 V and 250 mA for 90 min with transfer buffer (25 mM Tris-HCl [pH 8.3], 192 mM glycine, 20% methanol). Nonspecific binding was prevented by blocking for 1 h in 2% blocking reagent (Boehringer) dissolved in TBS (200 mM Tris-HCl [pH 7.6], 0.138 M NaCl). The blots were incubated with toxin (10 μg/ml) and antisera in 2% blocking reagent dissolved in TBS–0.05% Tween 20. Bound toxin was detected with 2,000-fold-diluted anti-CryIVD polyclonal antiserum. The antiserum was raised in a rabbit by five subcutaneous injections, at 3-week intervals, of 2 mg of purified and solubilized CryIVD crystals in incomplete Freund's adjuvant. Horseradish peroxidase (2,000-fold diluted) conjugated to donkey anti-rabbit immunoglobulin G (Amersham) was used as the secondary antibody. Peroxidase activity was detected by the enhanced chemiluminescence method (Amersham) as specified by the manufacturer, except for the washing steps. Washings were done between all incubations by rinsing the blot twice in 2% blocking reagent in TBS–0.05% Tween 20 followed by performing two 15-min incubations in the same solution. The blots were stripped by incubation at 50°C in a shaking water bath containing 62.5 mM Tris-HCl (pH 6.7)–100 mM β-mercaptoethanol–2% SDS. The specificity of the binding was checked on a parallel blot treated in an identical way except for the incubation with the respective toxins.

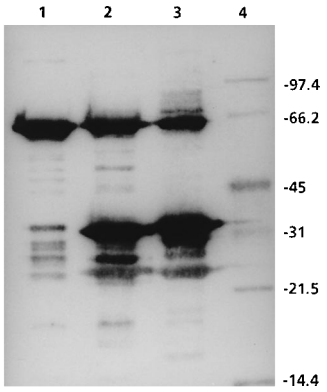


FIG. 1. Immunoblot (enhanced chemiluminescence luminogram) of sucrose gradient-purified CryIVD before (lane 1) and after proteolysis with trypsin (lane 2) or chymotrypsin (lane 3). Lanes: 1, 112 ng of CryIVD protein from the middle sucrose gradient fraction; 2, 250 ng of CryIVD after trypsin incubation; 3, 250 ng of CryIVD after chymotrypsin incubation; 4, biotinylated protein molecular weight markers (Bio-Rad), detected by enhanced chemiluminescence (Amersham) with streptavidin ligated to horseradish peroxidase.

**Insect toxicity assays.** *B. thuringiensis* subsp. *israelensis* IPS-82, 4Q7, and 759 were grown in minimal medium (31) at 30°C for several days. Sporulation of the cultures was monitored daily under the microscope, and spores and crystals were harvested when sporulation was complete. Spore counts were determined in a Bürkner-Türk chamber. The toxicity of *B. thuringiensis* subsp. *israelensis* toxins for *A. stephensi* larvae was determined by placing second-instar larvae ($n$ = 10) in a petri dish (30 mm in diameter) containing 5 ml of distilled water with various dilutions of washed crystal-spore suspensions from completely sporulated *B. thuringiensis* subsp. *israelensis* cultures. Each test was done in triplicate. The larvae were kept for 18 h at 30°C before survival was determined. The susceptibility of second-instar larvae of *T. oleracea* was assayed by spreading crystal-spore suspensions on 16-mm lettuce leaf disks as described previously (39). *M. sexta* second-instar larvae were tested for susceptibility to sucrose gradient-purified CryIVD toxin. The larvae were exposed to a 2-$cm^2$ surface of artificial medium that had been treated with known quantities CryIVD protein and placed in the wells of a macroplate (Greiner). Survival and weight were determined after 5 days at 20°C.

Logit transformation of the data was used to estimate the regression coefficient and the intercept of the dose-response curve, allowing the calculation of the spore-crystal number per ml causing 50% mortality ($LC_{50}$).

## RESULTS

**Binding studies of CryIVD and CryIA(b) to blotted larval midgut proteins of *A. stephensi*, *T. oleracea*, and *M. sexta*.** Proteolytic processing of CryIVD takes place in the insect midgut and may be a prerequisite for binding to receptor molecules (7). It was therefore important to test whether the anti-CryIVD antiserum also bound to the proteolytically derived fragments of CryIVD. Therefore, solubilized CryIVD and CryIVD treated with trypsin and chymotrypsin were analyzed by SDS-PAGE and immunoblotting (Fig. 1). The majority of the protein from the middle sucrose gradient fraction consisted of a 65-kDa protein, which could be processed by trypsin into major fragments of 33.5, 28, and 25 kDa and by chymotrypsin into major fragments of 35, 30, and 25 kDa. Figure 1 shows that the CryIVD antiserum bound to both the 65-kDa protein and the moieties obtained after proteolysis.

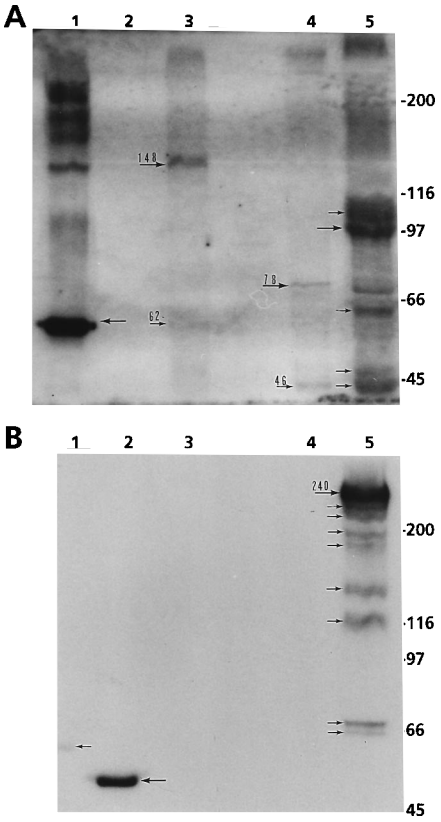


FIG. 2. Immunoblot demonstrating the binding of nontrypsinated CryIVD (A) and activated CryIA(b) (B) to midgut proteins of *A. stephensi* (lanes 3), *T. oleracea* (lanes 4), and *M. sexta* (lanes 5). A 10-μg sample of midgut protein was loaded per lane. Lanes 1 and 2 contain 500 ng of CryIVD and 300 ng of CryIA(b), respectively. Panel A was obtained after subsequent incubations with CryIVD as the ligand, rabbit anti-CryIVD as the primary antibody, and donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase. Panel B was obtained after stripping of the blot in panel A followed by reincubations with CryIA(b), anti-CryIA(b) antibodies, and the donkey anti-rabbit conjugate. Arrows indicate strong (large arrows) and weak (small arrows) binding signals. Dashes in the right margin indicate the positions of biotinylated marker proteins.

Immunoblotting of serially diluted CryIVD run on SDS-PAGE gel showed a detection limit of 200 pg (≈3.1 fmol) when a 25,000-fold-diluted primary antiserum and 2,500-fold-diluted secondary antiserum were used. In a dot blot assay, when these dilutions of antisera were used a detection level of 112 pg (≈1.7 fmol) was achieved (data not shown).

Figure 2A shows that nontrypsinated CryIVD bound to one or more proteins from the midguts of *A. stephensi*, *T. oleracea*, and *M. sexta* larvae. Trypsinated CryIVD did not show any binding to the midgut proteins of any of the species (data not shown). Nontrypsinated CryIVD toxin showed strong binding to a single protein in BBMVs of both the dipteran species, *A. stephensi* (148 kDa [Fig. 2A, lane 3]) and *T. oleracea* (78 kDa [lane 4]). Binding to *A. stephensi* BBMVs was stronger than binding to *T. oleracea* BBMVs. Minor binding signals were observed for a 62-kDa protein of *A. stephensi* and a 46-kDa protein of *T. oleracea*. Nontrypsinated CryIVD also bound to at least five midgut proteins of the nontarget insect *M. sexta*, ranging from 48 to 110 kDa (48, 52, 68, 103, and 110 kDa). The most intense binding signal was observed with a protein of 110 kDa (lane 5).

To demonstrate the specificity of the binding, the reciprocal experiment was also performed. Trypsinated CryIA(b) bound to nine proteins from the BBMVs of *M. sexta* larvae. These proteins had molecular masses ranging from 70 to 240 kDa (70, 76, 134, 148, 185, 195, 210, 230, and 240 kDa). The most intense signal was observed with the 240-kDa protein (Fig. 2B, lane 5). CryIA(b) did not bind to midgut proteins of the dipteran species *A. stephensi* (lane 3) or *T. oleracea* (lane 4).

The anti-CryIA(b) antiserum bound weakly to CryIVD (Fig. 2B, lane 1), and no signals were observed in the lanes with the insect BBMVs in the control blot (incubations without toxin [results not shown]). The anti-CryIVD antiserum did not bind to CryIA(b) (Fig. 2A, lane 2) but showed binding to *M. sexta* BBMV proteins of 265, 255, and 76 kDa in the control blot. These binding signals can also be seen in Fig. 2A, lane 5, and have been judged to be nonspecific. No such nonspecific binding was seen with the dipteran BBMVs.

TABLE 1. Mortality[a] of second-instar larvae of *T. oleracea* and *A. stephensi* induced by the insecticidal crystal proteins of *B. thuringiensis* subsp. *israelensis* IPS-82 and the CryIVD toxin from recombinant strain 759

| Strain | *T. oleracea*[b] | | *A. stephensi*[b] | |
|---|---|---|---|---|
| | $LC_{50}$ | *r* | $LC_{50}$ | *r* |
| IPS-82 | 7.16 | 5.77 | 3.29 | 3.50 |
| 759 | 8.80 | 3.15 | 4.81 | 2.18 |
| 4Q7 (control) | NT[c] | NT | NT | NT |

[a] Mortality was expressed as $LC_{50}$ values ($\log_{10}$ dose of spore-crystal mixture) corrected for control mortality and by the regression coefficients (*r*) of the dose-response curves.
[b] The least significant difference for the $LC_{50}$ values and regression coefficients is 0.49 and 1.1, respectively.
[c] NT, nontoxic ($>10^8$ spore-crystals).

**Effect of CryIVD on larval survival.** Washed spore-crystal suspensions of IPS-82, the acrystalliferous strain *B. thuringiensis* subsp. *israelensis* 4Q7, and *B. thuringiensis* subsp. *israelensis* 759 were tested for toxicity against second-instar larvae of *A. stephensi* and *T. oleracea* (Table 1). Spores of 4Q7 did not cause any mortality. Comparison within target species showed that the mortality ($LC_{50}$) caused by the CryIVD toxin (*B. thuringiensis* subsp. *israelensis* 759) was significantly lower than for the complete IPS-82 spore-crystal mixture ($P = 0.003$ for *T. oleracea*; $P = 0.006$ for *A. stephensi*). The *A. stephensi* larvae are 10,000-fold more susceptible than those of *T. oleracea* to the spore-crystal suspensions of both IPS-82 and *B. thuringiensis* subsp. *israelensis* 759. Because CryIVD showed strong binding to different midgut proteins of *M. sexta*, the possible susceptibility of *M. sexta* larvae to CryIVD was also tested. *B. thuringiensis* subsp. *israelensis* 759 did not exhibit an effect on either mortality or growth. In a second experiment, both solubilized and nonsolubilized sucrose gradient-purified CryIVD (1,000 ng/cm$^2$) were tested, but, again, no mortality or growth retardation was found in either application of CryIVD.

## DISCUSSION

This is the first report that shows by ligand-blotting experiments the binding of CryIVD protein to midgut proteins of the dipteran insects *A. stephensi* and *T. oleracea*. We observed that CryIVD also binds to the BBMVs of *M. sexta*. Binding of CryIA(b) was restricted to BBMVs of *M. sexta*. We observed that in both dipteran species, binding was observed predominantly with a single protein of 148 kDa in *A. stephensi* and 78 kDa in *T. oleracea*. Binding of CryIA toxins to BBMV proteins of different sizes has also been observed in studies on several lepidopteran species (13, 23, 32). We also found minor binding

signals with BBMV proteins with smaller molecular masses. Such minor binding signals could have arisen by proteolytic degradation of the putative receptor. However, this seems unlikely, since protease inhibitors were included during the dissection of the dipteran larvae and purification of the dipteran BBMVs. Further studies are needed to show if two different putative CryIVD receptors have been found in analogy with Sangadala et al., who identified and isolated a 120-kDa aminopeptidase and a 65-kDa protein, probably an alkaline phosphatase, as binding proteins for CryIA(c) in *M. sexta* (28).

In lepidopterans, CryI proteins are activated from protoxins into toxins by proteases. Similar activation of CryIVD has been shown to occur in dipteran midguts (5, 7). Chilcott and Ellar (5) found that solubilized CryIVD was not toxic for several dipteran and lepidopteran cell lines but found some toxicity with trypsinated CryIVD. The tryptic fragments of CryIVD, purified by gel filtration, were only slightly less toxic to *Aedes aegypti* larvae than was the intact CryIVD (5). The authors therefore concluded that CryIVD was a protoxin. Furthermore, solubilized CryIVD proteins treated with either trypsin, chymotrypsin, or thermolysin on the one hand or midgut proteases on the other were as toxic as the undigested CryIVD toward larvae of *Culex quinquefasciatus* (7). In a preliminary experiment, we found that high-pressure liquid chromatography-separated subunits of trypsinated CryIVD were not toxic to *A. stephensi* larvae, neither singly nor after mixing. In contrast, the trypsinated CryIVD was toxic (10a). This result can be explained by assuming that trypsinated CryIVD fragments are toxic only if they are in an aggregated configuration, as suggested previously (7). These data prompted us to analyze the binding of CryIVD after proteolysis with trypsin. The cleavage products obtained after treatment with trypsin and chymotrypsin had molecular sizes (Fig. 1) that correspond to earlier published data on the proteolysis of CryIVD (5, 7, 19). However, we were unable to show binding of the cleavage products to BBMVs. In view of these data, our results could be explained in three ways. First, processing of CryIVD in the midgut is essentially different from proteolysis by trypsin, as was shown by Dai and Gill for *C. quinquefasciatus* (7). Digestion of the *B. thuringiensis* subsp. *aizawai* IC1 δ endotoxin by insect gut proteases was associated with the insect specificity in toxic action and also with the binding properties of the different subunits of a given protoxin (2, 15). Second, ligand blotting was performed at neutral pH whereas binding, a prerequisite for toxicity, occurs in the insect midgut under alkaline conditions. Furthermore, the processing of CryIVD is affected both by the physical configuration (crystalline versus solubilized) and the pH (7). At pH 10, the pH of mosquito midguts (6), the solubilization of the CryIVD parasporal crystal proceeds only slowly and is simultaneously processed by the midgut proteases. Third, the receptor proteins in the BBMVs on the blot may have been degraded by residual trypsin activity during the incubation of the blot with the trypsinated CryIVD solution.

Our observation on the absence of binding of CryIA(b) with the dipteran BBMVs of *A. stephensi* and *T. oleracea* corresponds to data published previously (15, 32). Vadlamudi et al. (32) did not, however, observe binding of CryIVD toxins to *M. sexta* BBMVs, which is in contrast to our results.

The most striking difference we found in toxin binding between dipteran and lepidopteran species was that major binding in the dipteran species was restricted to a single midgut protein, whereas in *M. sexta* nine different proteins showed strong binding signals to CryIA(b) and five proteins showed binding to CryIVD. Vadlamudi et al. (32) found major binding of CryIA(b) to a 210-kDa protein in BBMVs of *M. sexta*, while we observed a 240-kDa protein as the major binding protein among nine different-sized proteins. This difference in the molecular mass of the major binding protein could be attributed to differences in electrophoresis conditions or to partial degradation of the putative receptor protein by endogenous proteases. *M. sexta* BBMV proteins of 210, 145, and 120 kDa have been identified previously as proteins with binding affinity for CryIA(b) (21, 32). None of the CryIA(b)-binding proteins also showed binding to CryIVD. The *M. sexta* binding proteins with lower molecular mass may be proteolytically derived from a larger precursor molecule without affecting the binding domain, since the *M. sexta* BBMVs were prepared without the use of proteinase inhibitors. Furthermore, BBMV proteins were stored at −80°C, which has been reported to affect the number of toxin-binding BBMV protein fractions (32). Another factor that might be important in causing variability in results among different laboratories is the origin of the toxins and target insects. Vadlamudi et al. (32) isolated the CryIVD protein from *B. thuringiensis* subsp. *israelensis* by selective solubilization of the parasporal inclusion body that contains different toxins (10, 19) followed by FPLC purification of the CryIVD toxin. Contamination with the CryIVC protein with almost the same molecular weight as CryIVD can then not be excluded. We deliberately obtained the CryIVD toxin from a *B. thuringiensis* subsp. *israelensis* recombinant to exclude this possibility. Differences in amino acid sequence among toxins from different strains may influence processing and consequently binding properties and specific toxicity (7, 32).

Binding of *B. thuringiensis* toxins to midgut proteins is in general positively correlated to toxicity (16, 34). But exceptions have also been found: CryIB binds to apical microvilli of *M. sexta* and is nontoxic for this species (4), and CryIA(c) binds to the BBMVs of *Spodoptera frugiperda* larvae but is nontoxic (12). We found no toxicity of CryIVD to *M. sexta*, but we did observe binding to its BBMVs. This strong binding makes the binding domain of the *cryIVD* gene a candidate for the construction of hybrid genes in conjunction with the pore-forming domain of lepidopteran-specific toxin genes. Such hybrid genes could code for insecticidal toxins with a different insecticidal action spectrum.

The observed difference in toxicity between IPS-82 and *B. thuringiensis* subsp. *israelensis* 759 (44-fold for *T. oleracea* and 33-fold for *A. stephensi*) was only slightly smaller than found by Ibarra and Federici (19), who obtained a 65-fold-lower $LC_{50}$ value for complete *B. thuringiensis* subsp. *israelensis* crystals (0.66 ng/ml) than for purified CryIVD (43 ng/ml) on first-instar *Aedes aegypti* larvae. Thus, for comparison purposes within the same experiment, the spore-crystal mixture can be used in a bioassay, as was done by us. We also have chosen the spore-crystal mixtures for accommodating the difference between the two species in larval feeding behavior. We compared filter-feeding aquatic mosquito larvae with terrestrially phytophagous tipulid larvae in toxicity assays. However, an exact comparison with literature data is prevented by the use of unknown quantities of the toxin, which is a disadvantage. We have found that *A. stephensi* larvae have a 10,000-fold-higher susceptibility ($LC_{50}$ value) to spore-crystal mixtures of IPS-82 and *B. thuringiensis* subsp. *israelensis* 759 (CryIVD) respectively than *T. oleracea* larvae do (Table 1). We also found a more intense binding signal of CryIVD to the putative receptor protein for *A. stephensi* than for *T. oleracea* BBMVs (Fig. 2A). This observation requires support by future competition experiments to determine the binding characteristics of CryIVD for the BBMVs of these species.

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