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Activation Process of the Mosquitocidal Delta-Endotoxin Cry39Aa Produced by *Bacillus thuringiensis* subsp. *aizawai* BUN1-14 and Binding Property to *Anopheles stephensi* BBMV

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The activation processes and the binding property to *Anopheles stephensi* BBMV of Cry39A were investigated. The 72 kDa of Cry39Aa protoxin was processed in vitro by trypsin and gut extract from *A. stephensi* larvae into a 60 kDa polypeptide by cleavage between Arg^{61} and Gly^{62}. Only in vivo, 40 and 25 kDa polypeptides were generated by intramolecular cleavage of the 60 kDa polypeptide in *A. stephensi.* Cry39A specifically bound to *A. stephensi* BBMV and did not compete with the Cry4Aa did. In addition, Cry39A associated preferentially with the Triton X-100 insoluble membrane fraction that was commonly defined as lipid rafts.

The gene for the 72 kDa protein from *B. thuringiensis* subsp. *aizawai* BUN1-14, cry39Aa, has been isolated, sequenced, and expressed. The resulting polypeptide is highly toxic to *Anopheles stephensi* (1). In order to know the mode of action of Cry39A, the activation processes of Cry39A and binding properties of Cry39A toxin to *A. stephensi* brush border membrane vesicles (BBMV) were investigated. The crystals containing Cry39A and ORF2-39A protein (the latter corresponds to the C-terminal half of the 130 kDa type of δ-endotoxin) were solubilized and digested with trypsin and *A. stephensi* gut extract. When digested by trypsin, the 72 kDa polypeptide of Cry39A was processed to a 60 kDa polypeptide (Fig. 1A). The N-terminus of the 60 kDa polypeptide was Gly^{62} of Cry39A. In contrast, 65 kDa of ORF2-39A appeared to be degraded since the ORF2-39A-derived polypeptide was not detected. Same results were obtained from digestion by the *A. stephensi* larvae gut extract (Fig. 1B). The 60 kDa fragment of Cry39A was not digested further when the concentration of trypsin or incubation time was increased (data not shown). To investigate *in vivo* processing of Cry39A and ORF2-39A, solubilized Cry39A and ORF2-39A were biotinylated and adsorbed to latex beads to use as a particulate suspension. *A. stephensi* larvae were fed with this suspension and the larvae were collected after an appropriate amount of time. The larvae were washed and dissected in PBS. The endoperitrophic spaces and ectoperitrophic spaces were placed directly into 5× Laemmli sample buffer and each sample was subjected to SDS-PAGE followed by detection of biotinylated polypeptides. Fig. 2 shows the *in vivo* processing of Cry39A protoxin in *A. stephensi* larvae. After exposure to Cry39A protoxin for 5 min, 60, 40, and 25 kDa polypeptides were detected in endoperitrophic space alone. With longer exposure, these polypeptides were detected not only endoperitrophic space and but also ectoperitrophic space. The 40 and 25 kDa polypeptides were also detected in the experiment using biotinylated Cry39A toxin (trypsinated 60 kDa Cry39A) (data not shown). These results indicated that *in vivo*, the 60 kDa polypeptide was processed into 40 and

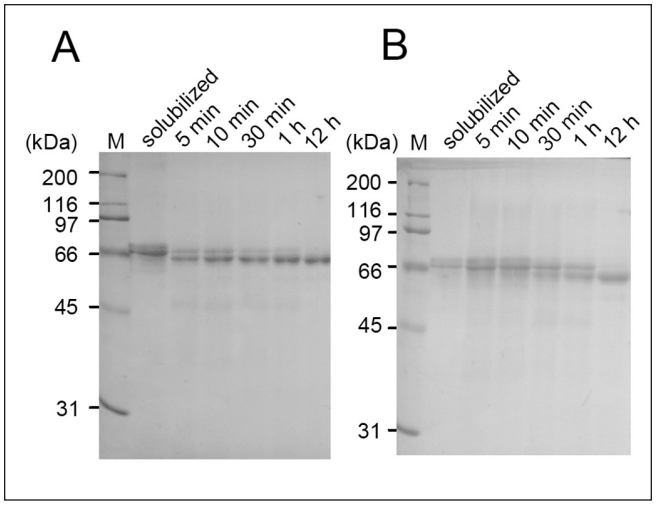

FIG. 1. *In vitro* processing of Cry39A. Twenty µg of solubilized Cry39A and ORF2-29A was digested with 10% (w/w) of trypsin (A) or *A. stephensi* gut extract (B). After an appropriate amount of time, aliquots of reaction mixture were subjected to SDS-PAGE.

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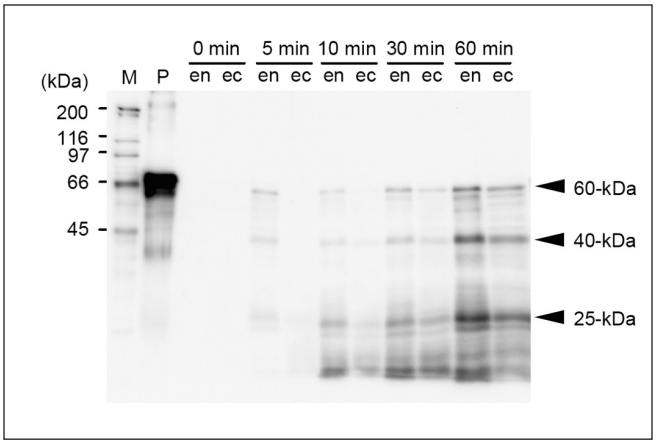

FIG. 2. *In vivo* processing of Cry39A. *A. stephensi* larvae were fed with biotinylated Cry39A protoxin suspension and were dissected after an appropriate amount of time. The endoperitrophic spaces and ectoperitrophic spaces were subjected to SDS-PAGE followed by detection of biotinylated polypeptides. Abbreviations, M, molecular weight marker; P; biotinylated Cry39A protoxin and ORF2-39A; en, endoperitrophic space; ec, ectoperitrophic space.

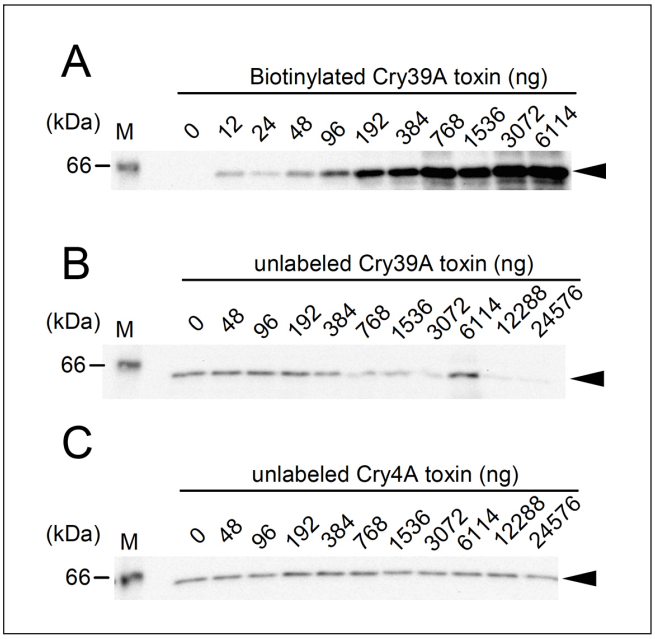

FIG. 3. Binding property of Cry39A toxin to *A. stephensi* BBMV. Twenty micrograms protein of *A. stephensi* BBMV was incubated with different amount of biotinylated Cry39A toxin (A), with 48 ng of biotinylated Cry39A toxin in the presence of different amount of unlabeled Cry39A toxin (B) or unlabeled Cry4A toxin (C).

25 kDa polypeptides by intramolecular cleavage. The intramolecular cleavage in toxin cores of approximately 60 kDa has also been reported in other mosquitocidal Cry toxins. The intramolecular cleavage of the 60 kDa intermediate of Cry4A into the two polypeptides was not essential for insecticidal activity (2). We also investigated the role of the intramolecular cleavage of the 60 kDa Cry39A polypeptide in the mode of action. Co-precipitation assay was performed to determine the binding property of 60 kDa Cry39A polypeptide to *A. stephensi* BBMV. For saturation binding assay, *A. stephensi* BBMV (20 µg protein) were incubated with an increasing concentration of biotinylated Cry39A toxin (Bio-39A) or Cry4A toxin (Bio-4A) for 30 min at 25°C. Unbound toxins were removed and the pellets were washed twice. Finally, the BBMV were resuspended in Laemmli sample buffer and subjected to SDS-PAGE followed by detection of Bio-39A and Bio-4A.

For competition binding assay, *A. stephensi* BBMV were incubated with 48 ng of Bio-39A in the absence or presence of different fold excesses of unlabeled Cry39A toxin or unlabeled Cry4A toxin. Both Bio-39A and Bio-4A bound to *A. stephensi* BBMV in a concentration-dependent manner, and binding was saturable (Cry39A; Fig. 3A, Cry4A; data not shown). In an homologous competition assay, as the concentration of unlabeled Cry39A toxin increased, Bio-39A binding was reduced and almost completely inhibited in the presence of 512-fold excess of unlabelled Cry39A toxin (Fig. 3B). In contrast, no inhibition of Bio-4A binding to *A. stephensi* BBMV was observed even in the presence of 512-fold excess of unlabelled Cry4A toxin (data not shown). This result was consistent with that obtained in a previous study (2). In an heterologous competition assay, Bio-39A binding was not reduced even in the presence of 512-fold excess of unlabelled Cry4A (Fig. 3C). Cry39A toxin binding may be mediated mainly by specific binding to a molecule other than that to which the Cry4A toxin binds. Lipid rafts are membrane microdomains enriched in GPI-anchored proteins, sphingolipids, and sterols, and they are defined by their insolubility in Triton X-100 at low temperature. Zhuang et al. recently reported that Cry1A toxins were associated with lipid rafts and that lipid raft integrity was essential for *in vitro* Cry1Ab pore-forming activity (4). In order to know if dipteran-specific Cry toxin is associated with lipid rafts on midgut epithelial cell membrane, biotinylated Cry39A toxin was incubated with *A. stephensi* BBMV and the detergent resistant membrane (DRM) was fractionated by Optiprep gradient centrifugation. Ten aliquots were collected from the top to the bottom of the gradient and subjected to SDS-PAGE followed by detection of Bio-39A. Cry39A toxin was detected only in the floated Triton X-100 insoluble fractions in the amount of 0.6 µg. As the

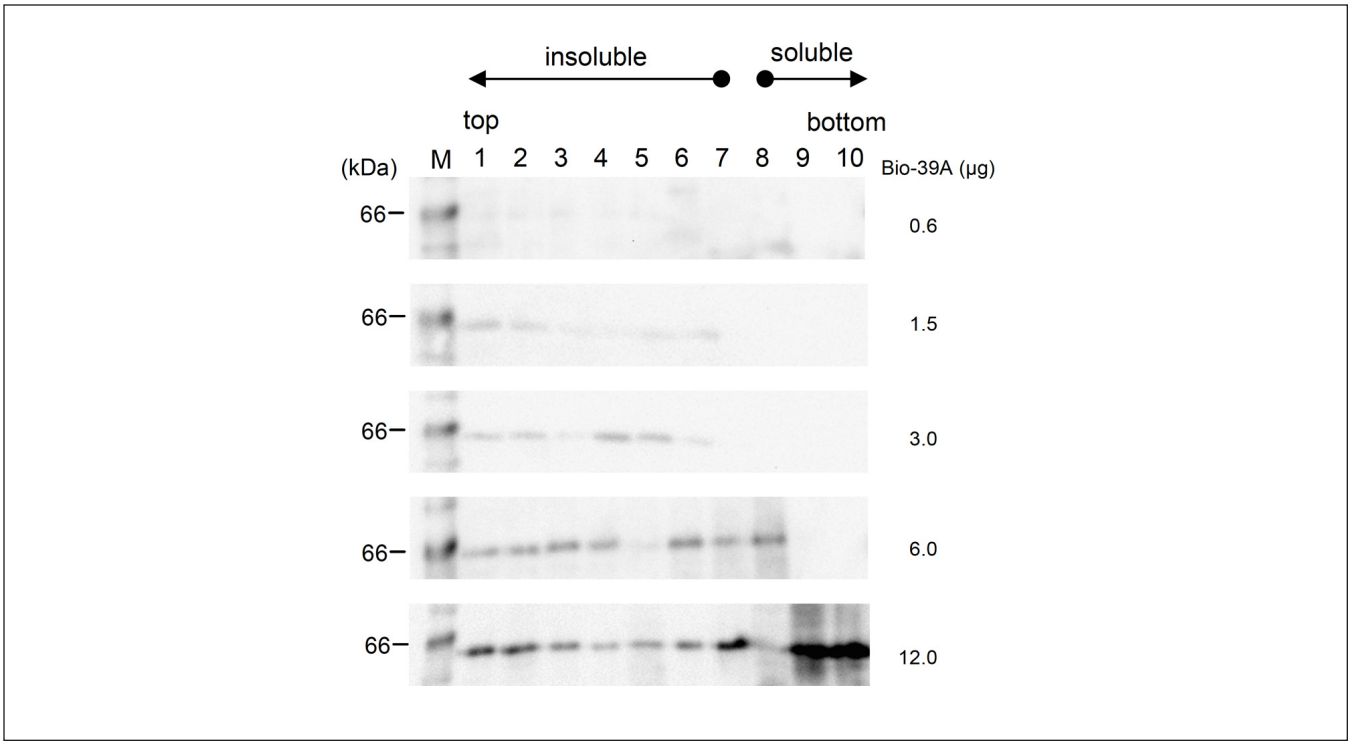

FIG. 4. Preferential association of Cry39A toxin with DRM of *A. stephensi* BBMV. Different amount of biotinylated Cry39A toxin-bound BBMV were treated with Triton X-100, subjected to a Optiprep gradient centrifugation, and fractionated from the top (lane 1-10). The distribution of biotinylated Cry39A toxin in the gradient fractions was analyzed.

amount of toxin increased, Cry39A toxin was detected in both the soluble and insoluble fractions (Fig. 4). Lipid rafts are commonly defined biochemically as membrane complexes that are insoluble in nonionic detergents at low temperatures. Therefore, this result might indicate that Cry39A toxin preferentially associated with lipid rafts of *A. stephensi* BBMV. To our knowledge, this is the first report that mosquitocidal Cry toxin is associated with DRM fraction of BBM of susceptible mosquito. It is difficult to conclude that Cry39A toxin is associated with lipid rafts because the "marker" for lipid rafts in *A. stephensi* BBM is unknown. The role of the DRM association of Cry39A toxin for toxicity to *A. stephensi* midgut epithelial cells is currently under investigation, and the molecules that interact with Cry39A toxin in DRM are also in the process of being identified.

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