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Title	Activation process of the mosquitocidal $\delta$ -endotoxin Cry39A produced by <i>Bacillus thuringiensis</i> subsp aizawai BUN1-14 and binding property to <i>Anopheles stephensi</i> BMW
Author(s)	Ito, Takeshi; Bando, Hisanori; Asano, Shin-ichiro
Citation	Journal of Invertebrate Pathology, 93(1), 29-35 <a href="https://doi.org/10.1016/j.jip.2006.05.007">https://doi.org/10.1016/j.jip.2006.05.007</a>
Issue Date	2006-09
Doc URL	<a href="https://hdl.handle.net/2115/14743">https://hdl.handle.net/2115/14743</a>
Type	journal article
File Information	JIP93-1.pdf



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Activation process of the mosquitocidal  $\delta$ -endotoxin Cry39A produced by *Bacillus thuringiensis* subsp. *aizawai* BUN1-14 and binding property to *Anopheles stephensi* BBMV.

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## Abstract

Most  $\delta$ -endotoxins produced by *Bacillus thuringiensis* require proteolytic processing in order to become active. The *in vitro* and *in vivo* activation processes of Cry39A, a  $\delta$ -endotoxin that is highly toxic to *Anopheles stephensi*, were investigated. Cry39A with a molecular mass of 72 kDa was processed *in vitro* into a 60 kDa fragment by trypsin and gut extract from *A. stephensi* larvae. N-terminal amino acid sequencing of the 60 kDa fragment revealed that trypsin and the protease(s) in the gut extract cleaved Cry39A between Arg<sup>61</sup> and Gly<sup>62</sup>. In contrast, 40 and 25 kDa polypeptides were generated *in vivo* by intramolecular cleavage of the 60 kDa fragment in *A. stephensi* larvae. Further, a co-precipitation assay was used to investigate the binding property of the activated Cry39A to *A. stephensi* BBMV. Cry39A bound to *A. stephensi* BBMV specifically and did not compete with the Cry4Aa toxin. This indicated that the binding molecule(s) for Cry39A might differ from those for Cry4A. In addition, Cry39A preferentially bound to the Triton X-100 insoluble membrane fraction that was commonly defined as lipid rafts.

Keywords: *Bacillus thuringiensis*;  $\delta$ -endotoxin; Mosquito; *Anopheles stephensi*; Processing; BBMV

## 1. Introduction

*Bacillus thuringiensis* (*Bt*) produces highly specific insecticidal proteins as parasporal crystalline inclusions during sporulation (Höfte and Whiteley, 1989). These crystalline inclusions are composed of one or several insecticidal proteins ( $\delta$ -endotoxins), which have been classified into two major families, Cry and Cyt toxins, based on the homology of their amino acid sequences (Crickmore et al., 1998). Most *Bt*  $\delta$ -endotoxins are produced as inactive protoxins within crystalline inclusions. After ingestion by susceptible insects, inclusions are solubilized in the alkaline environment of the midgut and are processed into active toxins by gut proteases. Lepidopteran-specific  $\delta$ -endotoxins of the 130

kDa type, such as Cry1, are processed into active toxins with a molecular mass of approximately 60 kDa. This relatively protease-resistant toxic core is derived from the N-terminal half of the protoxin by the removal of 500–600 amino acid residues from the C terminus and the first 27 to 29 N-terminal residues. As for dipteran-specific  $\delta$ -endotoxins such as Cry4A and Cry11A that are derived from *B. thuringiensis* subsp. *israelensis*, the 125 kDa protoxin of Cry4A is processed by trypsin and *Culex pipiens* midgut extract into two fragments of 20 and 45 kDa by the intramolecular cleavage of a 60 kDa intermediate (Yamagiwa et al., 1999). The 70 kDa protoxin of Cry11A is processed into fragments of 30–40 kDa by *C. quinquefasciatus* midgut extract (Dai and Gill, 1993) and into two fragments of 34–36 and 32 kDa by trypsin or *C. pipiens* gut extract (Yamagiwa et al., 2002). The active toxins bind to specific receptors on the midgut epithelial cell brush border membranes. Following the conformational change and/or oligomerization, the toxins insert into the membrane to form cation-permeable pores, which cause the swelling and death of epithelial cells by colloid-osmotic lysis (Höfte and Whiteley, 1989; Schnepf et al., 1998). In lepidopteran insects, aminopeptidase N (Knight et al., 1994; Gill et al., 1995; Lee et al., 1996) and a cadherin-like protein (Nagamatsu et al., 1998; Vadlamudi et al., 1995) are identified as specific receptors for lepidopteran-specific  $\delta$ -endotoxins such as Cry1A. However, in dipteran insects, the receptor molecule(s) of dipteran-specific  $\delta$ -endotoxins has (have) not yet been identified.

The gene for the 72 kDa protein from *B. thuringiensis* subsp. *aizawai* BUN1-14, *cry39A*, has been isolated, sequenced, and expressed (Ito et al., 2002). The resulting polypeptide is toxic to *Anopheles stephensi* and demonstrates a 50% lethal concentration ( $LC_{50}$ ) of 0.75  $\mu\text{g/ml}$  with purified inclusions against second instar larvae; however, it shows weak activity against *Culex pipiens pallens* (Ito et al., 2005) and *A. aegypti* (Ito, unpublished data). Cry39A belongs to a novel class of  $\delta$ -endotoxins that have no significant similarity to known *B. thuringiensis* toxins (Ito et al., 2002). In this study, we investigated the *in vitro* and *in vivo* activation processes of Cry39A toxin by trypsin and gut extract of

*A. stephensi* larvae as well as the binding property of activated Cry39A toxin to *A. stephensi* brush border membrane vesicles (BBMV).

## **2. Materials and Methods**

### *2.1. Preparation and solubilization of Cry39A protoxin*

Recombinant *B. thuringiensis* subsp. *kurstaki* HD-1 mutant Bt51 (pHY/IAaP-c39) (Ito et al., 2002) was used for the expression of Cry39A protoxin. This mutant produces parasporal inclusion bodies containing Cry39A and ORF2-39A protein; the latter corresponds to the C-terminal half of the 130 kDa type of  $\delta$ -endotoxin and is essential for crystallization. Recombinant *B. thuringiensis* subsp. *kurstaki* HD-1 mutant Bt51 (pHY/IAaP-IVA) (Hashimoto et al., 1996) was used for the expression of Cry4A. The crystals were purified on a sucrose gradient as described previously (Nishimoto et al., 1994) and solubilized in solubilization buffer (50 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM DTT, pH 10.5) for 1 h at 37°C with gentle shaking. Insoluble material was removed by centrifugation at 20,000 × g for 15 min at 4°C, and the supernatant was recovered. Protein concentration was determined using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as the standard.

### *2.2. In vitro processing of Cry39A protoxin*

The gut extract was prepared as described by Yamagiwa et al. (1999) with slight modifications. One hundred milligrams (wet weight) of early fourth instar *A. stephensi* larvae in 500 µl of 100 mM Na<sub>2</sub>CO<sub>3</sub>, pH 10.5 was sonicated on ice. After centrifugation at 20,000 × g for 5 min at 4°C, the supernatant was passed through a 0.22 µm filter and stored at -80°C until use. The protein concentration of the gut extract was determined as described above. Twenty micrograms of soluble Cry39A protoxin was treated with 10% (w/w) of trypsin (Sigma-Aldrich, St. Louis, MO) and *A. stephensi* gut extract in a volume of 100 µl of 100 mM Na<sub>2</sub>CO<sub>3</sub>, 10 mM DTT, pH 10.5 and incubated

at 25°C. After an appropriate amount of time, 10 µl of reaction mixture was taken and 2.5 µl of 5× Laemmli sample buffer supplemented with 4 mM phenylmethylsulfonyl fluoride (PMSF) was added and boiled for 5 min to stop the reaction. SDS-PAGE was carried out and the gel was stained with Coomassie brilliant blue.

### 2.3. Protein sequencing

N-terminal amino acid sequencing was performed at the Center for Instrumental Analysis, Hokkaido University, using Procise 492 cLC (Applied Biosystems, Foster City, CA).

### 2.4. Biotinylation of protoxin and toxin

The solubilized Cry39A protoxin was dialyzed against 40 mM sodium bicarbonate buffer, pH 8.6, concentrated to at least 1 mg/ml using centrifugal filter devices (Ultrafree-CL, Millipore, Billerica, MA), and biotinylated with the ECL protein biotinylation module (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. For the biotinylation of Cry39A and Cry4A toxins, the protoxins were solubilized as described above and activated by incubation with 5% *A. stephensi* gut extract (w/w) for 30 min at 37°C. PMSF was added to yield a final concentration of 0.1 mM, and the activated toxins (60 kDa fragment) were purified using a UNO-Q column (Bio-Rad Laboratories). Purified toxins were dialyzed, concentrated, and biotinylated as described above. Biotinylated toxins were centrifuged at  $20,000 \times g$  for 15 min at 4°C to remove the insoluble materials before use.

### 2.5. In vivo processing of Cry39A protoxin

Biotinylated Cry39A protoxin and toxin were adsorbed to 0.8-µm latex beads (Sigma-Aldrich) as described by Schnell et al. (1984) and used as a particulate suspension. Early fourth instar *A. stephensi*

larvae were placed in the wells of a 24-well titer plate with 475  $\mu$ l of deionized water. Twenty five microliters of biotinylated Cry39A suspension was added to each well to yield a final protein concentration of 10  $\mu$ g/ml, and the larvae were collected and washed with PBS after an appropriate amount of time. The larvae were dissected in PBS containing Complete Protease Inhibitor Cocktail (Roche Diagnostics). The peritrophic membrane was pulled out from the dissected midgut to divide the endoperitrophic and ectoperitrophic spaces. The dissected gut material was directly placed into 20  $\mu$ l of 5 $\times$  Laemmli sample buffer and boiled for 3 min. Five microliters of each sample was subjected to SDS-PAGE. For visualization, proteins were transferred to PVDF membranes and blocked in TBST buffer (100 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) containing 3% BSA and were kept overnight at 4°C. After washing with three changes of TBST buffer for 10 min each, the membranes were incubated with a 1:10,000 dilution of streptavidin-conjugated horseradish peroxidase (Amersham Biosciences) for 1 h at 25°C. After another set of washes, membranes were developed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL).

#### 2.6. Preparation of BBMV

BBMV were prepared from whole early fourth instar *A. stephensi* larvae by the magnesium precipitation method in the presence of the protease inhibitor described previously (Abdul-Rauf and Ellar, 1999). The final pellet representing the BBMV was resuspended in ice-cold TNE buffer (100 mM Tris-HCl, 150 mM NaCl, 0.2 mM EGTA, pH 7.5), and the protein concentration was determined using the 2-D Quant Kit (Amersham Biosciences) with BSA as the standard.

#### 2.7. Co-precipitation binding assay

For the saturation binding assay, *A. stephensi* BBMV (20  $\mu$ g protein) were incubated with an increasing concentration of biotinylated Cry39A toxin (Bio-39A) or Cry4A toxin (Bio-4A) in 100  $\mu$ l of

binding buffer (PBS, 0.1% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.4) for 30 min at 25°C. The unbound toxins were removed by centrifugation at  $20,000 \times g$  for 10 min at 4°C. The pellets were washed twice with the binding buffer. Finally, the BBMV were resuspended in 20  $\mu$ l of 1 $\times$  Laemmli sample buffer, boiled for 3 min, and subjected to SDS-PAGE. Western blotting and visualization of Bio-39A and Bio-4A that had bound to the BBMV were performed as described above. For the competition binding assay, *A. stephensi* BBMV (20  $\mu$ g protein) were incubated with 48 ng of Bio-39A in the absence or presence of different fold excesses of unlabeled-Cry39A toxin or Cry4A toxin. The Bio-39A that had bound to BBMV was visualized as described above.

#### 2.8. Fractionation of Triton X-100-Insoluble Membrane

The detergent-resistant membrane (DRM) was fractionated by Optiprep gradient centrifugation as described previously (Bravo et al., 2004) with minor modifications. *A. stephensi* BBMV (500  $\mu$ g protein) were incubated for 1 h at 25°C with various concentrations of Bio-39A in TNE buffer. After incubation, the unbound toxin was removed and washed with TNE buffer as described above. The BBMV pellet was suspended in 180  $\mu$ l of TNE buffer containing 1% Triton X-100 and incubated on ice for 30 min. The samples were mixed with 360  $\mu$ l of Optiprep (Sigma-Aldrich) in a 2.2 ml centrifuge tube and overlaid with 1.5 ml of 50% Optiprep in TNE buffer, followed by addition of 0.1 ml of TNE buffer. The gradients were centrifuged at  $160,000 \times g$  for 4 h at 4°C. Ten 210  $\mu$ l fractions were collected from the top to the bottom of the gradient and subjected to methanol precipitation (Wessel and Flugge, 1984). Proteins were suspended in Laemmli sample buffer and subjected to SDS-PAGE and Western blotting, followed by visualization of Bio-39A as described above.

### 3. Results

#### 3.1. *In vitro* proteolytic processing of Cry39A

The crystals containing Cry39A and ORF2-39A protein were solubilized and digested by trypsin and by the gut extract prepared from *A. stephensi* larvae. The N-terminal amino acid sequences of the resulting fragments were determined. When digested by trypsin, the 72 kDa polypeptide of Cry39A was processed to 60 kDa fragments (Fig. 1A). The N-terminus of the 60 kDa fragment was Gly<sup>62</sup> of Cry39A. The 60 kDa fragment of Cry39A was not digested further when the concentration of trypsin or incubation time was increased (data not shown). In contrast, 65 kDa of ORF2-39A appeared to be degraded since the ORF2-39A-derived polypeptide was not detected. The same result, that is, the Cry39A protoxin was processed into the 60 kDa polypeptide by the cleavage between Arg<sup>61</sup> and Gly<sup>62</sup>, was obtained from the digestion by the *A. stephensi* larvae gut extract (Fig. 1B). Under the same conditions of Cry39A digestion, the 125 kDa protoxin of Cry4A was processed into 45 and 20 kDa polypeptides by the intramolecular cleavage of the 60 kDa polypeptide by trypsin (Fig. 1C).

### 3.2. *In vivo* proteolytic processing of Cry39A

The *in vivo* processing of Cry39A protoxin in *A. stephensi* larvae is shown in Fig. 2A. After exposure to Cry39A protoxin for 5 min, 60, 40, and 25 kDa polypeptides were detected in the endoperitrophic space alone. With longer exposure, these polypeptides were detected not only in the endoperitrophic space but also in the ectoperitrophic space. The 40 and 25 kDa polypeptides were also detected in the experiment using biotinylated Cry39A toxin (trypsinated 60 kDa Cry39A) (Fig. 2B). These results clearly indicate that *in vivo*, the 60 kDa polypeptide was processed into 40 and 25 kDa polypeptides by intramolecular cleavage.

### 3.3. Binding property of Cry39A toxin to *A. stephensi* BBMV

Both biotinylated Cry39A toxin (Bio-39A) and biotinylated Cry4A toxin (Bio-4A) bound to *A. stephensi* BBMV in a concentration-dependent manner, and binding was saturable (Cry39A; Fig. 3A,

Cry4A; data not shown). In the 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 unlabeled 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; that is, the binding of digoxigenin-labeled Cry4A toxin to BBMV prepared from *Culex pipiens* larvae was not reduced even when the unlabeled Cry4A was added to yield a 1,000-fold concentration (Yamagiwa et al., 1999). In the heterologous competition assay, Bio-39A binding was not reduced even in the presence of 512-fold excess of unlabelled Cry4A (Fig. 3C).

#### 3.4. Preferential association of Cry39A toxin to DRM of *A. stephensi* BBMV

Cry39A toxin was detected only in the floated Triton X-100 insoluble fractions in the amount of 0.6 µg. As the amount of toxin increased, Cry39A toxin was detected in both the soluble and insoluble fractions (Fig. 4). With a few exceptions, lipid rafts are commonly defined biochemically as membrane complexes that are insoluble in nonionic detergents at low temperatures (Brown and Rose, 1992). Therefore, these results might indicate that Cry39A toxin preferentially associated with lipid rafts of *A. stephensi* BBMV.

## 4. Discussion

*B. thuringiensis* crystals ingested by susceptible insects are solubilized in the alkaline environment of the insect midgut. Solubilized protoxins are processed into active toxins by midgut proteases. Appropriate processing is very important for Cry toxins to achieve their full toxicity in the midgut of the susceptible insect. In this paper, we investigated the activation processes of Cry39A, which is highly toxic to *A. stephensi*. The *in vitro* processing of the Cry39A protoxin by trypsin and by the *A.*

*stephensi* midgut extract yielded the same results; the 72 kDa of Cry39A was processed into a 60 kDa polypeptide that was resistant to further digestion due to the removal of N-terminal 61 residues. However, *in vivo*, the 40 and 25 kDa polypeptides were generated by the intramolecular cleavage of a 60 kDa polypeptide. In addition, we detected a small amount of 40 and 25 kDa polypeptides when a biotinylated 60 kDa polypeptide of Cry39A (Bio-39A) was incubated with *A. stephensi* BBMV (data not shown). Therefore, the membrane-bound protease that was insoluble in the carbonate buffer might be involved in the intramolecular cleavage of the 60 kDa polypeptide. Is the intramolecular cleavage of 60 kDa polypeptide essential to the insecticidal activity of Cry39A? The intramolecular cleavage in toxin cores of approximately 60 kDa has also been reported in other mosquitocidal Cry toxins, namely, Cry4A (Yamagiwa et al., 1999), Cry4B (Angsuthanasombat et al., 1993), Cry11A (Dai and Gill, 1993; Yamagiwa et al., 2002), and Cry11B (Segura et al., 2000). The intramolecular cleavage of the 60 kDa intermediate of Cry4A into the two polypeptides was not essential for insecticidal activity (Yamagiwa et al., 1999). A similar result—that the intramolecular cleavage of 70 kDa Cry11A into two polypeptides, did not promote insecticidal activity—has also been reported (Yamagiwa et al., 2002). In the case of Cry4B, the blockage of the intramolecular cleavage at R<sup>203</sup> showed an increase in toxicity against *A. aegypti* larvae (Angsuthanasombat et al., 1993). Therefore, it is likely that the intramolecular cleavage of the 60 kDa Cry39A polypeptide was not essential for insecticidal activity. We are investigating the role of the intramolecular cleavage of the 60 kDa Cry39A polypeptide in the mode of action.

It is difficult to refer to the difference between the modes of actions of Cry4A and Cry39A because the precise mode of action of mosquitocidal Cry toxins, including Cry4A, has not been understood. However, binding of a Cry toxin to a specific receptor in the epithelial cell membrane is a crucial step in any mode of action. The 60 kDa Cry39A toxin specifically bound to the *A. stephensi* BBMV and did not compete with the Cry4A toxin. Yamagiwa et al. (2001) suggested that Cry4A toxin has two

binding pathways that lead to irreversible binding to a cell surface. The first pathway depends on a specific binding protein and follows the two-step mode of interaction, which results in the correct orientation of the insertion of the toxin molecule into the membrane, leading to the formation of functional channels. The second pathway depends on nonspecific and direct binding to the membrane, which may not be involved in determining the toxicity. This proposed binding property of the Cry4A toxin may explain why Cry39A toxin does not compete with Cry4A toxin in binding to *A. stephensi* BBMV. 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 temperatures (Brown and Rose, 1992). There is a growing body of evidence suggesting that lipid rafts exist in biological membranes and that the preferential association of proteins with rafts is involved in crucial cellular functions such as cell signaling and membrane traffic and fusion (Simons and Toomre, 2000; Sprong et al., 2001). In addition, several bacterial and viral pathogens appear to use raft binding as a means of entry into their target cells (Nichols and Lippincott-Schwartz, 2001; van der Goot and Harder, 2001), and a variety of toxins, such as perfringolysin O (Waheed et al., 2001), lysenin (Yamaji-Hasegawa et al., 2003), *Vibrio cholerae* cytolysin (Zitser et al., 1999), aerolysin (Abrami and van Der Goot, 1999), and cholera toxin (Orlandi and Fishman, 1998), also appear to require raft association for cytotoxic activity. It has been shown that lipid rafts act as concentration platforms for the oligomerization of the pore-forming toxin aerolysin from *Aeromonas hydrophila* (Abrami and van Der Goot, 1999). Zhuang et al. (2002) recently reported that Cry1A toxins were also associated with lipid rafts and that lipid raft integrity was essential for *in vitro* Cry1Ab pore-forming activity. In this study, we demonstrated that the Cry39A toxin preferentially associated with the DRM fraction on *A. stephensi* BBMV. To our knowledge, this is the first report that mosquitocidal Cry toxin associates with the DRM fraction of the

BBMV of susceptible mosquitoes. It is difficult to conclude that Cry39A toxin associates with lipid rafts because the “marker” for lipid rafts in *A. stephensi* BBMV 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. These studies will clarify the mode of action of Cry39A toxin and serve as molecular evidence that Cry39A shows promise as an alternative agent for *B. thuringiensis* subsp. *israelensis*-derived Cry toxin.

### **Acknowledgments**

This work was supported in part by the Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists (T. I).

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### Figure legends

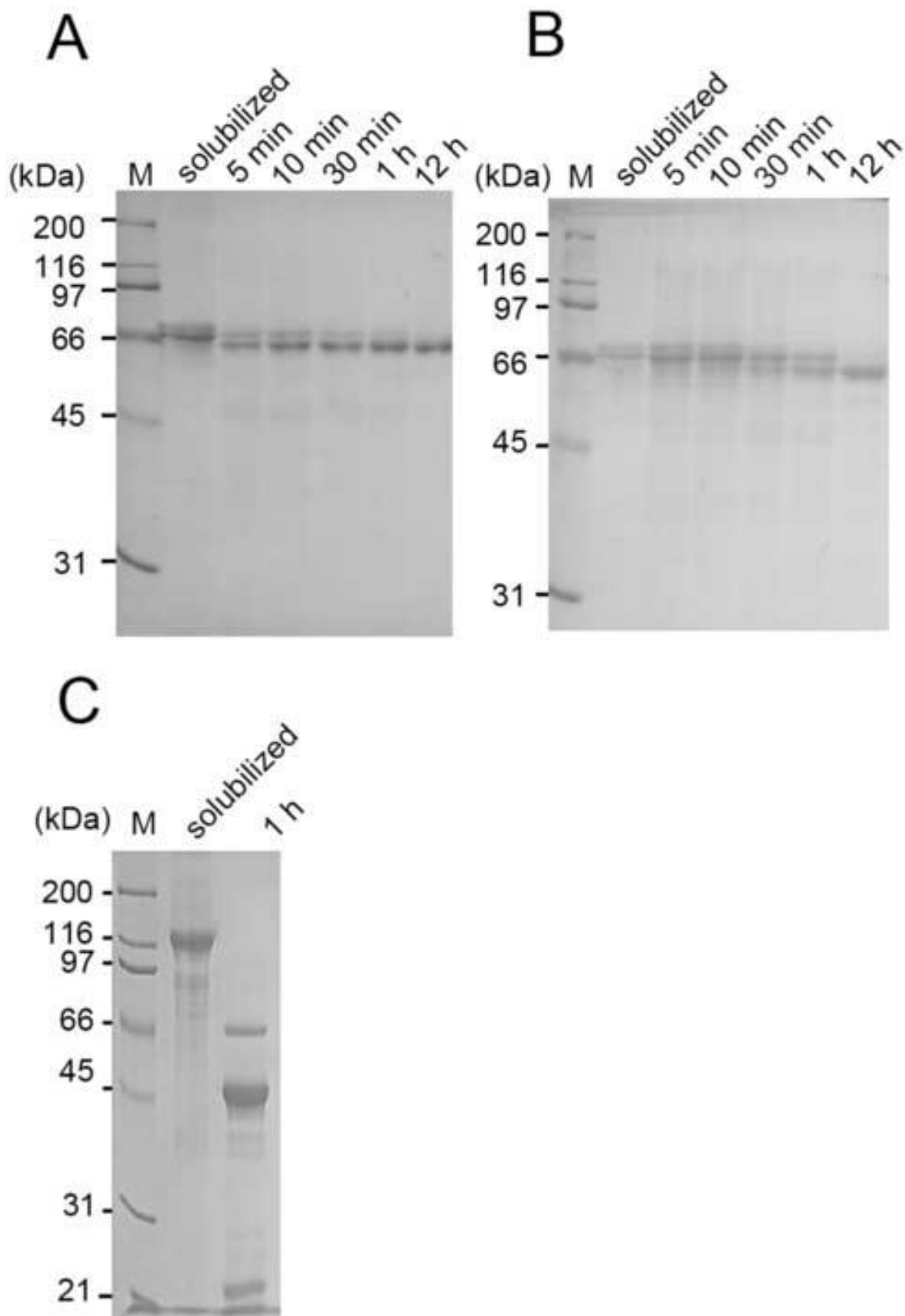
Fig. 1. *In vitro* processing of Cry39A. Twenty micrograms of solubilized Cry39A and ORF2-29A was digested with 10% (w/w) of trypsin (A) or *A. stephensi* gut extract (B). After an appropriate amount of time, aliquots of reaction mixture were subjected to SDS-PAGE. Solubilized Cry4A was digested with trypsin under the same conditions of Cry39A digestion (C).

Fig. 2. *In vivo* processing of Cry39A. *A. stephensi* larvae were fed with biotinylated Cry39A protoxin suspension and the larvae were collected and dissected after an appropriate amount of time. The endoperitrophic spaces and ectoperitrophic spaces were subjected to SDS-PAGE, Western blotting followed by detection of biotinylated polypeptides (A). Similar experiment was performed using biotinylated trypsin-resistant 60 kDa polypeptide of Cry39A (B). M, molecular weight marker; P; biotinylated Cry39A protoxin and ORF2-39A; en, endoperitrophic space; ec, ectoperitrophic space; Bio-39A, biotinylated trypsin-resistant 60 kDa polypeptide of Cry39A.

Fig. 3. Binding property of Cry39A toxin to *A. stephensi* BBMV. Twenty micrograms protein of *A. stephensi* BBMV was incubated at 25 °C for 30 min with different amount of biotinylated Cry39A toxin (A) and with 48 ng of biotinylated Cry39A toxin in the presence of different amount of unlabeled Cry39A toxin (B) or unlabeled Cry4A toxin (C). The membrane-bound Cry39A toxins were detected with the streptavidin-conjugated horseradish peroxidase.

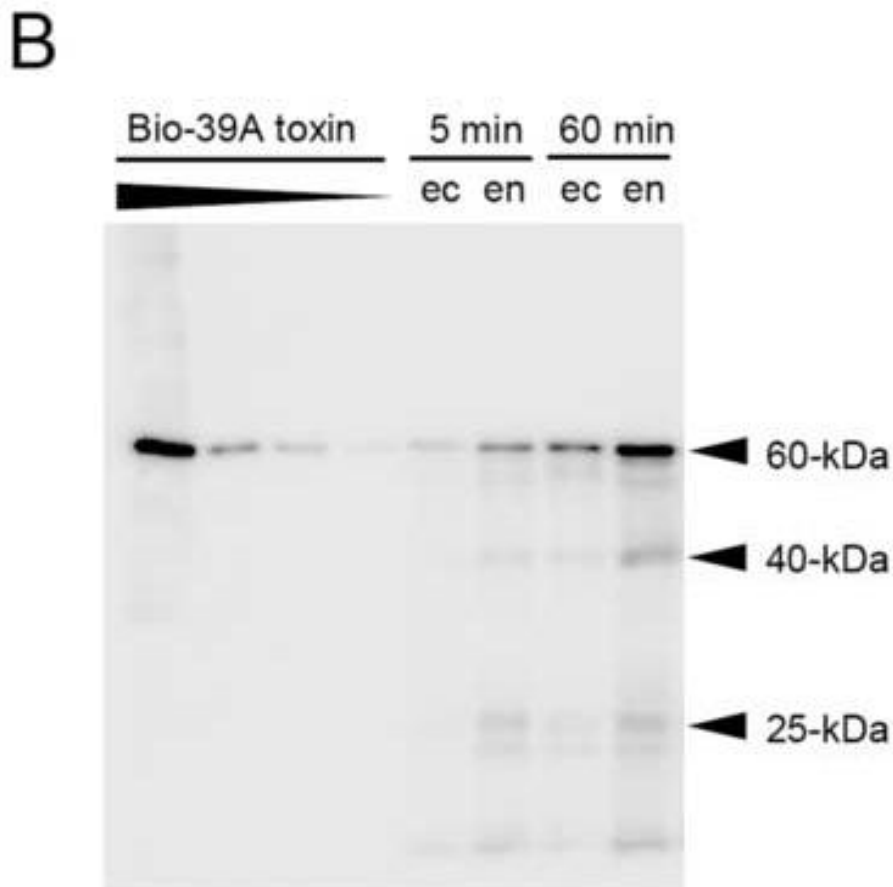
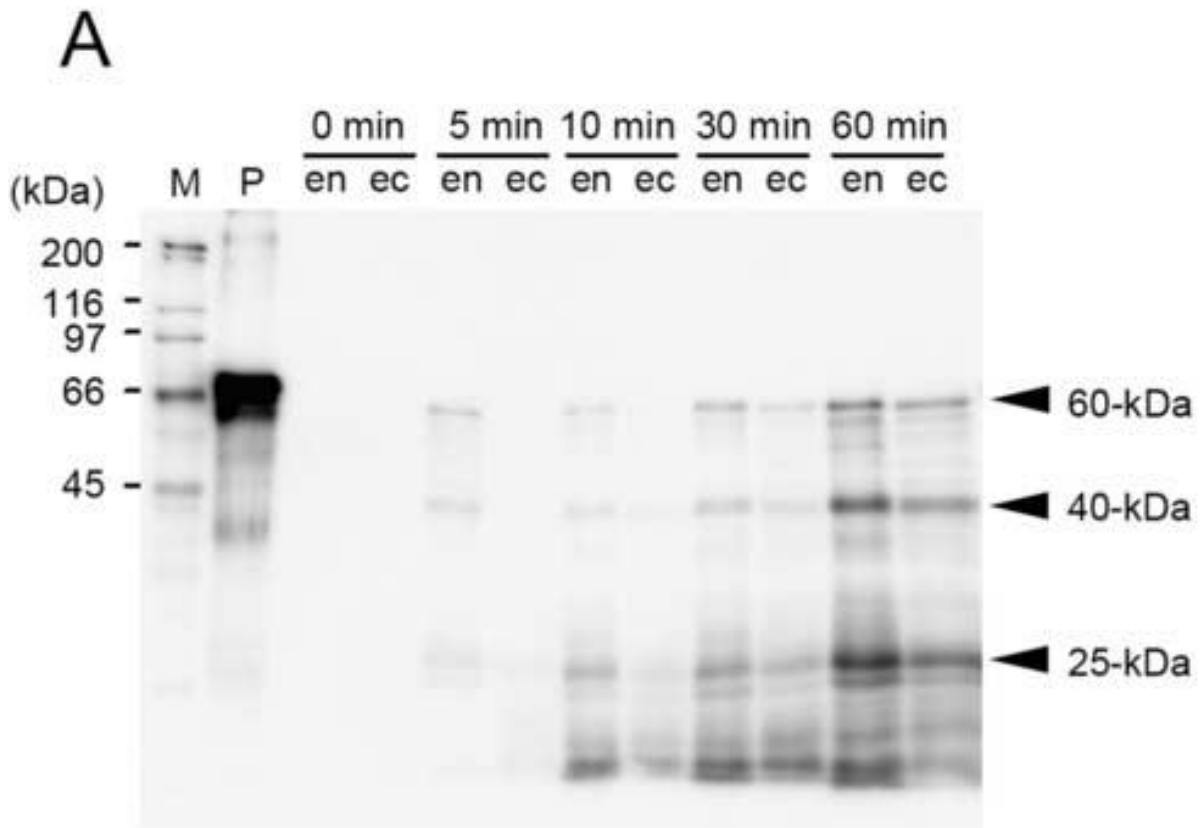
Fig. 4. Preferential association of Cry39A toxin with DRM of *A. stephensi* BBMV. Different amount of biotinylated Cry39A toxin-bound BBMV were treated with Triton X-100, subjected to a Optiprep gradient centrifugation, and fractionated from the top (lane 1-10). The distribution of biotinylated Cry39A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

**Figure 1**  
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**Figure 2**

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**Figure 3**  
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