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Determination of a Region of Cry1Aa Inserted into *Bombyx mori* BBMV

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Cry1Aa penetrates into the brush border membrane (BBM) of insect midgut, and causes cell lysis by pore formation. We detected various digests of Cry1Aa inserted into BBMV using various part-specific antisera of Cry1Aa and advocated the plausible insertion region of oligomeric and monomeric Cry1Aa. In oligomeric Cry1Aa, a 15 kDa fragment presumed to be dimeric α-4,5 helices was detected. On the other hand, the digests of monomeric Cry1Aa was quite different from that of oligomer. α-2-7 helices as well as domain III were inserted into BBM whereas α-1-5 sheets were on the membrane surface.

Cry1Aa is an insecticidal protein that specifically kills lepidopteran insects by forming pores on BBM of insect midgut. The umbrella model has been recognized as an insertion and pore forming model of Cry1A (1). Several Cry1A toxin molecules gather as oligomerized form on BBM; α-4,5 helices may be subsequently inserted into the membrane to form a pore (2). However, this hypothesis has several disadvantages. One of the most important question is that not only tetrameric Cry1Ab but also monomeric Cry1Ab has pore forming activity and the umbrella model can not explain the latter (3). Furthermore, in the umbrella model, the bulky uninserted region might cause steric hindrance and thereby membrane insertion with assembled molecules might be inhibited. Then, pore forming monomeric Cry1Aa model may differ from that of oligomeric Cry1Aa.

We performed pronase digestion of *Bombyx mori* BBMV bound Cry1Aa to identify the region. Membrane inserted region may not be digested by pronase, whereas the uninserted region must be vigorously done. Antisera specific to various Cry1Aa region, such as anti α-2,3, anti α-4,5, anti α-6,7, anti β-1-5, anti β-6-11 and anti domain III antisera were prepared to detect fragments of Cry1Aa remaining even after the digestion. All antisera recognized each specific site of Cry1Aa individually, and did not show non-specific binding to *B. mori* BBMV proteins (data not shown).

Digestion was done at 37°C for 24 hours on various pronase concentrations. Many digests were detected even in 1 mg/ml treatment (Fig. 1 all panels, lane 1). It is clear that ultra-high proteinase resistant peptides reside in BBMV bound Cry1Aa. Dominant fragments of 30-35 kDa were detected by anti α-4,5 and anti α-6,7 antisera (Fig. 1 B and C, lane 2-5), but not by anti α-2,3 antiserum (Fig. 1 A). These fragments recognized by those two antisera seemed to be identical to each other based on the SDS-PAGE pattern. Along with these peptides of higher molecular size, a 7.5 kDa fragment was detected by anti α-2,3 antiserum and was not digested further in even 2 mg/ml treatment (Fig. 1 A, lane 6). The molecular weight of the fragment closely accorded with that of α-2,3 helices. This result indicates that intact α-2,3 helices are removed from BBMV bound Cry1Aa, thereafter it is still in the lipid bilayer as stable form. On the other hand, a 30 kDa fragments were detected with anti β-6-11 and anti domain III antisera and these patterns in SDS-PAGE

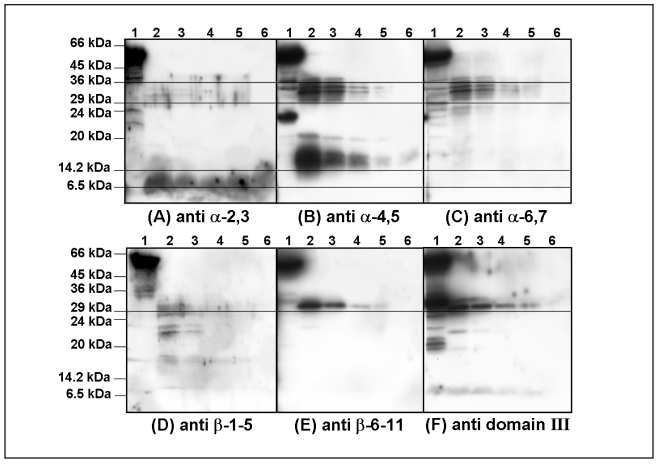

FIG. 1. Pronase digestion assay of BBMV bound CryAa. Lane 1 : non-treated BBMV bound Cry1Aa; lane 2: 1 mg/mL pronase digestion; lane 3: 1.25 mg/mL pronase; lane 4: 1.75 mg/mL pronase; lane 5: 1.75 mg/mL pronase; lane 6: 2 mg/mL pronase.

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were mutually similar (Fig. 1 E and F, lane 2-5). Thus, they appeared to be the same fragments composed of β-6-11 sheets and domain III. In contrast, almost no fragments were detected using anti β-1-5 antiserum (Fig. 1 D). As expected, total molecular weight summed up the three fragments size, i.e., 30-35, 7.5 and 30 kDa, were nearly equal to that of activated Cry1Aa. This clearly suggests that these fragments are generated from the "same" Cry1Aa molecule. On the other hand, a significant 15 kDa fragment was recognized only by anti α-4,5 antiserum was also observed (Fig. 1 B, lane 2-6). We confirmed the specificity of anti α-2,3 and anti α-6,7 antisera, in which α-3 helix and α-6 helix were respectively recognized (data not shown). Therefore, this result together with the specificity suggests that 15 kDa fragment does not include both α-3 and α-6 helices. In a previous report, α-4,5 helices were shown to have oligomerization as well as pore formation activities (2). We therefore analogize that the 15 kDa fragment must be dimeric α-4,5 helices. Interestingly, 15 kDa fragment of dimeric α-4,5 helices appeared together with 30-35 kDa fragments at the same time and the same intensity (Fig. 1 B). This suggested that the dimer of α-4,5 helices may not derive from the Cry1Aa molecule that produced 7.5, 30-35 and 30 kDa fragments. All results described above suggest that a single molecule of Cry1Aa inserts into BBM and forms pore, and that oligomerized α-4,5 helices also penetrates simultaneously.

Our new pore forming model is shown on Fig. 2. The pore forming region of monomeric Cry1Aa is thought to be from α-2 to α-7 helix because this region was not digested and kept in the BBMV even after vigorous Pronase digestion (Fig. 2 A). Conversely, the region between α-1 and α-2, α-3 and α-4, and β-1 and β-5 were digested. These digested portions may expose themselves to the membrane surface. In addition, a 30 kDa fragment including β-6 and domain III region was also was conserved in BBMV. Domains II and III have been speculated to be on the membrane surface with binding to receptor. But our results do not clearly support the above speculation; rather they suggest that these regions are also buried in the membrane by interaction with lipids. Based on our results, we proposed a "Buried dragon model". The main characteristic of this model feature is whole parts of Cry1Aa are inserted or buried into the membrane. Moreover, not only the membrane inserted region but also buried regions must be important for pore forming in some way. Alternatively, pore forming region of tetrameric Cry1Aa should be α-4,5 helices (Fig. 2 B). In a pronase digestion assay, a detected fragment was actually dimeric α-4,5 helices although it may be too small to form pore. Cry1A toxin has been known to normally oligomerize to tetramer. Therefore, it is reasonable to analogize that pore forming unit must be dimer of dimeric α-4,5 helices or tetramer of the helices. However, as indicated above, Cry1Aa tetramer must cause steric hindrance as it is, and thus bulky extra portions should be removed during oligomerization. Our observation that only α-4,5 helices dimer was detected matched to our hypothesis.

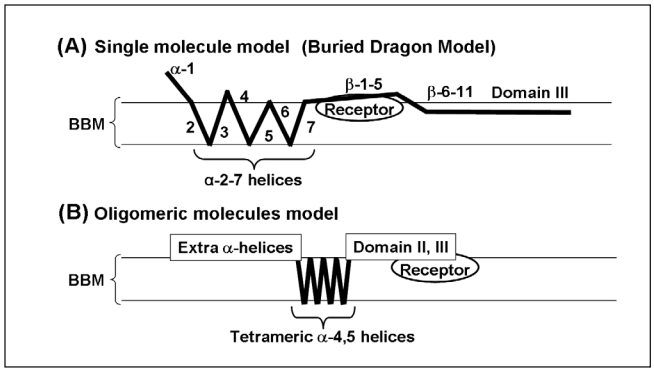

FIG. 2. Hypothesis of membrane insertion region and pore forming model of Cry1Aa.

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