GalNAc pretreatment inhibits trapping of *Bacillus thuringiensis* Cry1Ac on the peritrophic membrane of *Bombyx mori*

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Abstract ***Bombyx mori*** **(Shunrei×Shogetsu) is sensitive to Cry1Aa and resistant to Cry1Ac, both insecticidal proteins of *Bacillus thuringiensis*. Cry1Aa passed through the peritrophic membrane (PM) much faster (0.37 μg/mm² PM/h) than Cry1Ac (0.05 μg/mm² PM/h) during the initial observation period. Both Cry1Aa and Cry1Ac bound to the PM but only the binding of Cry1Ac was specifically inhibited by *N*-acetylgalactosamine (GalNAc). When Cry1Ac was pretreated with GalNAc, Cry1Ac permeated the PM much faster. These results suggested that Cry1Ac bound a PM protein via GalNAc on a sugar side chain. The role of the PM on Cry1Ac resistance of *B. mori* was briefly discussed.**

Keywords: δ-Endotoxin; Peritrophic membrane protein; Permeability of peritrophic membrane; *N*-acetylgalactosamine; *Bacillus thuringiensis*; *Bombyx mori*

1. Introduction

Insecticidal proteins, Cry toxins, encoded by *Bacillus thuringiensis* show specific insecticidal spectrum and are widely used as agent of insect pest control. Genes encoding Cry toxins have been introduced into various transgenic crops in order to protect them from insect pests [1]. Insects have evolved resistance mechanism to Cry toxins both as a result of laboratory selection and in agricultural fields [2]. Cry toxin resistance in the field has also been reported primarily in *Plutella xylostella* [2]. The evolution of resistance poses a major threat to the future use of Cry toxins. The most common mechanism for resistance to Cry toxin is altered binding to the brush border membrane (BBM) of the larval midgut [2–11], however, the resistances without altered BBM binding have also been reported in various insects [10,12–14].

Mechanism of Cry toxin resistance in insects is seemed to be complicated. We found that, using surface plasmon resonance analysis, Cry1Aa and Cry1Ac equally bound to solubilized BBMV proteins from both *P. xylostella*, which were highly resistance to Cry1Aa and Cry1Ac and sensitive to both Cry1Aa and Cry1Ac, respectively. And furthermore, although Cry1Ab effectively killed the both insects, binding of Cry1Ab to the proteins was almost negligible in both strains [15]. Contrarily to our findings, Jenkins and Dean showed in *Bombyx mori* using surface plasmon resonance analysis, that immobilized aminopeptidase (APN) and cadherin-like protein had high affinity to Cry1Aa but not to Cry1Ab and Cry1Ac and these characteristics matched to native sensitivity of the insect to Cry toxins [16]. Although our experiment and theirs were different from each other in insects and proteins used, in our case, it was difficult to understand the reason why Cry1Ac equally bound to BBMV proteins from both highly resistant and very susceptible *P. xylostella* without consideration of another factors along with those two tentative receptor proteins.

To understand the mechanism of resistance, the peritrophic membrane (PM) is seemed to be important as well as receptor proteins, since the PM is a major barrier that Cry toxins must cross before binding to the BBM. The PM is a semi-permeable membrane composed of chitin and proteins that lines the entire midgut of insect larvae [17–19]. The PM is associated with food digestion due to the presence of hydrolytic enzymes and protects midgut from physical damage and attack from microorganisms. The treatment with chitinase [20–22] or proteases such as enhancin from *Trichoplusia nigranulovirus* [23] disrupted the PM and significantly increased susceptibility to Cry toxins.

Here, we described the interactions between Cry toxins and the PM, and briefly discussed the roles of the PM in Cry toxin susceptibility.

2. Materials and methods

2.1. Insect and toxins

Bombyx mori larvae, hybrid Shunrei×Shogetsu, were reared with an artificial diet (Silk mate, Nosan Kogyo, Yokohama, Japan) at 27 °C in the dark and second day, fifth instar larvae were used for preparation of PM.

Cry1Aa and Cry1Ac were prepared from *B. thuringiensis* serovar *sotto* strain T84A1, a gift from Prof. M. Ohba, Kyushu University, and serovar *kurstaki* strain HD-73, respectively. The 130-kDa Cry toxins were activated with trypsin [24] and the active 60-kDa Cry1Aa and Cry1Ac were purified with DEAE–Sepharose [25].

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Abbreviations: PM, peritrophic membrane; GalNAc, *N*-acetylgalactosamine; BBM(V), brush border membrane (vesicle) of the larval midgut; Cry, insecticidal toxin of *Bacillus thuringiensis*; APN, aminopeptidase N; CA, carbonic anhydrase

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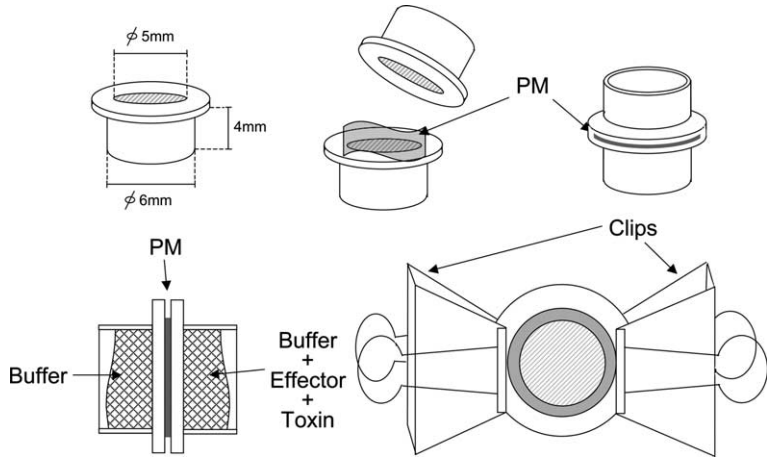

Fig. 1. Schematic diagram of the apparatus used to measure PM permeability. The PM was stretched over the opening (5 mm diameter) of plastic cups, sandwiched and secured by two clips. Seventy microliters of PBS containing toxin proteins and effectors was placed on the luminal face of the PM. Seventy microliters of buffer without effectors was placed on the midgut face of the PM.

2.2. Apparatus for measurement of PM permeability

An apparatus consisting of two plastic cups held together by clips was used to measure the permeability of the PM (Fig. 1). The diameter of the opening of each cup was 5 mm, generating an area of 19.6 mm^2.

The PM was prepared by longitudinal dissection of the midgut and residual artificial diet was rinsed off with PBS (8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl). The PM with an area about 1 cm^2 was stretched over the opening of the apparatus, sandwiched and immediately used for experiments (Fig. 1).

2.3. Measurement of PM permeability

Seventy mictoliters of PBS was applied to the midgut side of the apparatus and PBS containing Cry toxin or effectors (100 μg/ml) was applied to the lumenal side of the apparatus and allowed to stand for a designated period. At 1, 2 and 3 h after the setting, the PBS in the chamber on midgut side of the membrane was analyzed by SDS–7.5% PAGE and stained with Coomassie brilliant blue (CBB) as previously [26]. Bovine serum albumin (BSA), 66 kDa, and carbonic anhydrase (CA), 29 kDa, were used as penetrants in control experiments. Cry1Aa and Cry1Ac separated by SDS–PAGE were transferred onto PVDF membranes and analyzed by western blotting using anti-Cry1Aa antiserum [25]. ECL (Amersham–Pharmacia Biotech, Piscataway, USA) was used for visualization and proteins were quantified with an image analyzer (PD Quest, Bio-Rad Lab, USA).

2.4. Solubilization of PM proteins

PM proteins were categorized into four classes [27] based on their solubility in physiological saline (class 1), a mild detergent (class 2) or strong denaturant (class 3); class 4 proteins included non-extractable proteins. Proteins in class 1 and class 4 are more difficult to analyze due to contamination from various diet proteins adsorbed onto the PM and insolublility, respectively. Therefore, class 2 and 3 proteins were used for this analysis. The PM was washed with 10 ml of PBS using gentle shaking and washes were discarded. Subsequently, class 2 proteins were extracted by shaking with 10 ml of PBS containing 1% Triton X-100 and separated from insoluble PM pellet by centrifugation at $10\,000\times g$, for 30 min. Class 3 proteins were extracted from the Triton X-100 insoluble pellet using sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol and 1% bromophenol blue). The solubilized PM proteins were separated by 7.5% SDS–PAGE.

2.5. Ligand blot assay

Peritrophic membrane proteins blotted onto a PVDF membrane were incubated for 1 h with PBS containing 0.1% Tween 20 and Cry1Aa or Cry1Ac (6 μg/ml). Cry1Aa and Cry1Ac that bound to the PM proteins were detected with anti-Cry1Aa antiserum and visualized as described above. Sugars such as GalNAc, *N*-acetylglucosamine (GlcNAc), mannose, fucose and galactose were added at 100 mM to the blot 30 min prior to the reaction with Cry1A toxins to determine their effect on the binding of Cry1Aa/PM or Cry1Ac/PM and analyzed by dot blot assay.

2.6. Lectin binding assay

Peroxidase-conjugated concanavalin A (Con A), soybean agglutinin (SBA), wheat germ agglutinin (WGA), *Phaseolus vulgaris* agglutinin (PHA-E_4) and peanut agglutinin (PNA) (Seikagaku Corporation, Tokyo, Japan) were used for the assay [25]. The bound lectins were visualized and analyzed as described above.

3. Results

3.1. Passage of Cry1Aa and Cry1Ac through PM

BSA and CA passed through the PM at a rate of 0.37 μg/mm^2 PM/h for 2 h. This rate was slightly reduced after this point (Fig. 2(a)). The rate of Cry1Aa passage was similar to that of BSA and CA (Fig. 2(b)). In contrast, almost no Cry1Ac passage was observed for first 2 h. However, during the third hour, Cry1Ac passed through the PM at a rate of 0.34 μg/mm^2 PM/h (Fig. 2(b)).

3.2. Analysis of PM proteins bound to Cry1Aa and Cry1Ac

SDS–PAGE detected various PM proteins from class 2 and class 3 (Fig. 3(a)). Ligand blots demonstrated binding of Cry1Aa primarily to class 3 proteins with molecular sizes of 250 kDa (P250), 190 kDa (P190) and 150 kDa (P150) and the proteins migrating at 60–90 kDa (P60-90) (Fig. 3(b), lane 2). Cry1Ac also bound to these proteins, but the binding intensity with P60-90 was substantially lower compared with that of Cry1Aa. In addition, Cry1Ac bound to proteins with molecular size of 125 kDa (P125) from class 2 and 165 kDa (P165) from class 3 (Fig. 3(c)).

3.3. Effect of sugars on binding of Cry toxins to the PM proteins

Sugars, such as GalNAc, GlcNAc and others, were added at 100 mM to determine their effect on Cry1Aa or Cry1Ac binding to the PM and analyzed by dot blot. The addition of each of the sugars had little effect on binding of Cry1Aa to PM proteins, however, the presence of GalNAc, mannose or galactose inhibited binding of Cry1Ac to PM proteins. Binding of Cry1Ac to the PM was inhibited by approximately 70% in

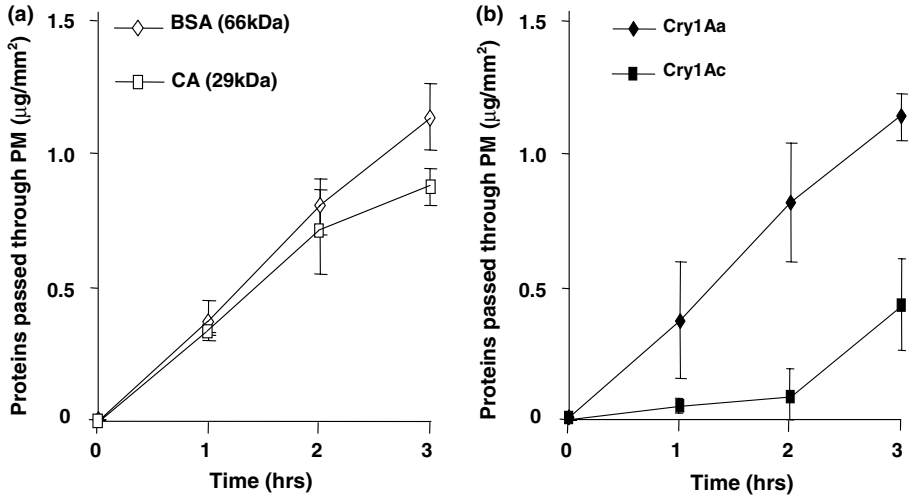

Fig. 2. Permeability of the PM to various proteins. Permeability of the PM to BSA, CA (a), Cry1Aa and Cry1Ac (b) was measured. Seventy microliters of PBS containing 100 μg/ml of BSA, CA, Cry1Aa or Cry1Ac was placed in the luminal face to start the reaction. The PBS solution in chamber on the midgut face was collected hourly. The concentration of effectors that passed through the PM was visualized by SDS–PAGE with CBB staining or Western blotting (see Section 2). Error bars represent the standard deviation of three or more replicate experiments.

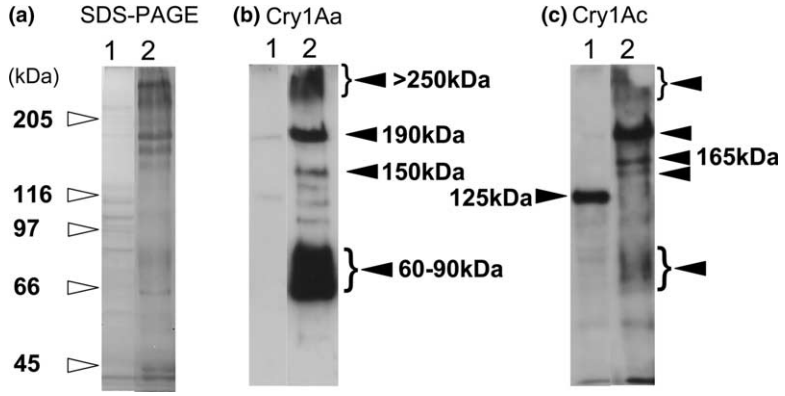

Fig. 3. Ligand blot analysis of PM proteins from *B. mori* treated with Cry1Aa and Cry1Ac. Class 2 (lane 1) and class 3 (lane 2) PM proteins were separated by SDS 7.5%–PAGE and stained with CBB (a). PM proteins were then transferred to a PVDF membrane and analyzed by ligand blot using Cry1Aa (b) and Cry1Ac (c). The migration of molecular weight markers is indicated by open arrow heads. Major PM proteins bound to Cry1Aa and/or Cry1Ac are indicated by filled arrow heads.

the presence of GalNAc (Fig. 4(a)). Minor reductions in binding of Cry1Ac to the PM (less than 25%) were observed when mannose or galactose was added (Fig. 4(a)).

The effect of GalNAc on binding of Cry toxins to PM proteins was further analyzed by ligand blotting after SDS–PAGE. Almost no binding inhibition was observed for Cry1Aa in the presence of GalNAc (Fig. 4(b)). In contrast, the addition of GalNAc significantly inhibited binding of Cry1Ac to some PM proteins (Fig. 4(b)). Bindings of Cry1Ac to P190, P165 and P150 was inhibited by 75%, 90% and 90%, respectively, and interestingly, the binding of Cry1Ac to P125 was almost completely inhibited.

The effect of GalNAc on the passage of Cry1Ac through the PM was estimated (Fig. 4(c)). As we expected, co-inoculation of Cry1Ac and 100 mM GalNAc facilitated the passage of Cry1Ac through the PM and the rate was increased to 0.45 μg/mm^2 PM/h (Fig. 4(c)).

3.4. Lectin binding assay

Lectin binding assays were used to deduce the structure of possible sugar side chains on the PM proteins of which binding with Cry1Ac was inhibited by GalNAc (Fig. 5). The major class 3 proteins of P250, P190 and P60-90 strongly bound to PNA and P190 also bound to SBA. The class 2 protein, P125, bound Con A (Fig. 5). These assays suggested that P250, P190 and P60-90 contained O-linked mucin-type sugar structure such as Galβ1-3GalNAcα1-Ser/Thr and GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr. The P125 bound to Con A was suggested to have N-linked high mannose-type sugar side chains.

4. Discussions

The larvae of *B. mori* used in this study are highly sensitive to Cry1Aa and highly resistant to Cry1Ac with LC_{50}(s) of 0.23 and >734 μg/g diet, respectively (K. Miyamoto et al., unpublished data).

Passage of Cry1Aa, BSA and CA through the PM occurred at approximately the same rate of about 0.4 μg/mm^2 PM/h for 3 h. This rate may reflect physicochemical diffusion through the PM. The passage of Cry1Ac, however, was negligible

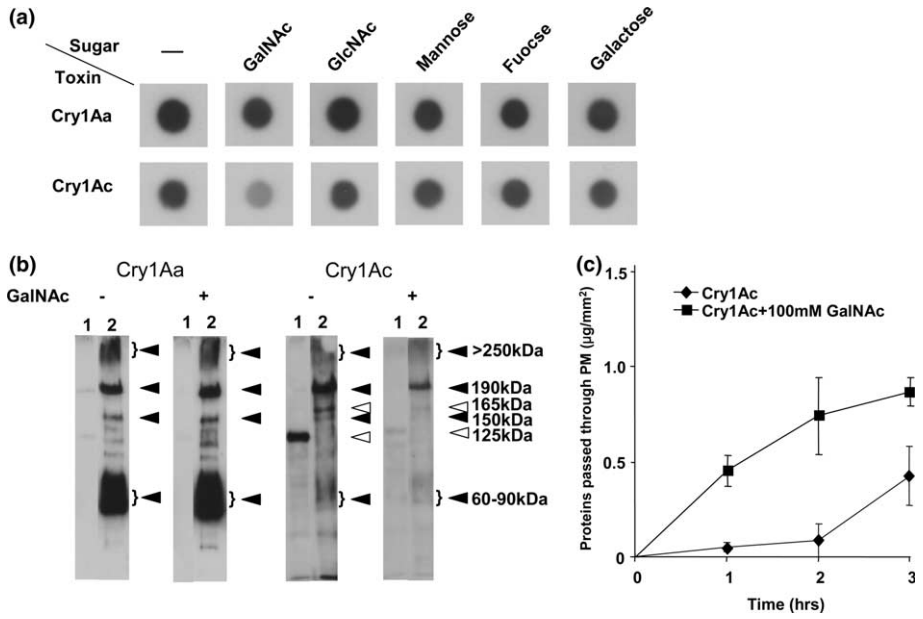

Fig. 4. Effect of monosaccharides on the interaction between the PM and Cry1A. (a) PM proteins (a mixture of class 2 and class 3) were blotted onto a PVDF membrane and binding of Cry1Aa and Cry1Ac was analyzed by ligand blot in the presence or absence of 100 mM of the following monosaccharides: GalNAc, GlcNAc, mannose, fucose and galactose. (b) Class 2 (lane 1) and class 3 (lane 2) PM proteins were separated by SDS 7.5%–PAGE and the binding of Cry1Aa and Cry1Ac was analyzed by ligand blot analysis with or without 100 mM of GalNAc. Protein bound was detected with Western blot analysis as described in Section 2. (c) The permeability of the PM to Cry1Ac with or without 100 mM GalNAc was measured as described in Fig. 2. Error bars represent the standard deviation of three or more replicate experiments.

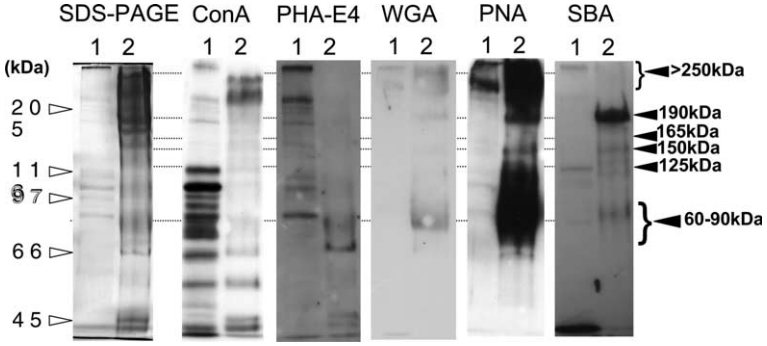

Fig. 5. Lectin binding assay with *B. mori* PM proteins. Class 2 (lane 1) and class 3 (lane 2) PM proteins were separated by SDS 7.5%–PAGE and transferred to PVDF membranes. The membranes were incubated with peroxidase-conjugated lectins, Con A, PHA-E4, WGA, PNA and SBA and visualized by ECL. Molecular weight markers are indicated by open arrow heads. Major PM proteins bound to Cry1Aa and/or Cry1Ac are indicated by filled arrow heads.

during the first 2 h and then occurred at a rate of roughly 0.4 μg/mm^2 PM/h in the third hour. This suggested that there were Cry1Ac binding sites on the PM and Cry1Ac could pass through the PM after these sites were saturated.

The binding of Cry1Ac to several PM proteins was inhibited by GalNAc. On the other hand, many PM proteins bound to Cry1Aa, which passed through the PM, but the interactions were not inhibited by GalNAc. This suggested that GalNAc was a key factor for understanding Cry1Ac binding to these PM proteins. The lectin-like domain III of Cry1Ac recognized GalNAc residues on *Manduca sexta* aminopeptidase [28,29]. Lectin binding assays suggested that some major PM proteins such as P250, P190 and P60-90 had mucin-type sugar side chains such as Galβ1-3GalNAcα1-Ser/Thr and/or GalNAcα1-3Galβ1-3GalNAcα1-Ser/Thr. The GalNAc-recognition site on Cry1Ac was likely bound by the GalNAc residues of the sugar side chains on these PM proteins. After these sites were saturated, passage through the PM occurred at a normal rate of 0.4 μg/mm^2 PM/h. If this was the case, we could expect ordinary passage of Cry1Ac when the site was occupied with free GalNAc. As expected, when Cry1Ac was incubated with GalNAc, Cry1Ac passed through the PM at normal rate. It is not yet clear how to interpret the result that Cry1Aa/PM binding was not inhibited by GalNAc. However, the passage of Cry1Aa was similar to that of BSA and CA. Binding of Cry1Aa to PM proteins observed in ligand blot analysis may not have a direct relationship with penetration of Cry1Aa through PM. The interactions of Cry1Aa and Cry1Ac with the PM warrant further investigation of the functional significance of the binding.

Recently, suggestive and informative theories regarding the insecticidal mechanism of Cry toxins have been reported. A tetrameric oligomer of Cry1Ac was observed in synthetic membranes [30] and it was proposed that the Cry oligomer formation was necessary for efficient pore formation. These oligomers were observed only in Cry toxins activated by BBMV, whereas Cry toxins activated by trypsin or insect midgut juice showed only monomeric forms [31]. In this hypothesis, quick passage of Cry toxins through the PM is necessary to generate insecticidal activity. The concentration of the Cry toxins used in our study was likely to be extremely high compared with that in the lumen of insect larvae ingested the toxin from the insecticidal *B. thuringiensis* formulation. Thus, Cry1Ac will saturate the binding sites on PM in the insect lumen more slowly and Cry1Ac will likely be retained in the PM for longer periods under conditions in the field. Therefore, it is likely that Cry1Ac is thoroughly processed before it can permeate the PM and perhaps cleavage of the N-terminus occurs at a slightly more internal location in silkworm midgut fluid. Indeed, midgut fluids intensively modified Cry1Ac within minutes and excess digestion of N-terminal amino acids was suggested to lead to inactivation of Cry1Ac in common cutworm [32].

The binding of Cry toxins to the PM has been reported in many lepidopteran insects [4,32–35]. But, to our knowledge, we are the first to discover that (i) binding of Cry1Ac to the PM was completely inhibited by GalNAc and (ii) co-incubation of Cry1Ac and GalNAc completely restored Cry1Ac passage. We believe that these findings are an important contribution to the study of the PM.

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