FUNCTIONAL STRUCTURES OF CRY4AA TOXIN THAT ARE RESPONSIBLE FOR THE MOSQUITOCIDAL ACTIVITY AGAINST CULEX PIPIENS

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ABSTRACT

The Cry4Aa produced by *Bacillus thuringiensis* subsp. *israelensis* is a protein of special interest because of its specific toxicity towards *Anopheles*, *Aedes*, and *Culex* mosquito larvae. The detail mechanisms of its molecular mode of action and the functional structures of Cry4Aa related to toxicity are not yet identified. To determine the functional structures of Cry4Aa involving in mosquitocidal activity, the polypeptides derived from domains II and III of Cry4Aa were expressed as inclusions and purified. Binding affinity between the polypeptides and the brush border membrane (BBM) proteins prepared from *C. pipiens* larvae were analyzed using quartz crystal microbalance (QCM) device. The results revealed relatively high affinities of β1-α8, β2-3, and domain III polypeptides to BBM proteins and their KD were estimated as 59, 54, and 63nM, respectively. Thus, it is revealed that multiple functional subsites are present in Cry4Aa which are spreading out in domain II and III. Involvement of various monosaccharide sugars on Cry4Aa toxicity was also analyzed by bioassay against *C. pipiens* larvae. It was found that GalNAc pretreatment with Cry4Aa caused acceleration of toxicity and fucose pretreatment caused inhibition of Cry4Aa toxicity.

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INTRODUCTION

The Cry4Aa is a 130 kDa protein produced as protoxin by *Bacillus thuringiensis* subsp. *israelensis* (Bti). It has drawn great interest for developing a bioinsecticide since it shows potent and specific toxicity towards the larvae of *Anopheles*, *Aedes*, and *Culex* mosquito species, which are the vectors of serious diseases, such as malaria and viral hemorrhagic fevers, respectively. However, the molecular modes of mechanisms of its toxicity are still unidentified.

The three dimensional structure of Cry4Aa (Fig.1) was determined by Boonserm et al [1] in 2006, which showing similar structure like other Cry toxins. Three-dimensional structure of Cry4Aa consists of three domains I, II, and III. The domain I is consists of seven α helices and is assumed to be involved in membrane insertion and pore formation. Domain II, the receptor binding domain, is composed of three antiparallel β sheets connected by loops, which are believed to be responsible for receptor binding. Domain III, a sandwich of two antiparallel β sheets, is believed to involve in structural stability, toxicity and also participates in pore formation. This similarity in structure with the other Cry toxins' structures may result in similar mode of actions, but the functional sites of Cry4Aa remain to be elucidated.

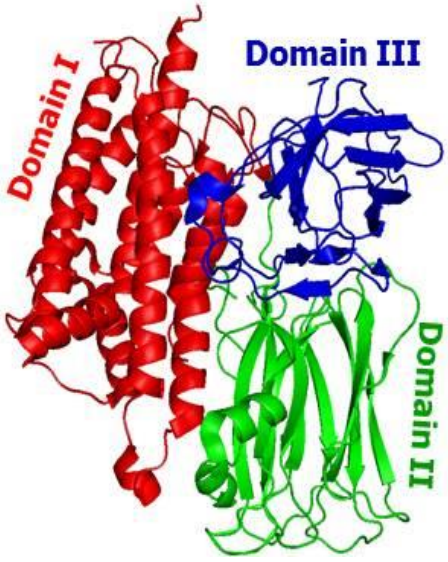

Fig. 1. The three-dimensional structure of the Cry4Aa showing its three domains. Like structures of previously determined Cry proteins, Cry4Aa also have three domains, domain

I, II, and III. Domain I is responsible for pore formation, domain II for specificity, receptor binding. The function of domain III is clearly understand but is thought that this is responsible for structural stability, specificity. The illustration was created using the Cry4Aa PDB code[1] with PyMOL software [2].

By using alanine scanning and loop replacement technique, Howlader et al [3, 4] analyzed three typical loops (loops 1, 2 and 3) of domain II to look after the functional structures and results suggested that, unlike the receptor binding site of the well-characterized Cry1 toxins, the receptor-binding site of Cry4Aa is different from the loops 1, 2 and 3 or that there may be multiple binding sites present that may work cooperatively for receptor binding.

In this study, we created six small polypeptides in such a manner so that each of which contain an individual loop structure of domain II exposed on the surface of Cry4Aa and another polypeptide consisting of domain III. All peptides were successfully expressed in *Escherichia coli* and purified. Using these polypeptides, we analyzed the interactions with the brush border membrane (BBM) proteins extracted from *C. pipiens* larvae. Further, the effect of monosaccharaides on the Cry4Aa toxicity against *C. pipiens* larvae was also analyzed. Our results suggested that multiple sites may present in Cry4Aa which may work as functional structures related to toxicity and sugars may also have possess role in Cry4Aa toxicity.

MATERIALS AND METHODS

DESIGN, CONSTRUCTION OF CRY4AA DOMAIN II AND III PEPTIDES

The Cry4Aa wild-type (WT) were expressed and purified as GST fusions as per the protocol reported earlier [3, 4, 5]. A synthetic *cry4Aa* (Cry4Aa-S1) gene as reported by Hayakawa et al [5], which showed hyper-expression in *Escherichia coli*, was used in this study. Based on the sequence and three-dimensional structures, as previously determined and reported, six small polypeptides each which contains an individual loop structure of domain II exposed on the surface of Cry4Aa and one single polypeptide of domain III were designed. All the peptides were constructs as 4ACter-expresstion vector as reported by Hayakawa et al [6] in 2010. Constructions were confirmed by DNA sequencing using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

EXPRESSION AND PURIFICATION OF THE CRY4AA DOMAIN II PEPTIDES

The loop-polypeptides were successfully expressed in *E. coli* BL21 cells as fusions with 4ACter-tag that facilitates the formation of alkali-soluble protein inclusions. Expression of

the peptides was induced by adding isopropyl-β-d-thiogalactopyranoside (IPTG) at a concentration of 0.06 mM. The bacterial cells were disrupted by sonication after harvesting by centrifugation. The pellets containing the polypeptide inclusion bodies were collected and solubilized in denaturing buffer (PBS with 8M urea and 10mM imidazole; pH 7.4). The solubilized proteins were then purified through elusion by using HisPur^{TM} Ni-NTA spin column.

The protein concentration was determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as a standard. The proteins were analyzed by 14% SDS-PAGE followed by visualization with Coomassie brilliant blue (CBB) staining.

EXTRACTION OF BRUSH BORDER MEMBRANE PROTEINS

Brush border membrane vesicles (BBMVs) were prepared from 7- to 10-day-old *C. pipiens* 4th instar larvae using previously described protocols[(7)]. The brush border membrane proteins were extracted from the prepared BBMVs using detergent cocktail (0.4% Saponin plus, 2% Triton X-100 in 1mM PMSF in PBS buffer) for 1hr on ice. The supernatant containing soluble proteins were obtained by ultra-centrifugation (48000rpm, 30min, 4°C) and stored at -30°C until use.

AFFINITY STUDIES BY QUARTZ CRYSTAL MICROBALANCE (QCM) ASSAY

Analysis of binding affinity of the purified domain II and III polypeptides of Cry4Aa against brush border membrane proteins was conducted by using single Q0500 (AsOne), a quartz crystal microbalance (QCM) device following the manufacturer's guidelines. The sensor chip was smeared with brush border membrane proteins (5µg), washed with PBS buffer and then treated with respective peptides in blocking solution. The dissociation constant (Kd) of the reaction kinetics was calculated using a software, Q-up analyzer ver. 1.02 of Scinics corporation.

SUGAR INHIBITION BIOASSAY

GST-Cry4Aa was purified as per previously reported method and concentration was determined by BIO-RAD assay as per the protocol. Effect of five monosaccharaides on the mosquitocidal activity of *C. pipiens* was tested by absorbing the proteins in latex beads and then applied to *C. pipiens* larvae for mortality assessment. Briefly, 100mM of *N*-Acetyl-*D*-Galactosamine (GalNAc), *N*-Acetyl-*D*-Glucosamine (GlcNAc), Galactose, Mannose and having 100mM Tris-HCL (pH 7.4) buffer solution. The cocktail is then thoroughly mixed at room temperature for one hour and then applied to *C. pipiens* larvae.

Mortality of the larvae was recorded after 24 and 48hr post-incubation and 50% lethal doses (LC_{50}) were determined using PROBIT analysis [8].

RESULTS AND DISCUSSIONS:

CONSTRUCTION, EXPRESSION AND PURIFICATION OF CRY4AA DOMAIN II AND II PEPTIDES

Domain II of Cry4Aa is considered to be responsible for receptor binding. However, our previous studies have shown that loop structures have limited functions on toxicity of Cry4Aa and the functional structures may be located in other locations rather not confined only in loop areas. Therefore, domain II was splitted in six more peptides in such a manner that each of which should incorporate a single loop of domain II exposed on the surface of Cry4Aa. In addition, domain III was also considered to identify its functions, if any. Domain II and III peptides were constructed as 4AaCter fusion protein. Domain II and III of GST-Cry4Aa was cloned, inserted into MCS of the 4ACter expression vector and finally successfully constructed the 4ACter fusion expression vector.

The domain II and III polypeptides of Cry4Aa were successfully expressed as 4AaCter fusion protein in *E. coli* BL21 cells. Upon disruption of *E. coli* cells by sonication, total cell lysate were analyzed by SDS–12%PAGE (Fig. 2).

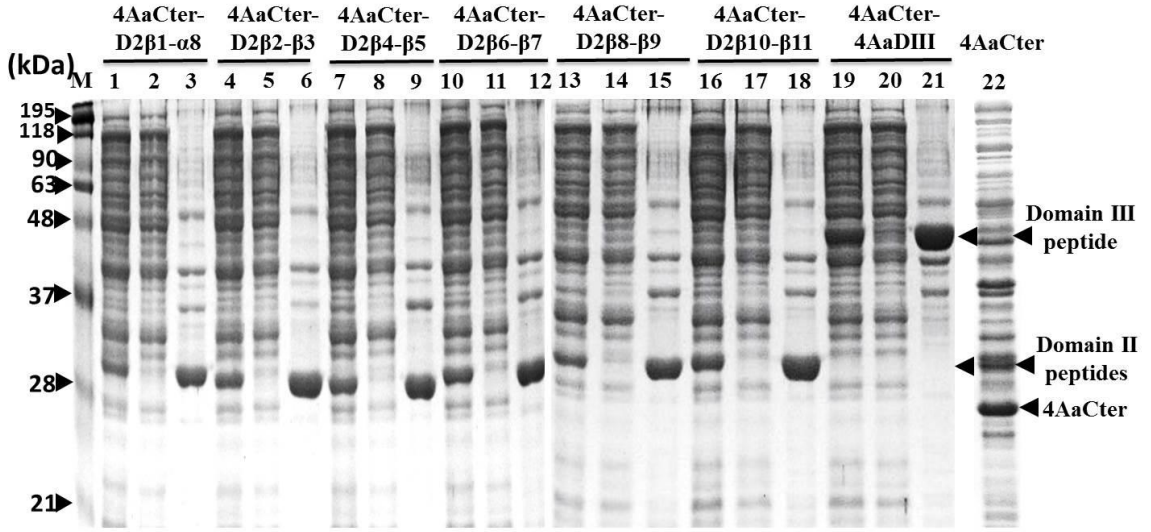

Fig. 2. The expression of the Cry4Aa domain II and III polypeptides was analyzed in SDS–12%PAGE. All the peptides were constructed as 4AaCter fusion protein and expressed in *E. coli* BL21 cells. Lanes 1, 4, 7, 10, 13, 16, 19 represents the total cell lysate (TCL) of the polypeptides, 4AaCter-D2β1-α8, 4AaCter-D2β2-β3, 4AaCter-D2β4-β5,

4AaCter-D2β6-β7, 4AaCter- D2β8-β9, 4AaCter-D2β10-β11and 4AaCter-DIII, respectively. On the other hand, Lanes 2, 5, 8, 11, 14, 17, 20 and the lanes 3, 6, 9, 12, 15, 18, 21 represents the soluble and insoluble fractions of the peptides mentioned above. Lane 22 represents the expression of the 4AaCter expression vector. The 4AaCter is a peptide-tag that facilitates the formation of protein inclusion bodies in *E. coli*. Therefore, all the polypeptides were expressed and formed as protein inclusion bodies. The protein inclusion bodies were separated as insoluble fraction from soluble fractions by centrifuge and were analyzed by SDS–12%PAGE (Fig. 2).

The molecular mass of six domain II polypeptides, 4AaCter-D2β1-α8, 4AaCter-D2β2-β3, 4AaCter-D2β4-β5, 4AaCter-D2β6-β7, 4AaCter- D2β8-β9 and 4AaCter-D2β10-β11 was estimated to be 28, 27, 26.5, 27, 27, 27kDa, respectively, which is slightly higher than that predicted from the deduced amino acid sequence (Fig. 2). The molecular mass of 4AaCter-domain III polypeptide and 4AaCter-tag only was estimated to be 44 and 26kDa, which is also slightly higher than that predicted from the deduced amino acid sequence (22kDa). The particular cause for the variance between the expected and observed size of the 4AaCter fusion proteins is unidentified, similar observations using same expression vector were previously reported [(9)]**.** Given their molecular weights, the expression level of 4AaCter-domain II and III polypeptides were efficiently expressed as protein inclusion bodies**.**

The insoluble protein inclusions of all the polypeptides were solubilized in 8M urea and were purified using HisPur^{TM} Ni-NTA Spin Columns under denatured conditions. Finally, successfully purified polypeptides were aliquoted and stored at -30^{o}C until further use.

CALCULATIONS OF BINDING AFFINITY OF CRY4AA DOMAIN II AND III PEPTIDES BY QCM ASSAY

The binding affinities of above mentioned purified Cry4Aa domain II and III peptides with the brush border membrane (BBM) proteins was analyzed using a QCM device and the affinity as dissociation constant (KD) was calculated. The results of affinity studies are presented at the Table 1. The data revealed relatively high affinities of the domain II polypeptides; D2β1-α8, D2β2-3, and domain III to BBM proteins, and their KD were estimated as 59, 54, and 63 nM, respectively. The D2β1-α8, D2β2-3 polypeptides incorporate loop α8 and loop 1 of domain II respectively. Thus, these two sites as well as the domain III of Cry4Aa may act as functional site for receptor binding studies. This data

also suggested that multiple sites may be present in case of Cry4Aa as we suggested in our previous studies. Our[3,4] previous reports indicated that the loop 1 sequences have limited role on Cry4Aa toxicity. Hence, considering our present data, the up and/or downstream sequences of loop 1 may some role on Cry4Aa toxicity.

Table 1. The binding affinity (Kd) of the GST-Cry4Aa wild-type and 4AaCter-domain II and III polypeptides to BBM proteins as determined by QCM device.

Peptides	Dissociation constant (KD, nM with SD)
4AaCter-D2β1-α8	59.25±5.54
4AaCter-D2β2-3	54.11±10.31
4AaCter-D2β4-5	216.67±7.37
4AaCter-D2β6-7	159.50±39.88
4AaCter-D2β8-9	718.90±18.63
4AaCter-D2β10-11	207.70±11.97
4AaCter-Domain III	62.82±5.67
GST-Cry4Aa (Wild-type)	49.20±5.54

ROLE OF SUGARS ON CRY4AA TOXICITY

Effect of simple monosaccharide sugar on Cry4Aa toxicity was assessed by sugar inhibition bioassay. The bioassay data revealed that the GalNAc pretreatment enhanced the toxicity of Cry4Aa and the pretreatment with fucose inhibited the toxicity of Cry4Aa (Fig. 3). Other monosaccharaides have limited or no effect on Cry4Aa toxicity.

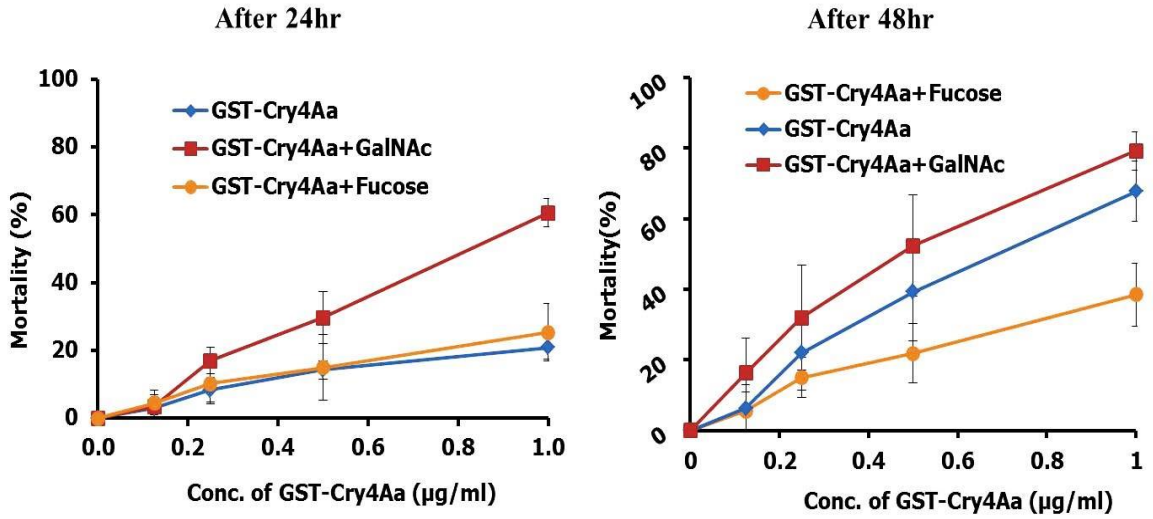

Fig. 3. The effect of GalNAc and fucose monosaccharide sugars on the toxicity of Cry4Aa against *C. pipiens* larvae. The Cry4Aa was pretreated with the respective sugars and then applied to *C. pipiens* larvae and the mortality was recorded after 24h and 48hr.

CONCLUSIONS

Our results suggested that multiple subsites that work cooperatively for receptor binding may be spread out in domain II and III of Cry4Aa. Monosaccharide sugars such as GalNAc and fucose have distinct role of Cry4Aa toxicity. Thus, the mechanisms of Cry4Aa is a unique and may be quite different from that of well-characterized other Cry toxins.

ACKNOWLEDGEMENT

The first author, MTH Howlader is awarded a fellowship by the Japan Society for the Promotion of Science to conduct the research mentioned here.

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