Interaction between *Bacillus thuringiensis* Cyt2Aa Toxin and Phosphatidylcholine-liposome

Naoki KOYAMA[1], Kohsuke HAGINOYA[1], Miki TANAKA[1], Boonhiang PROMODONKOY[2], Chanan ANGSUTHANASOMBAT[3], Toshiaki MITSUI[1], Masayuki TANIGUCHI[4], Tomoaki KHOYA[1], Kimiko ITOH[1] and Hidetaka HORI[1*]

(Received January 14, 2011)

Summary

Insecticidal toxin of Cyt2Aa produced with *Bacillus thuringiensis* was proteolytically activated with Proteinase K for 60 min or 90 min. Digests from each proteinase K-treatment were analyzed with protein sequencer and it was shown that in the 60 min-digested peptide, linkage of amino acid residues between 33rd Lysine and 34th Threonine was segregated. On the other hand, in the case of 90 min digestion, a few amino acid residues were further chopped off till 38th Serine. After purification of the activated Cyt2Aa, pore-formation activity of it on Phosphatidylcholine-liposome (PC-Lipo) was determined with calcein release assay. Cyt2Aa was shown to interact with PC-Lipo with high affinity and the reaction speed was quickly saturated during 30 min. This fast saturation of the reaction speed is different from that of Cry1A insecticidal toxins, and further suggested the different fashion of interactions between Cry1A(s) and PC-Lipo, and Cyt2Aa and PC-Lipo.

Bull.Facul.Agric.Niigata Univ., 63(2):69-76, 2011

Key words : *Bacillus thuringiensis,* cytotoxic protein, Phosphatidylcholine-liposome, kinetics of binding of PC-Lipo to Cyt2Aa

Bacillus thuringiensis is Gram positive soil living bacteria and produces parasporal inclusion body containing insecticidal crystal proteins. The insecticidal crystal proteins (ICP), *B. thuringiensis* toxin (BT toxin), show the kill activity to spodoptera, diptera and coleoptera insects, and have been used as BT formulation for more than 60 years for crops etc., since they are biodegradable and less remain in soil (Pigott and Ellar, 2007). The bacteria also have been used as gene sources for insect resistant gene modified crops and vegetables (GM Plant) and play an important role to development for sustainable agriculture.

BT toxin is generally recognized as Cry toxin but Cyt toxin which has cytotoxic activity is also produced but these have no direct relationships each other in gene structure (Crickmore *et al*., 1998). However both toxins resemble each other in insecticidal mechanism, thus when they are ingested into susceptible insects, both are solubilized in midgut fluid and kill susceptible insect by pore formation in plasma membrane of midgut epithelial cells. Although the precise mechanism to open pore on the plasma membrane are still in argument (Bravo *et al*., 2004; Tomimoto *et al*., 2006; Nair and Dean, 2008; Nair *et al*., 2008; Groulx *et al*., 2009), pore formation theory are widely accepted (Knowles and Ellar, 1987; Höfte and Whiteley, 1989; Schnepf *et al*., 1998). BT toxin has very specific insecticidal spectra and Cry1A attacks susceptible coleopteran insects, Cry4B, on the other hand, shows insecticidal activity for diptera, and Cry8C is known to kill only coleopteran scarabaeid family (Ogiwara *et al*., 1995). On the other hand, Cyt has been known to have insecticidal activity against mosquito and fly belonging to dipterous insect (Chilcott and Ellar, 1988; Fernández *et al*., 2005; Promdonkoy *et al*., 2005). Its principle mechanism is cytotoxicity by cell-lyses (Chilcott *et al*., 1998).

The structures of Cry and Cyt toxins are completely different from each other. Cry toxin is composed of three domains and structure of Cry3Aa (Li *et al*., 1991), Cry1Aa (Grochulski *et al*., 1995), and Cry4Ba (Boonserm *et al*., 2005) was shown to be almost the same each other by X-ray three dimensional analysis but Cyt toxin has single domain structure. Interestingly various Cry toxins have low homology in amino acid level with each other (Crickmore *et al*., 1998; Morse *et al*., 2001) but their three dimensional structure are very similar and activated Cry toxins are composed of three domains, i.e., domain I, II and III, each other. Domain I composed of seven α-helices and centrally positioned α-helix5 seemed to be surrounded by 6 α-helices.

[1] Laboratory of Bioscience, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan

[2] Biotec Central Research Unit, Thailand Science Park 113 Paholyothin Road, Klong Luang, Pathumthani 12120, Thailand

[3] Institute of Molecular Biosciences, Mahidol University, Nakornpathom 73170, Thailand

[4] Laboratory of Chemistry and Chemical Engineering, Department of Technology, Niigata University, Niigata 950-2181, Japan

* Corresponding author: hide-hri@gs.niigata-u.ac.jp

This domain I is very hydrophobic and resemble to that of hemolysin and colicin which are inserted into plasma membrane (de Maagd *et al*., 2003). Domain II is composed of three anti parallel *β*-sheets and named Greek key motif (de Maagd *et al*., 2003) and Domain III contains two anti parallel *β*-sheets and also shows *β*-sandwich structure with jelly roll topology (Li *et al*., 1991; Grochulski *et al*., 1995; Boonserm *et al*., 2006).

On the other hand, whole Cyt toxins show high homology in amino acid level and therefore they are thought to have highly resembled three dimensional structures (Guerchicoff *et al*., 2001). Cyt2 structure has been clarified and structure composed of 6 α-helices and 7 *β*-sheets in which anti parallel *β*-sheets are surrounded with two hairpin-like α-helices was proposed (Li *et al*., 1996). But the structure of active Cyt toxin has not been cleared yet.

Cyt toxin is grouped into Cyt1 and Cyt2 and these show nonspecific cytotoxicity. They were shown to have insecticidal activity against dipteran insects and especially Cyt1Aa and Cyt2Aa are thought to have high activity against those insects. Cyt2Aa is produced by *B. thuringiensis* subspecies darmstadiensis and solubilized in insect alkaline midgut fluid to 29 kDa protoxin. N-terminal and C-terminal regions are partially degraded to 23 kDa active-toxin (Promdonkoy *et al*., 2003) and activities to *Aedes aegypti* and *Culex pipiens* were observed. The precise kill mechanism, however, has not been clarified yet but two models have been proposed, i.e., pore formation theory (Promdonkoy and Ellar, 2000; 2003) and detergent like theory (Butko *et al*., 1996; Butko, 2003). In the latter model, the toxin was thought to coagulate on the cell surface to make thin layer structure spreading on plasma membrane surface. The toxin composing thin layered structure bind to lipid to make complex capable of destroying the lipid ordered structure.

Compare to the high activity of Cry4 and Cry11 against dipteran insects, that of Cyt has been estimated to be lower (Chilcott and Ellar, 1988; Fernández *et al*., 2005; Promdonkoy *et al*., 2005), however, when it was offered to target insect with Cry4 or Cry11, it was synergistically increased the activity to those toxins (Promdonkoy *et al*., 2005; Oestergaard *et al*., 2007). Therefore, it will be a good helper to control the occurrence of resistant dipteran insects such as *C. pipiens, C. quinquefasciatus, A. aegypti, Tipula paludosa* etc.

It is important to elucidate the insecticidal mechanism of Cyt toxin to use it as helper or insecticide. We activated Cyt2Aa with Proteinase K and purified with chromatography to characterize Cyt2Aa using phosphatidylcholine (PC) liposome (PC-Lipo). Calcein, fluorescent chemical, was simultaneously enclosed into the PC-Lipo. Here we report the fact that Cyt2Aa had affinity to PC-Lipo and some kinetics showing the difference of the interaction from ordinary pore formation by Cry1A are also shown.

MATERIALS AND METHODS

Preparation of Cyt2Aa

1. Culture of transformed *E. coli* cells

Expression vector *pGEM-Teasy* harboring *cyt2Aa* gene was gift from Dr. B. Promdonkoy, National Institute of Biotechnology, Bangkok, Thailand (Promdonkoy and Ellar, 2003).

E. coli competent cell, JM109 and *pGEM-Teasy* were mixed, and allowed to stand on ice for 5 min. The mixture was warmed at 42℃ for a min with water bath and replaced on ice for 5 min. The mixture was mixed with 900 μl of SOC medium in a 1.7 ml Eppendorf tube and cultured at 37℃ for 60 min. After 5 min-centrifugation, 100 μl supernatant was discarded and precipitate was suspended with pipetting. A hundred μl of the suspension was spread on Luria-Bertani (LB medium) agar plate (1.5%, w/w, agar) containing 10 g of Bacto tryptone, 5 g of Bacto yeast extract and 10 g of NaCl each per liter of water and cultured for 16 h at 37℃. The agar plates were autoclaved beforehand at 121℃ for 20 min and after the temperature cool down to 50℃, ampicillin was added at 50 μg/ml final concentration.

A hundred ml of SOC, Super Optimal broth with Catabolite repression medium, was composed of 2 g Bacto tryptone, 0.5 g Bacto yeast extract, 0.36 g glucose, 0.05 g NaCl, 0.02 g KCl, 0.1 g $MgCl_2$ and 0.12 g $MgSO_4$.

A single colony of *E. coli* transformed obtained as above was taken and cultured with LB agar plate at 37℃ for 16 h with a incubator (FI-610: Advantec Toyo Roshi Co., Ltd., Tokyo, Japan). Again a single colony was cultured in 1.7 ml of Eppendorf tube containing 1 ml of LB medium with 50 μg of Ampicillin at 37℃ for 16 h in a rotary shaker at 180 rpm (Bio Shaker BR-300L, Taitec Co., Ltd., Koshigaya, Saitama, Japan). One ml of the culture and ampicillin at final concentration of 50 μg/ml were added to 2 L triangle flask containing 1 L LB medium, and cultured with a rotary shaker at 180 rpm for 24 h. On half way of the culture, when optical density of the medium at 600 nm was reached to 0.4-0.5, IPTG (Isopropyl-*β*-D-thiogalactopyranoside) was added at final concentration of 0.5 mM.

2. Preparation of Cyt2Aa crystal and solubilization

Toxin crystal produced in *E. coli* was recovered by a method of Promdonkoy and Ellar (2000; 2003). The culture was centrifuged at 9,500 × *g* for 20 min at 4℃ with Hitachi himac (CR20G, Hitachi, Tokyo, Japan) and resulted precipitate was suspended in 30 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM KCl and 0.01% (v/v) Triton X-100 with a glass-Teflon homogenizer. Further, the suspension was thoroughly suspended with sonication (VCX 130PB, Sonics & Materials, Inc., Newtown, PV, USA) at 20 kHz for 30 min with 1 min interval in each 5 min sonication. After the centrifugation at 10,000 × *g*, for 10 min, the resulted precipitate was suspended to Gradient buffer and centrifuged again. This washing was performed three times and the final precipitate was suspended in 10 ml of DDW (Deionized

distilled water). The crystals were precipitated with centrifugation at 10,000 × *g* for 10 min at 4℃ and protein concentration of the suspension was determined with Bradford method with bovine serum albumin, BSA, as standard.

Crystals corresponding to 2 mg protein was suspended with 1 ml of solubilization liquid composed of 50 mM Na_2CO_3 and 10 ml dithiothreitol, pH 10, and shaken at 80 rpm for 2 h at 37℃ in a rotary shaker. The solubilized protein, protoxin, was recovered in the supernatant after the centrifugation at 14,000 × *g* for 20 min at 4℃ . The supernatant was dialyzed overnight with cellulose tubing (Seamless Cellulose Tubing, Viskase Sales, Chicago, USA) against 30 times volume of 50 mM Tris-HCl, pH 8.3, at 4℃ .

3. Activation of Cyt2Aa

Solubilized toxin corresponding 100 mg of protein was added with 31.7 μl of Proteinase K (31.5 mg/ml) and shaken at 180 rpm with a rotary shaker for various period till 150 min. The proteolytic digestion was ceased by the addition of PMSF (phenylmethylsulfonyl fluoride) at 1 mM final concentration.

4. Purification of active Cyt2Aa with DEAE Sepharose Fast Flow column chromatography

The solubilized Cyt2Aa was purified with a DEAE Sepharose Fast Flow column, ϕ1.5 × 20 cm (GE Healthcare, UK Ltd., Buckinghamshire, England). The adsorbed Cyt2Aa was eluted with the linear gradient made with 50 mM Tris-HCl 50 mM NaCl (pH 8.3) and 50 mM Tris-HCl 400 mM NaCl (pH 8.3) at 1.5 ml/min effluent speed, and each 1 ml fraction was collected. Protein was monitored with photo absorbance at 280 nm. Sample in every fraction was analyzed with SDS-PAGE. The fractions containing about 23 kDa active Cyt2Aa were collected and dialyzed as described above except against 50 mM Tris-HCl containing 50 mM NaCl. The protein was determined with Bradford method.

5. Determination of protein concentration

Protein concentration was determined with Bradford method (Bradford, 1976) using calibration curve with BSA. Occasionally, photo absorbance at 280 nm was used for the protein concentration.

6. SDS-Polyacrylamide electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Indrasith *et al.* (1991) and Laemmli (1970) using an apparatus AE-6500 (Atto, Tokyo, Japan). The sample was denatured at 95℃ for 3 min with 10% (w/v) SDS, 40% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, 0.08% (w/v) Bromophenol Blue, 50 mM Tris-HCl, pH 6.8. Protein marker, broad range (2-212 kDa, New England Biolabs, Beverly, USA) was used and protein was stained with 0.2% (w/v) Coomassie brilliant blue R-250.

7. Determination of N-terminal amino acid of active Cyt2Aa

After SDS-PAGE, activated Cyt2Aa was blotted to PVDF (PolyVinylidene DiFluoride) membrane (Hybond-P, GE healthcare Bio-sciences, Uppsala, Sweden) with an electro blotter (Horizontal Stack Gel Blottin Device, Atto, Tokyo, Japan). Stained protein on the PVDF membrane was cut out and applied to protein sequencer (Shimadzu PPSQ-21A, Kyoto, Japan).

Analysis of interaction between Cyt2Aa and PC-liposome

1. Preparation of liposome

Liposome was made with eggyolk phosphatidylcholine (Sigma-Aldrich Chemicals, St. Louis, MO, USA) as Haginoya *et al.* (2010). Calcein, a fluorescence dye was introduced into the liposome according to Haginoya *et al.* (2005; 2010) and liposome containing calcein was separated from one without calcein with sucrose density gradient centrifugation as Haginoya *et al.* (2005).

2. Pore-formation assay

Pore-formation assay was performed as Kato *et al.* (2006) and Haginoya *et al.* (2010). Appropriate but equal volume of Cyt2Aa at 2 μM as final concentration, in 50 mM Tris-HCl, pH 8.3 containing 50 mM NaCl, and purified PC-Lipo suspension were mixed and placed in a 15 ml test tube. The maximum fluorescence intensity (FI) of the final suspension was adjusted around 400 before the start of the test.

Pore-formation activity by Cyt2Aa was monitored for 3 h and evaluated by Fluorescence Recovery (FR). FR was expressed as the ratio of measured FI to the maximum FI observed by addition of Triton X-100 (final concentration of 1%) into PC-Lipo suspension. In control experiment, only the buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8.3) was added. FI in control was subtracted from each FI measured to obtain FR of Cyt2Aa.

To study the pore-formation kinetics of the interaction with Cyt2Aa and PC-Lipo, FI was monitored in a microcuvette every 30 min for 3 h and FI at 520 nm was directly plotted against the corresponding incubation period. The reaction was started by the addition of 200 μl Cyt2Aa toxin at 0.2-5.0 μM in 50 mM Tris-HCl, pH 8.3 containing 50 mM NaCl to 200 μl PC-Lipo in the same buffer with a maximum FI of about 400. The slope of each tangential line of the saturation curve in the first 30 min was plotted against the concentration of Cry1A employed.

RESULTS AND DISCUSSION

Proteolytic activation of Cyt2A

Cyt2Aa produced by *B. thuringiensis* must be partially digested with appropriate proteinase(s) in the susceptible insect midgut to express its insecticidal activity (Knowles and Ellar, 1987; Höfte and Whiteley, 1989; Schnepf *et al.*, 1998).

Cyt2Aa corresponding to 100 mg were digested with 1

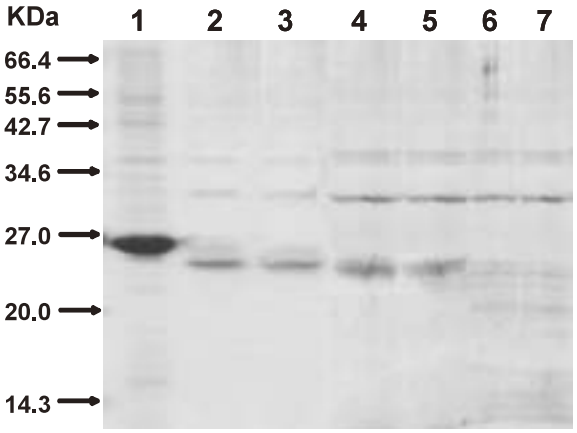


Fig. 1 SDS-PAGE of Cyt2Aa proteolytically activated for various periods. Proteins were polyacrylamide gel electrophoresed with 15% gel and stained with CBB-R250. Lane1; Cyt2Aa protoxin, Lane2; 45-digestion, Lane3; 60 min-digestion, Lane4; 75 min-digestion, Lane5; 90 min-digestion, Lane6; 120 min-digestion, Lane7; 150 min-digestion. The figures on left side of the picture are size of molecular markers in kDa.

mg of Proteinase K at 37℃ for 45, 60, 75, 90, 120 and 150 min, and each digest was analyzed with SDS-PAGE (Fig. 1). Beyond 120 min, excess digestion was observed and 60 min-digestion was employed for the activation. Promdonkoy and Ellar (2000; 2003) once purified Cyt2Aa with 1% (w/w) Proteinase K and we obeyed their way and noticed that 120 min-incubation over digested Cyt2Aa and 23 kDa activated peptide was not detected (Fig. 1, Lane 6). Proteolytic cleavage sites of the enzyme are wide and linkage of aromatic amino acid and alkyl amino acid can be chopped off, therefore, 120 min-digestion resulted in very small peptides.

N-terminal amino acid sequence of activated Cyt2Aa

Cyt2Aa digested for 60 min with Proteinase K was applied to protein sequence analysis to determine N-terminal amino acid. Fifteen amino acid residues were determined as TVPSSDLDNFNTVFY (Fig. 2). On the other hand, the sequence of the peptide digested for 90 min was shown to be SDLDNFNTVFYVQPQ. The known amino acid sequence of Cyt2Aa was compared to those sequences we determined and it was evidenced that in 60 min digestion, N-terminal peptide of Cyt2Aa was chopped off at between 33rd Lysine and 34th Threonine. On the other hand, in the case of 90 min digestion, a few amino acid residues further chopped off till 38th Serine. Cyt2Aa protoxin is composed of 259 amino acid residues and we digested with the method of Promdonkoy and Ellar (2000; 2003). We thought that the peptide started from 38th Serine residue at N-terminal peptide must be the smallest active Cyt2Aa.

Purification of activated Cyt2Aa

Activated Cyt2Aa toxin was purified by DEAE

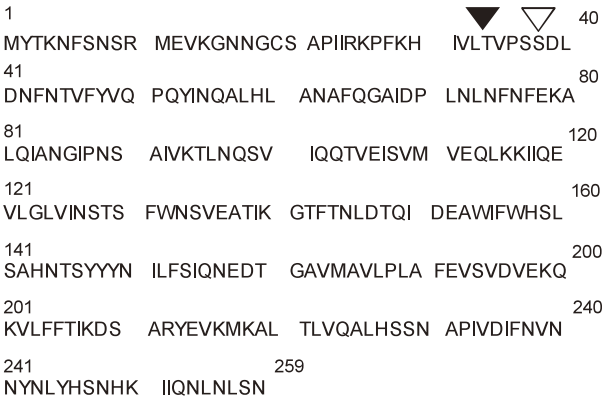


Fig. 2 Cleavage sites of Cyt2Aa digested with Proteinase K for 60 min and 90 min. Cleavage sites with Proteinase K on Cyt2Aa-digestion was determined. Cyt2Aa was digested for 60 min and 90 min, and N-terminal amino acid sequence of these digests were determined and each cleavage site was marked with a symbol of ▼ and ▽, respectively.

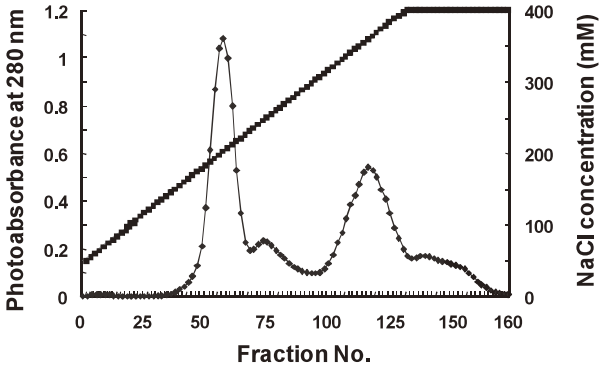


Fig. 3 DEAE Sepharose Fast Flow column chromatography of Cyt2Aa digested with Proteinase K. (- ◆ -); Photo absorbance at 280 nm. (- ■ -) NaCl concentration for the protein elution. The solubilized Cyt2Aa was purified with a DEAE Sepharose Fast Flow column, ϕ1.5 × 20 cm (GE Healthcare, UK Ltd., Buckinghamshire, England). The adsorbed Cyt2Aa was eluted with the linear gradient made with 50 mM Tris-HCl 50 mM NaCl (pH 8.3) and 50 mM Tris-HCl 400 mM NaCl (pH 8.3) at 1.5 ml/min effluent speed, and each 1 ml fraction was collected.

Sepharose fast flow column chromatography with 50-400 mM linear NaCl gradient (Fig. 3) and was eluted at around fraction 60 as a single peak. Fraction 50 to 64 were collected and applied to SDS-PAGE (Fig. 4). As shown in Fig. 4, Cyt2Aa without Proteinase K was purified. In this report we could separate Proteinase K from purified Cyt2Aa using Sepharose Fast Flow column. The peak containing about 25 kDa peptide was eluted as first appeared peak at 170-220 mM NaCl in the column chromatography. Proteinase K was eluted in non

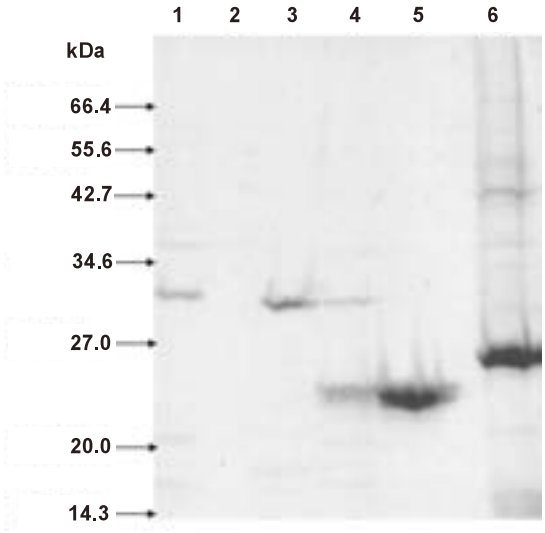
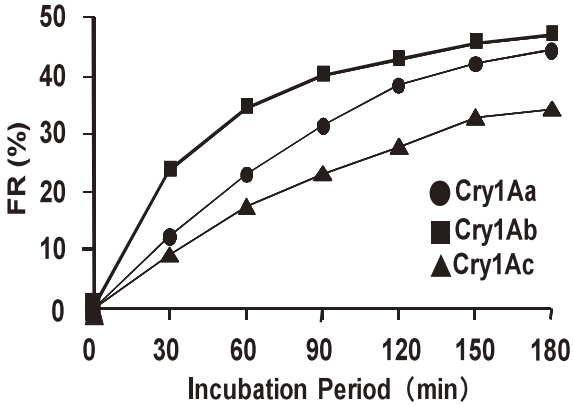


Fig. 4 SDS-PAGE of several main fractions separated in DEAE Sepharose Fast Flow column chromatography. The proteins separated were collected and polyacrylamide gel electrophoresed with 15% gel and proteins were stained with CBB-R250. Lane 1; The through fraction in DEAE Sepharose column chromatography. Lane 2; Washings of the column with 500 mM NaCl, Lane 3; Proteinase K. Lane 4; Digests of Cyt2A with Proteinase K. Lane 5; Peak fraction No.58 in DEAE Sepharose column chromatography shown in Fig. 3. Lane 6; Protoxin of Cyt2Aa.



adsorbed fraction (Fig. 4, Lanes 1 and 3) and completely eliminated from the activated Cyt2Aa fraction. Therefore, since the sample hereafter used in the interaction with PC-Lipo had no Proteinase K, which is main contaminants, the data from interaction experiments are thought to be reliable.

Interaction of Cyt2Aa and PC-liposome

1. Calcein release assay

PC-liposome (PC-Lipo) containing calcein fluorescence indicator was mixed with Cyt2Aa at 2 μM final concentration. The activity to release calcein form PC-Lipo was evaluated with Fluorescence Recovery (FR) as described in materials and methods. In first 30 sec, FR was reached 15% and beyond this point, released fluorescence was increased slowly and reached to 30% at 20 min-reaction (Fig. 5, Upper panel). When the reaction period was prolonged to 3 h, the FR rapidly increased to 30% FR till 30 min and after the point the FR gradually decreased to 26% (Fig. 5, Lower panel). Thus the tendency where FR increased rapidly to 20% during a few minutes and increased further to 30% during 20-30 min, was essentially observed in both the incubation experiments. These rapid saturations of calcein release in a short period were different from that of Cry1A toxins reported by Haginoya *et al.* (2010), thus they showed the release curve as a function of incubation period in which FR of calcein was slowly increased during 3 h in the reaction with Cry1A. These different incubation curves suggested that pore formation by Cyt2Aa and Cry1A on PC-Lipo must be achieved with fairly different mechanisms. It is noteworthy that the pore-formation speed with three kinds of Cry1A toxins were saturated slowly during around 180 min, but in the case of the pore-formation with Cyt2Aa, as shown in Fig. 5, during 30 min at 2 μM of Cyt2Aa the reaction reached to plateau.

2. Brief kinetic analysis of pore-formation activity by Cyt2Aa on PC-Lipo

Cyt2Aa saturation curve as a function of concentration of the toxin was determined (Fig. 6). Increase of FI was measured for 2 min with various concentration of Cyt2Aa and initial velocity was expressed as FI increased/min and as

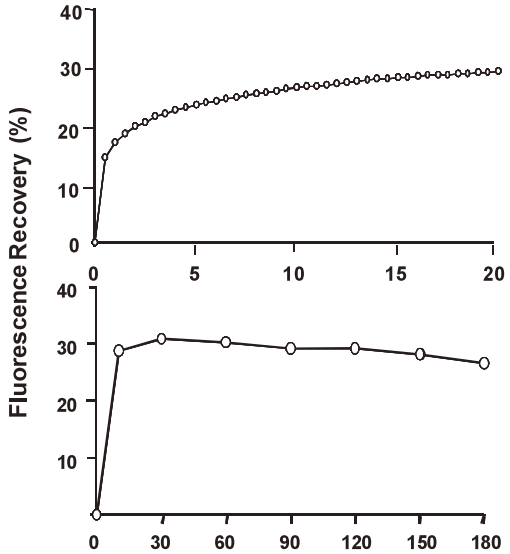


Fig. 5 Pore-formation activity of Cyt2Aa on PC-Lipo as a function of incubation period. Upper figure: Pore formation was performed for 20 min and every half min the released calcein was determined with calcein release assay. Lower figure: Calcein release assay was performed for 180 min. For the experimental detail, see section materials and methods.

shown in Fig. 6, the velocity was reached plateau at around 2.4 μM Cyt2Aa. Lineweaver-Burk plots were calculated from the data shown in Fig. 6, and V_{max} and K_m values were determined as 7.8 nM calcein released/min and 0.24 μM Cyt2Aa, respectively (Fig. 6, superimposed figure). These data clearly suggested that Cyt2Aa had high affinity to PC-Lipo. However if we compare these data to that between PC-Lipo and Cry1A toxins, the reaction in former was much more rapid than that of later. The reactions of the PC-Lipo with Cyt2Aa and Cry1A were different from each other. PC is mainly made of *β*-structure, then we think that Cyt2Aa interact with PC-Lipo with a fashion in which the toxin destroys the liposome quickly as a sort of detergent.

Cyt2 pro-toxin is composed of 6 α-helices and 7 *β*-sheets and hydrophobic portion which is composed of anti-parallel *β*-sheets is exposed on surface of the Cyt2 molecule (Li *et al.*, 1996). Activation of pro-toxin of Cyt2Aa must make two α-helical structures away from the hydrophilic portion which is composed of anti-parallel *β*-sheets and as a result, the hydrophobicity may increase. And then, this portion will interact with hydrophobic PC.

To evidence this hypothesis, the structure of activated Cyt2Aa must be compare with that of pro-toxin of Cyt2Aa. And furthermore Pro-toxin of Cyt2Aa must be interacted with PC-Liposome.

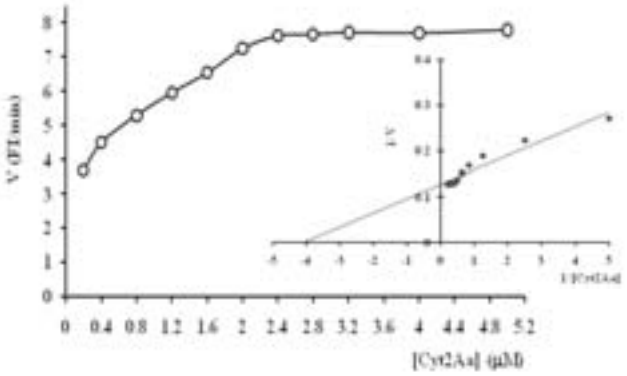


Fig. 6 Pore-formation activity of Cyt2Aa on PC-Lipo as a function of the concentration. The maximum fluorescence intensity (FI) of the final suspension was adjusted around 400 before the start of the test. Pore-formation activity was monitored for 30 min with various concentration of Cyt2Aa and initial velocity in each concentration was plotted. Pore formation activity was evaluated by Fluorescence Recovery (FR) which was expressed as the ratio of measured FI to the maximum FI observed by addition of Triton X-100 (final concentration of 1%) into PC-Lipo suspension. In control experiment, only the buffer (50 mM NaCl, 50 mM Tris-HCl, pH 8.3) was added. FI in control was subtracted from each FI measured to obtain FR of Cry1A toxins. Super imposed figure is double reciprocal plot from the Cyt2Aa-saturation curve.

REFERENCES

Boonserm, P., P. Davis, D.J. Ellar and J. Li. 2005. Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. *J. Mol. Biol.*, **348**: 363-382.

Boonserm, P., M. Mo, C. Angsuthanasombat and J. Lescar. 2006. Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. *J. Bacteriol.*, **188**: 3391-3401.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**: 248-254.

Bravo, A., I. Gómez, J. Conde, C. Muñoz-Garay, J. Sánchez, R. Miranda, M. Zhuang, S.S. Gill and M. Soberón. 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim. Biophys. Acta.*, **1667**: 38-46.

Butko, P., F. Huang, M. Pusztai-Carey and W.K. Surewicz. 1996. Membrane permeabilization induced by cytolytic δ-endotoxin CytA from *Bacillus thuringiensis* var. *israelensis*. *Biochemistry*, **35**: 11355-11360.

Butko, P. 2003 Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. *Appl. Environ. Microbiol.*, **69**: 2415-2422.

Chilcott, C.N. and D.J. Ellar. 1988. Comparative toxicity of *Bacillus thuringiensis* var. *israelensis* Crystal Proteins *in vivo* and *in vitro*. *J. Gene. Micobiol.*, **134**: 2551-2558.

Chilcott, C.N., P.J. Wigley, A.H. Broadwell, D.J. Park and D.J. Ellar. 1998. Activities of *Bacillus thuringiensis* insecticidal crystal proteins Cyt1Aa and Cyt2Aa against three species of sheep blowfly. *Appl. Environ. Microbiol.*, **64**: 4060-4061.

Crickmore, N., D.R. Zeigler, J. Feitelson, E. Schnepf, J.Van Rie, D. Lereclus, J. Baum, and D.H. Dean. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.*, **62**: 807-813.

de Maagd, R.A., A. Bravo, C. Berry, N. Crickmore and H.E. Schnepf. 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Ann. Rev. Gene.*, **37**: 409-433.

Fernández, L.E., C. Pérez, L. Segovia, M.H. Rodríguez, S.S. Gill, A. Bravo and M. Soberón. 2005. Cry11Aa toxin from *Bacillus thuringiensis* binds its receptor in *Aedes aegypti* mosquito larvae through loop α-8 of domain II. *FEBS Lett.*, **579**: 3508-3514.

Grochulski, P., L. Masson, S. Borisova, M. Pusztai-Carey, J.-L. Schwartz, R. Brousseau and M. Cygler. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J. Mol. Biol.*, **254**: 447-464.

Groulx, N., M. Juteau, J.L. Schwartz, R. Laprade, R. and Blunck. 2009. Rapid topology determination of membrane proteins: pore-forming mechanism of Bt toxin Cry1Aa. *Biophys. J.*, **96**: 535a, 2751-Pos Board B721.

Guerchicoff, A., A. Delécluse and C.P. Rubinstein. 2001. The

Bacillus thuringiensis cyt genes for hemolytic endotoxins constitute a gene family. *Appl. Environ. Microbiol.*, **67**: 1090-1096.

Haginoya, K., T. Kato, M. Higuchi, Y. Shitomi, T. Asakura, T. Hayakawa, T. Mistui and H. Hori. 2005. Preparation of stable liposomes using sucrose density gradient centrifugation and their interaction with insecticidal Cry1A toxin of *Bacillus thuringiensis*. *Bull. Facul. Agric. Niigata Univ.*, **57**: 115-120.

Haginoya, K., V. Thangavel, G.N. Pandian, K. Tomimoto, Y. Shitomi, M. Azuma, C. Angsuthanasombat and H. Hori. 2010. Investigation of physicochemical condition to stabilize phosphatidylcholine-liposome enclosing fluorescent calcein and its exploitation for analysis of pore formation with Cry1A toxins of *Bacillus thuringiensis*. *Appl. Entomol. Zool.*, **45**: 477-488.

Höfte, H. and H.R. Whiteley. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis Microbiol. Mol. Biol. Rev.*, **53**: 242-255.

Indrasith, L.S., K. Ogiwara, M. Minami, T. Iwasa, T. Maruyama, N. Suzuki, S. Asano, K. Sakanaka and H. Hori. 1991. Processing of delta endotoxin from *Bacillus thuringiensis* subspp. *Kurstaki* HD-1 and HD-73 by immobilized trypsin and chymotrypsin. *Appl. Entomol. Zool.*, **26**: 485-492.

Kato, T., M. Higuchi, R. Endo, T. Maruyama, K. Haginoya, Y. Shitomi, T. Hayakawa, T. Mitsui, R. Sato and H. Hori. 2006. *Bacillus thuringiensis* Cry1Ab, but not Cry1Aa or Cry1Ac, disrupts liposomes. *Pestic. Biochem. Phys.*, **84**: 1-9.

Knowles, B.H. and D.J. Ellar. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. *Biochem. Biophys. Acta.*, **924**: 509-518.

Laemmlli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Li, J., J. Carroll and D.J. Ellar. 1991. Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. *Nature*, **353**: 815-821.

Li, J., P.A. Koni and D.J. Ellar. 1996. Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensi*s and implications for membrane pore formation. *J. Mol. Biol.*, **257**: 129-152.

Morse, R.J., T. Yamamoto and R.M. Stroud. 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure*, **9**: 409-417.

Nair, M.S. and D.H. Dean. 2008. All domains of Cry1A toxins insert into insect brush border membranes. *J. Biol. Chem.*, **283**: 26324-26331.

Nair, M.S., X.S. Liu and D.H. Dean. 2008. Membrane insertion of the *Bacillus thuringiensis* Cry1Ab toxin: single mutation in domain II block partitioning of the toxin into the brush border membrane. *Biochemistry*, **47**: 5814-5822.

Oestergaard, J., R.-U. Ehlers, A.C.Martinez-Ramírez and M.D. Real. 2007. Binding of Cyt1Aa and Cry11Aa toxins of *Bacillus thuringiensis* serovar *israelensi*s to brush border membrane vesicles of *Tipula paludosa* (Diptera: Nematocera) and subsequent pore formation. *Appl. Environ. Microbiol.*, **73**: 3623-3629.

Ogiwara, K., H. Hori, M. Minami, K. Takeuchi, R. Sato, M. Ohba and H. Iwahana. 1995. Nucleotide sequence of the gene encoding novel delta-endotoxin from *Bacillus thuringiensis* serovar *japonensis* strain buibui specific to scarabaeid beetles. *Curr. Microbiol.*, **30**: 227-235.

Pigott, C.R. and D.J. Ellar. 2007. Role of receptors in *Bacillus thuringiensis* crystal toxin activity. *Microbiol. Mol. Biol. Rev.*, **71**: 255-281.

Promdonkoy, B. and D.J. Ellar. 2000. Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*. *Biochem. J.*, **350**: 275-282.

Promdonkoy, B., N. Chewawiwat, S. Tanapongpipat, P. Luxananil and S. Panyim. 2003. Cloning and characterization of a cytolytic and mosquito larvicidal δ-endotoxin from *Bacillus thuringiensis* subsp. *darmstadiensis*. *Curr. Microbiol.*, **46**: 94-98.

Promdonkoy, B. and D.J. Ellar. 2003. Investigation of the pore-forming mechanism of a cytolytic δ-endotoxin from *Bacillus thuringiensis*. *Biochem. J.*, **374**: 255-259.

Promdonkoy, B., P. Promdonkoy and S. Panyim. 2005. Co-expression of *Bacillus thuringiensis* Cry4Ba and Cyt2Aa2 in *Escherichia coli* revealed high synergism against *Aedes aegypti* and *Culex quinquefasciatus* larvae. *FEMS Microbiol. Lett.*, **252**: 121-126.

Schnepf, E., N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler and D.H. Dean. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.*, **62**: 775-806.

Tomimoto, K., T. Hayakawa and H. Hori. 2006. Pronase digestion of brush border membrane-bound Cry1Aa shows that almost the whole activated Cry1Aa molecule penetrates into the membrane. *Comp. Biochem. Phys. B.*, **144**: 413-422.

Bacillus thuringiensis Cyt2Aa トキシンとホスファチジルコリンリポソームの相互作用

小山尚生[1]・萩野谷功輔[1]・田中未希[1]・ブーヒアン プロモドンコイ[2]・チャナン アングスタナソンバット[3]・三ツ井敏明[1]・谷口正之[4]・高屋朋彰[1]・伊藤紀美子[1]・堀秀隆[1*]

（平成23年１月14日受付）

要　約

Bacillus thuringiensis が生産する Cyt2Aa は、双翅目昆虫を殺虫する細胞毒性殺虫タンパク質で、その殺虫メカニズムを詳細に解明することは持続可能な農業を実現するために重要である。活性型 Cyt2Aa を得るために、プロテネース K により部分分解を行ったところ、反応時間が120分を超えると過度な分解が進行し、活性化断片と考えられる23 kDa の断片が消失した。60分間の反応で得られた23 kDa の消化断片はアミノ酸配列解析の結果、33番目のリシンと34番目のスレオニンの間で切断されていた。一方、90分間の消化処理で得られたペプチドは、さらに38番目のセリンまで4残基が消化切断されていたが、これを最小活性断片であるとし、蛍光物質カルセインを内部に取り込んだ人工脂質膜リポソームを作製し小孔形成活性を測定した。精製した Cyt2Aa を、ホスファチジルコリンリポソーム（PC-Lipo）と反応させ、その小孔形成活性を放出されるカルセインの蛍光量から評価した。反応開始30分で小孔形成活性は最大となった。３時間かけて小孔形成活性が最大値に達する Cry1A との反応に比べて、Cyt2Aa が PC-Lipo に対して早い破壊活性を持つことが示唆され、Cyt2A と Cry1A は異なる反応様式で PC-Lipo に小孔を形成し、Cyt2A は脂質膜とより早く反応すると考えられた。

新大農研報, 63(2):69-76, 2011

キーワード：*Bacillus thuringiensis*、細胞毒性タンパク質、ホスファチジルコリンリポソーム（PC-Lipo）、PC-Lipo に対するCyt2Aa 結合キネティクス

[1] 新潟大学大学院自然科学研究科
[2] タイ王国バイオテック中央研究ユニット
[3] タイ王国マヒドン大学サラヤキャンパス分子生物化学研究所
[4] 新潟大学工学部
*代表著者：hide-hri@gs.niigata-u.ac.jp