Potency of the mosquitocidal Cry46Ab toxin produced using a 4AaCter-tag, which facilitates formation of protein inclusion bodies in *Escherichia coli*

Tomoaki Okazaki[1]·Junya Ichinose[1]·So Takebe[2]·Toru Ide[1]·Tohru Hayakawa[1, *]

[1]Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan, [2]Graduate School of Biology-Oriented Science and Technology, Kindai University, 930 Nishimitani, Kinokawa 649-6493, Japan

***Corresponding author**
Tohru Hayakawa, Email address: hayaka-t@cc.okayama-u.ac.jp
Phone: +81862518205, Fax: +81862518264

Abstract

Cry46Ab toxin derived from *Bacillus thuringiensis* strain TK-E6 shows mosquitocidal activity against *Culex pipiens pallens* Coquillett (Diptera: Culicidae) larvae as well as preferential cytotoxicity against human cancer cells. In *B. thuringiensis* cells, Cry46Ab is produced and accumulates as a protein crystal that is processed into the active 29-kDa toxin upon solubilization in the alkaline environment of the insect midgut. The Cry46Ab protoxin is 30 kDa and is therefore thought to require an accessory protein such as P20 and/or ORF2 for efficient crystal formation. For efficient production of alkali-soluble inclusion bodies of recombinant Cry46Ab in *Escherichia coli*, the potency of the 4AaCter-tag was assessed in the present study. The 4AaCter-tag is a polypeptide derived from the C-terminal region of *B. thuringiensis* Cry4Aa toxin and facilitates the formation of alkali-soluble protein inclusion bodies in *E. coli*. Fusion with the 4AaCter-tag enhanced both Cry46Ab production and the formation of Cry46Ab inclusion bodies. In addition, upon optimization of protein expression procedures, the Cry46Ab-4AaCter inclusion bodies showed mosquitocidal activity and stability in aqueous environments comparable to Cry46Ab without the 4AaCter-tag. Our study suggests that use of the 4AaCter-tag is a straightforward approach for preparing formulations of smaller-sized Cry toxins such as Cry46Ab in *E. coli*.

Keywords
Bacillus thuringiensis· mosquitocidal Cry46Ab toxin· 4AaCter-tag· formation of protein inclusion· *Escherichia coli*

Introduction

Bacillus thuringiensis is a gram-positive soil bacterium that produces specific insecticidal proteins (Cry toxins) used widely as insect-pest control agents (Schnepf et al. 1998). Cry toxins are produced as protoxins during sporulation and accumulate in the form of protein crystals. After ingestion by susceptible insect larvae, the protein crystals are solubilized in the alkaline midgut juice and then processed into the active toxin by midgut proteases. Why Cry toxins are produced in the form of protein crystals is not clear. However, it is reasonable to speculate that formation of protein crystals allows for packaging of a large amount of Cry toxins into the limited intracellular space, protection of Cry toxins from proteolytic degradation, and protection of the host cell from the toxicity of Cry toxins. In addition, the formation of protein crystals increases the dose of Cry toxin to which susceptible insect larvae are exposed. Formation of protein crystals thus enhances the efficiency of Cry toxin production and their potential application as bioinsecticides.

The mode of crystallization seems to be complex and varies with the type of Cry toxin. For example, crystallization of Cry toxins derived from larger-sized protoxins (130 kDa–type) is thought to involve self-assembly. The recombinant Cry 1 toxin, with a 130 kDa–type protoxin, forms protein inclusions containing biologically active toxin

in *Escherichia coli* (Chak and Ellar 1987; Oeda et al. 1987). Furthermore, bipyramidal crystals denatured in 8 M urea revert to their original crystal shape when the urea is removed by dialysis (Lecadet 1967). It has been suggested that the cysteines located in the C-terminal half of the 130 kDa–type protoxin, which is removed during toxin activation, form intermolecular disulfide bridges in the crystal lattice, leading to solubilization in the presence of reducing agents at the high pH that is typical of the midgut juice of lepidopteran and dipteran larvae (Bietlot et al. 1990; Nickerson 1980; Yu et al. 2002). In addition, the C-terminal half of the protoxin contains highly conserved block sequence -6, -7 and -8 that are thought to be important for crystallization (Schnepf et al. 1998). A new peptide-tag derived from the C-terminal half of the 130 kDa–type protoxin was recently developed. This peptide-tag, designated the '4AaCter-tag' is a polypeptide (I^{696} to P^{851}) derived from the C-terminal half of the Cry4Aa protoxin and contains conserved block sequence -6 and -7 (Hayakawa et al. 2010). It was demonstrated that fusion with the 4AaCter-tag facilitates the formation of protein inclusion bodies composed of fused heterologous protein in *E. coli* (Hayakawa et al. 2010). Fusion with the 4AaCter-tag was shown to dramatically increase protein production and simplify product purification for a number of recombinant proteins, including syphilis antigens, TpNs (Hayakawa et al. 2010), markers of renal dysfunction,

cystatin C (Hayashi et al. 2013), and scorpion toxin as a bioinsecticide (Matsumoto et al. 2014). By contrast, Cry toxins with smaller-sized protoxins (70 and 30 kDa–types) do not contain the polypeptide corresponding to the C-terminal half of 130 kDa–type protoxin, and crystallization of these proteins is thus thought to require an accessory protein such as P20 and/or ORF2 (Agaisse and Lereclus 1995). It is not known whether the polypeptides derived from the C-terminal half of 130 kDa–type protoxins such as 4AaCter enhance the crystallization of smaller-sized Cry toxins.

Cry46Ab, derived from *B. thuringiensis* strain TK-E6, is a Cry toxin with a 30 kDa–type protoxin and shows preferential cytotoxicity against human cancer cells (Hayakawa et al. 2007). In addition, mosquitocidal activity against *Culex pipiens pallens* Coquillett (Diptera: Culicidae) larvae was recently reported for Cry46Ab (Hayakawa et al. 2017). The three-dimensional structure of Cry46Aa, which is closely related to Cry46Ab (84% identity), was determined and shown to be similar to that of aerolysin type-β pore-forming toxins (Akiba et al. 2009). Cry46Ab is a new mosquitocidal toxin with an aerolysin-type architecture that is expected to be useful as a bioinsecticide to control mosquito larvae.

In this study, we produced protein inclusion bodies consisting of Cry46Ab in *E. coli*. Formation of inclusion bodies, production level, mosquitocidal activity, solubility

in alkaline buffer, and stability in aqueous environments were compared for inclusion bodies formed with and without a 4AaCter-tag. The results of our study suggest that use of the 4AaCter-tag is a straightforward approach for preparing formulations of smaller-sized Cry toxins in *E. coli*.

Materials and methods

Construction of expression vectors

The DNA fragment encoding the 4AaCter-tag was previously inserted in frame into the *Xho*I site of the expression vector pGEX-6P-1 (GE Healthcare, Little Chalfont, UK) to generate pGST-4AaCter 696-851 (Hayakawa et al. 2010); pGST-4AaCter 696-851 is thus an expression vector for producing glutathione *S*-transferase (GST) fused with the 4AaCter-tag at the C-terminal end. In this study, the *Xho*I site at the 3′ end of the *4AaCter* gene was removed from pGST-4AaCter 696-851 by site-directed mutagenesis using a set of specific primer pairs (4AaCter-deltaX-f, CGGCCGCATCGTGACTGACTG; 4AaCter-deltaX-r, CGGCACATTCATGATTGCATC) with back-to-back orientation. Furthermore, additional site-directed mutagenesis using a different set of primer pairs (deltaGST-f, CTGGAAGTGCTGTTCCAGGGTCCGATCATCAACACCTTCTACGCA; deltaGST-r,

CTCGAGTCGACCCGGGAATTCGGGGATCCCATGAATACTGTTTCCTGTG) was performed. In the resulting recombinant plasmid, the DNA sequence encoding GST was replaced with the DNA sequence of a multi-cloning site followed by the cleavage site for PreScission protease (GE Healthcare). The nucleotide sequence (Fig. 1) of the constructed plasmid, p4AaCter-C, was confirmed using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The DNA fragment encoding *cry46Ab-S1* (Hayakawa et al. 2017) without the stop codon was amplified by PCR using a set of specific primer pairs (Cry46Ab-f, GCGGATCCATGTATTACACTACCCAGGTG; Cry46Ab-r, GCCTCGAGCAGGCCAATGGTTTGCTGAAT) and was inserted between the *Bam*HI and *Xho*I sites of p4AaCter-C to generate pCry46Ab-4AaCter. Recombinant Cry46Ab without the 4AaCter-tag was produced using the expression vector pCry46Ab. In the expression vector pCry46Ab, the DNA fragment encoding *cry46Ab-S1* was inserted in-frame between the *Bam*HI and *Xho*I sites of pGEX-ΔGST (Hayakawa et al. 2010).

Expression of recombinant Cry46Ab

Recombinant Cry46Abs were expressed in *E. coli* BL21. Bacteria were cultured at 37°C in TB medium containing ampicillin (100 µg/ml) until the OD_{600}

reached 0.6-0.7. Recombinant Cry46Ab expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37°C for 4 h, unless otherwise stated. Formation of inclusion bodies containing recombinant Cry46Ab was observed under a light microscope (Axio Observer A1; Carl Zeiss, Göttingen, Germany). The pellet of *E. coli* cells harvested by centrifugation was washed twice with phosphate-buffered saline (PBS) and disrupted using an Ultrasonic disrupter (UD-211; TOMY SEIKO CO. LTD., Tokyo, Japan). After disruption by sonication, soluble and insoluble *E. coli* proteins were separated by centrifugation (15,000 *g*, 5 min, 4°C), and were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a 10 or 15% gel as described previously (Laemmli 1970). Proteins were visualized with coomassie brilliant blue (CBB). Protein concentration was estimated using a protein assay kit (Bio-Rad Laboratories, Inc. Hercules, CA) with bovine serum albumin (BSA) as the standard, or by densitometric scanning with a cooled CCD camera system (Ez-Capture; ATTO, Tokyo, Japan) and image analysis using CS analyzer software (ver. 3.0; ATTO).

Detection of recombinant Cry46Ab

After disruption of *E. coli* cells by sonication, soluble and insoluble proteins were separated by centrifugation (15,000 *g*, 5 min, 4ºC). Proteins were separated by

10% SDS-PAGE and then transferred onto a nitrocellulose membrane using an iBlot® 2 gel transfer device (Life Technologies, Carlsbad, CA). The membrane was blocked overnight with 4% (w/v) Block Ace (DS Pharma Biomedical Co. Ltd. Osaka, Japan) in PBS containing 0.05% (v/v) Tween 20. Recombinant Cry46Ab on the membrane was detected using anti-Cry46Ab rabbit antiserum followed by anti-rabbit IgG goat antibodies conjugated with horseradish peroxidase (American Qualex Antibodies, San Clemente, CA) and visualized using ECL Western blotting substrates (Promega Corp. Madison, WI). To generate anti-Cry46Ab rabbit antiserum, GST-Cry46Ab (Hayakawa et al. 2017) was treated with thrombin, and released GST and undigested GST-Cry46Ab were removed using glutathione-Sepharose 4B (GE Healthcare). About 0.5 mg of the purified Cry46Ab was used to immunize rabbits with 4 shots a week each. Expression level was determined by densitometric scanning with a cooled CCD camera system (Ez-Capture; ATTO).

The yield of recombinant Cry46Ab produced by *E. coli* cells was estimated by dot blot analysis. Briefly, lysate of *E. coli* cells expressing recombinant Cry46Ab was spotted onto a polyvinylidene difluoride membrane using a vacuum-based dot blot apparatus (Bio-Dot® microfiltration apparatus; Bio-Rad Laboratories, Inc.). Recombinant Cry46Ab in the *E. coli* lysate was detected and visualized as described

above.

Determination of insecticidal activity

The mosquitocidal activity of recombinant Cry46Ab was analyzed using a bioassay with third-instar larvae of *C. pipiens*. Mosquito larvae were reared from eggs supplied by the Research and Development Laboratory at Dainihon Jochugiku Co., Ltd. (Osaka, Japan), as described previously (Hayakawa et al. 2008).

After disruption of *E. coli* cells by sonication, inclusion bodies consisting of recombinant Cry46Ab were harvested by centrifugation (15,000 *g*, 5 min, 4°C). The pelleted inclusion bodies were resuspended in sterile water and fed to *C. pipiens* larvae. The concentration of recombinant Cry46Ab was estimated by densitometric scanning of the protein bands. Mosquito mortality was recorded 48 h after inoculation, and the 50% lethal dose (LC_{50}) was determined by Probit analysis (Finney 1971).

Solubility of inclusion bodies in alkaline buffer

A total of 100 µg of inclusion bodies consisting of recombinant Cry46Ab was resuspended in 500 µl of alkaline buffer (50 mM $NaHCO_3$, NaOH) over the pH range 9-12 and incubated at room temperature for 30 min. Soluble and insoluble proteins

separated by centrifugation (15,000 *g*, 5 min, 4°C) were analyzed by 15% SDS-PAGE.

Results and discussion

Production of recombinant Cry46Abs in *E. coli*

In this study, recombinant Cry46Ab and Cry46Ab fused with the 4AaCter-tag at the C-terminal end (Cry46Ab-4AaCter) were produced in *E. coli*. Analysis using 15% SDS-PAGE revealed that Cry46Ab and Cry46Ab-4AaCter were 32 and 53 kDa, respectively (Fig. 2A). The molecular masses of Cry46Ab and Cry46Ab-4AaCter were very similar to those predicted from their deduced amino acid sequences.

To assess formation of protein inclusion bodies, soluble and insoluble protein fractions were separated by centrifugation and analyzed by Western blotting using anti-Cry46Ab antiserum. Interestingly, about 61% of recombinant Cry46Ab expressed in *E. coli* was localized in the insoluble protein fraction (Fig. 2B). Although crystallization of 30 kDa–type Cry toxins such as Cry46Ab is thought to require an accessory protein from *B. thuringiensis*, Cry46Ab alone apparently forms protein inclusion bodies at lower efficiency. By contrast, Cry46Ab-4AaCter was found only in the insoluble protein fraction (Fig. 2B). This suggests that fusion with the 4AaCter-tag facilitates the formation of Cry46Ab inclusion bodies in *E. coli* cells. This was

supported by microscopic observations. The inclusion bodies that formed in cells expressing Cry46Ab-4AaCter (0.5-0.7 μm in diameter) were larger than those formed in cells expressing Cry46Ab (0.3-0.5 μm in diameter) (Fig. 2C).

Fusion with the 4AaCter-tag seemed to enhance the yield of recombinant Cry46Ab. Dot blot analysis using anti-Cry46Ab antiserum demonstrated that *E. coli* cells produced 4 times more Cry46Ab-4AaCter than Cry46Ab (Fig. 2D). The final yield of Cry46Ab and Cry46Ab-4AaCter inclusion bodies was estimated at 31 ± 3 and 118 ± 8 mg/l culture, respectively (data not shown). Our results thus demonstrate that fusion with the 4AaCter-tag enhances both the yield of Cry46Ab and the formation of Cry46Ab inclusion bodies.

Mosquitocidal activity of recombinant Cry46Abs

To assess the potential for application to mosquito control, the toxicity of Cry46Ab and Cry46Ab-4AaCter inclusion bodies to *C. pipiens* larvae was analyzed. The bioassay results demonstrated significant toxicity of Cry46Ab inclusion bodies, with an LC_{50} value (95% confidence limits) of 102.3 (98.8–105.9) nM (Fig. 3A). Contrary to our expectation, however, the toxicity of Cry46Ab-4AaCter inclusion bodies was low, with an LC_{50} value (95% confidence limits) of 193.4 (187.0–200.0) nM

(Fig. 3A), suggesting that fusion with the 4AaCter-tag decreases the toxicity of Cry46Ab. To investigate this result further, the solubility of inclusion bodies in alkaline buffer (50 mM $NaHCO_3$, NaOH) was analyzed over the pH range 9-12. Cry46Ab inclusion bodies exhibited high alkaline solubility, with 65 ± 17% of the inclusion bodies solubilized even at pH 9 (Fig. 4). The solubility at pH 10, 11, and 12 was estimated at 83 ± 12, 95 ± 1, and 99 ± 3%, respectively (Fig. 3B). However, Cry46Ab-4AaCter inclusion bodies were less alkaline soluble, with solubility values at pH 9, 10, 11, and 12 estimated at 20 ± 7, 32 ± 16, 67 ± 12, and 95 ± 6%, respectively (Fig. 3B). As toxin crystals produced by *B. thuringiensis* are generally solubilized in the alkaline midgut juice of insect larvae, differences in the degree of toxicity can sometimes be explained by differences in alkaline solubility (Aronson et al. 1991; Du et al. 1994). It was therefore reasonable to hypothesize that the low alkaline solubility of Cry46Ab-4AaCter inclusion bodies is associated with their lower toxicity. Incidentally, it was reported that inclusion bodies consisting solely of 4AaCter-tag exhibit 48, 71, 100, and 100% solubility at pH 9, 10, 11, and 12, respectively (Matsumoto et al. 2014). The alkaline solubility of Cry46Ab-4AaCter inclusion bodies was lower than that of inclusion bodies consisting solely of 4AaCter-tag. Considering that the level of Cry46Ab-4AaCter expression was 4 times higher than that of Cry46Ab (Fig. 2D),

overexpression of Cry46Ab-4AaCter could adversely affect its folding fidelity, rendering it less toxic.

Effect of production at low temperature

To avoid overexpression in *E. coli*, Cry46Ab-4AaCter expression was induced at a lower temperature (30ºC), and cells expressing Cry46Ab-4AaCter were harvested at 4 and 8 h after induction. The cells were disrupted by sonication, and then soluble and insoluble proteins were separated by centrifugation. Analysis by Western blotting using anti-Cry46Ab antiserum demonstrated that Cry46Ab-4AaCter expressed at 30ºC was present only in the insoluble protein fraction (Fig. 4A). As expected, lower temperature (30ºC) affected the expression level of Cry46Ab-4AaCter. The yield of Cry46Ab-4AaCter inclusion bodies 4 h after induction was 47 ± 14 mg/l culture, which was significantly lower than that obtained at 37ºC (118 ± 8 mg/l culture); the yield was 156 ± 12 mg/l culture 8 h after induction (data not shown).

The alkaline solubility of Cry46Ab-4AaCter inclusion bodies was improved by culture at 30ºC. The alkaline solubility of Cry46Ab-4AaCter inclusion bodies produced at 30ºC for 4 h was estimated at 44 ± 14% at pH 9, 67 ± 23% at pH 10, 99 ± 2% at pH 11, and 100% at pH 12 (Fig. 4B). At 8 h after induction, Cry46Ab-4AaCter inclusion

bodies exhibited slightly lower alkaline solubility, estimated at 40 ± 14% at pH 9, 50 ± 20% at pH 10, 97 ± 4% at pH 11, and 99 ± 2% at pH 12 (Fig. 4B). Results of the bioassay using *C. pipiens* larvae demonstrated that the toxicity of Cry46Ab-4AaCter inclusion bodies improved with culture at 30ºC, with LC_{50} values (95% confidence limits) after 4 h estimated at 85.7 (83.1–88.3) nM and 103.9 (100.7–107.2) nM after 8 h (Fig. 4C).

Stability of inclusion bodies in aqueous environments

Cry46Ab (37ºC, 4 h) and Cry46Ab-4AaCter (30ºC, 8 h) inclusion bodies were resuspended in water and left at room temperature. After 14 and 28 days, the biological activity of the inclusion bodies was evaluated by bioassay using *C. pipiens* larvae. Both inclusion body types appeared to be stable in water and exhibited toxicity comparable to (or sometimes higher than) that of inclusion bodies examined immediately after preparation. The relative activity (LC_{50} value immediately after preparation/LC_{50} value after incubation in water) of Cry46Ab (37ºC, 4 h) and Cry46Ab-4AaCter (30ºC, 8 h) inclusion bodies was 1.9 ± 0.9 and 2.5 ± 2.0, respectively, after 14 days and 1.6 ± 0.5 and 2.0 ± 1.2, respectively, after 28 days (Fig. 5). Thus, both Cry46Ab-4AaCter and Cry46Ab inclusion bodies maintain their biological activity in aqueous environments

for at least 28 days. Since inclusion bodies composed of Cry46Ab tend to aggregate in water, possibility is remained that increase size of toxin inclusion bodies may affect the dose of toxin to which *C. pipiens* larvae are exposed.

In conclusion, fusion with the 4AaCter-tag enhances both the production of Cry46Ab and the formation of Cry46Ab inclusion bodies. In addition, Cry46Ab-4AaCter inclusion bodies produced under optimized conditions exhibit mosquitocidal activity and stability in aqueous environments comparable to Cry46Ab without the 4AaCter-tag. The results of our study suggest that use of the 4AaCter-tag is a straightforward approach for preparing formulations of smaller-sized Cry toxins such as Cry46Ab in *E. coli*.

Acknowledgments

Culex pipiens eggs were kindly supplied by the Research and Development Laboratory at Dainihon Jochugiku, Osaka, Japan. This work was supported by the Japan Society for the Promotion of Science KAKENHI (Grant nos. JP24380034 and JP26660268).

References

Agaisse H, Lereclus D (1995) How *Bacillus thuringiensis* produce so much insecticidal crystal protein? J Bacteriol 177:6027–6032

Akiba T, Abe Y, Kitada S, Kusaka Y, Ito A, Ichimatsu T, Katayama H, Akao T, Higuchi K, Mizuki E, Ohba M, Kanai R, Harata K (2009) Crystal structure of the parasporin-2 *Bacillus thuringiensis* toxin that recognizes cancer cells. J Mol Biol 386:121–133. doi: 10.1016/j.jmb.2008.12.002

Aronson A I, Han ES, McGaughey W, Johnson D (1991) The solubility of inclusion proteins from *Bacillus thuringiensis* is dependent upon protoxin composition and is a factor in toxicity to insects. Appl Environ Microbiol 57:981–986

Bietlot HP, Vishnubhatla I, Carey PR, Pozsgay M, Kaplan H (1990) Characterization of the cysteine residues and disulphide linkages in the protein crystal of *Bacillus thuringiensis*. Biochem J 267:309-315

Chak KF, Ellar DJ (1987) Cloning and expression in *Escherichia coli* of an insecticidal crystal protein gene from *Bacillus thuringiensis* var. aizawai HD-133. J Gen Microbiol 133:2921–2931. doi: 10.1099/00221287-133-10-2921

Du C, Martin PAW, Nickerson KW (1994) Comparison of disulfide contents and solubility at alkaline pH of insecticidal and noninsecticidal *Bacillus thuringiensis* protein crystals. Appl Environ Microbiol 60:3847–3853

Finney DJ (1971) Probit analysis, 3rd edn. Cambridge University Press, London

Hayakawa T, Kanagawa R, Kotani Y, Kimura M, Yamagiwa M, Yamane Y, Takebe S, Sakai H (2007) Parasporin-2Ab, a newly isolated cytotoxic crystal protein from *Bacillus thuringiensis*. Curr Microbiol 55:278-283. doi: 10.1007/s00284-006-0351-8

Hayakawa T, Howlader MTH, Yamagiwa M, Sakai H (2008) Design and construction of a synthetic *Bacillus thuringiensis* Cry4Aa gene-Hyperexpression in *Escherichia coli*. Appl Microbiol Biotechnol 80:1033–1037. doi: 10.1007/s00253-008-1560-9

Hayakawa T, Sato S, Iwamoto S, Sudo S, Sakamoto Y, Yamashita T, Uchida M, Matsushima K, Kashino Y, Sakai H (2010) Novel strategy for protein production using a peptide tag derived from *Bacillus thuringiensis* Cry4Aa. FEBS J 277:2883–2891. doi: 10.1111/j.1742-4658.2010.07704.x

Hayakawa T, Sakakibara A, Ueda S, Azuma Y, Ide T, Takebe S (2017) Cry46Ab from *Bacillus thuringiensis* TK-E6 is a new mosquitocidal toxin with aerolysin-architecture. Insect Biochem Mol Biol: 87:100-106. doi: 10.1016/j.ibmb.2017.06.015

Hayashi M, Iwamoto S, Sato S, Sudo S, Takagi M, Sakai H, Hayakawa T (2013) Efficient production of recombinant cystatin C using a peptide-tag, 4AaCter, that facilitates formation of insoluble protein inclusion bodies in *Escherichia coli*. Protein Expr Purif 88:230-234. doi: 10.1016/j.pep.2013.01.011

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lecadet MM (1967) Action comparée de l'urée et du thioglycolate sur la toxine figurée de *Bacillus thuringiensis*. C R Acad Sci Hebd Seances Acad Sci D 264:2847– 2850

Matsumoto R, Shimizu Y, Howlader MT, Namba M, Iwamoto A, Sakai H, Hayakawa T. (2014) Potency of insect-specific scorpion toxins on mosquito control using *Bacillus thuringiensis* Cry4Aa. J Biosci Bioeng 117:680-683. doi: 10.1016/j.jbiosc.2013.12.004

Nickerson KW (1980) Structure and function of the *Bacillus thuringiensis* protein crystal. Biotechnol Bioeng 22:1305–1333. doi: 10.1002/bit.260220704

Oeda K, Oshie K, Shimizu M, Nakamura K, Yamamoto H, Nakayama I, Ohkawa H (1987) Nucleotide sequence of the insecticidal protein gene of *Bacillus thuringiensis* strain aizawai IPL-7 and its high-level expression in *Escherichia coli*. Gene 53:113–119

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

Yu J, Xie R, Tan L, Xu W, Zeng S, Chen J, Tang M, Pang Y (2002) Expression of the full-length and 3'-spliced cry1Ab gene in the 135-kDa crystal protein minus derivative of *Bacillus thuringiensis* subsp. *kyushuensis*. Curr Microbiol 45:133–138. doi: 10.1007/s00284-001-0092-7

Figure legends

Fig. 1 The expression vector, p4AaCter-C. (A) Schematic structure of the expression vector, p4AaCter-C. The promoter P_{tac} is IPTG inducible. (B) Nucleotide sequence around the multi-cloning site of p4AaCter-C. The expression vector, pGST-4AaCter 696-851, which produces GST fused with the 4AaCter-tag at the C-terminal end, was modified by site-directed mutagenesis. The DNA sequence encoding GST was replaced with the DNA sequence of the multi-cloning site followed by the cleavage site for

PreScission protease. Amino acid sequences of the cleavage site and 4AaCter-tag (shaded) are shown above the nucleotide sequence.

Fig. 2 Production of recombinant Cry46Abs. (A) *Escherichia coli* cells were disrupted by sonication and separated into soluble (S) and insoluble (P) protein fractions by centrifugation. Proteins were analyzed by 15% SDS-PAGE and visualized with CBB stain. (B) Western blotting analysis using anti-Cry46Ab antiserum. Ratios of the recombinant Cry46Abs found in the insoluble protein fraction are indicated. (C) Micrographs of *E. coli* cells expressing Cry46Ab and Cry46Ab-4AaCter. Spherical inclusion bodies are indicated by arrows. Bar: 1 μm. (D) Dot blot analysis. Yield of recombinant Cry46Ab in *E. coli* cells was estimated by dot blot analysis using anti-Cry46Ab antiserum.

Fig. 3 Mosquitocidal activity and alkaline solubility of inclusion bodies composed of Cry46Ab or Cry46Ab-4AaCter. (A) The bioassay experiments were repeated independently more than four times, and the average and standard deviation of the mortality observed at 48 h after administration are shown. (B) Inclusion bodies composed of Cry46Ab or Cry46Ab-4AaCter were incubated in alkaline buffer over the

pH range 9-12. Soluble and insoluble proteins were separated by centrifugation. Soluble proteins were analyzed by 15% SDS-PAGE and visualized with CBB stain.

Fig. 4 Effect of culture at low temperature. (A) *Escherichia coli* cells expressing Cry46Ab-4AaCter were disrupted by sonication and separated into soluble (S) and insoluble (P) protein fractions by centrifugation. Proteins were separated by 15% SDS-PAGE and analyzed by Western blotting using anti-Cry46Ab antiserum. Culture conditions are indicated at left. (B) Alkaline solubility of inclusion bodies. Inclusion bodies of Cry46Ab-4AaCter prepared using different condition were incubated in alkaline buffer over the pH range 9-12. The percentage of soluble protein was estimated by densitometric scanning using image analysis software. The experiments were repeated independently more than three times, and the average and standard deviation are shown. (C) Mosquitocidal activity of Cry46Ab-4AaCter inclusion bodies prepared at 30ºC. The experiment was repeated independently more than three times, and the average and standard deviation of the mortality observed at 48 h after administration are shown.

Fig. 5 Stability of inclusion bodies in aqueous environments. Cry46Ab and

Cry46Ab-4AaCter inclusion bodies prepared using the indicated conditions were resuspended in water. After 14 and 28 days of incubation at room temperature, the mosquitocidal activity of the inclusion bodies was analyzed by bioassay using *C. pipiens* larvae. The experiment was repeated independently more than three times, and the average and standard deviation of the relative activity (LC_{50} value immediately after preparation/LC_{50} value after incubation in water) are shown.