氏　　名	Mohammad Tofazzal Hossain Howlader
授与した学位	博　士
専攻分野の名称	学　術
学位授与番号	博甲第４１４４号
学位授与の日付	平成２２年　３月２５日
学位授与の要件	自然科学研究科　機能分子化学専攻 （学位規則第５条第１項該当）
学位論文の題目	Characterization of functional motifs in Cry4Aa mosquitocidal δ-endotoxin of *Bacillus thuringiensis* （*Bacillus thuringiensis* が産生する殺虫蛋白質 Cry4Aa の機能構造解析）
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学位論文内容の要旨

Cry4Aa produced by *Bacillus thuringiensis* subsp. *israelensis* (Bti) is a mosquitocidal toxin that exhibits a specific high toxicity to *Anopheles*, *Aedes,* and *Culex* larvae. This work describes the hyper-expression and the functional roles of three major loops in domain II of Cry4Aa.

The expression of wild-type Cry4Aa in *E. coli* is relatively low, which is a major disadvantage in its development as a bioinsecticide. To establish an effective production system, a 1914-bp modified synthetic gene (*cry4Aa-S1*) encoding the Cry4Aa protein was designed in accordance with the G+C content and codon preference of *E. coli* genes without altering the encoded amino acid sequence. The *cry4Aa-S1* gene allowed a significant improvement in the expression level, over 5-fold, compared to that of the original *cry4Aa* gene. The *cry4Aa-S1* gene product showed the same level of insecticidal activity against *Culex pipiens* larvae as that from *cry4Aa*. This suggested that unfavorable codon usage was one of the reasons for poor expression of *cry4Aa* in *E. coli*, and therefore, changing the *cry4Aa* codons to accord with the codon usage in *E. coli* led to efficient production of Cry4Aa. The efficient production in *E. coli* can be a powerful measure to prepare a sufficient amount of Cry4Aa protein for both analytical and applied researches.

The molecular mode of action of mosquitocidal Cry4Aa is poorly understood in comparison with the lepidopteran specific protein. In general, domain II of the Cry toxin is believed to be important for insecticidal specificity. To understand the toxin-receptor interactions and to identify the epitopes in the Cry4Aa molecule responsible for those interactions, three loops in the domain II of Cry4Aa were targeted and the mosquitocidal activities were analyzed both by loop-exchange and site directed mutagenesis methods.

To analyze the biological functions of loops 1, 2, and 3 of Cry4Aa, mutants were constructed in which one of the loops was replaced with either of the other two loops. A bioassay using *Culex pipiens* larvae revealed that the mosquitocidal activity was virtually lost upon replacement of the loop 2. The mutants in which the loops 1 and/or 3 were replaced showed decreased but some significant insecticidal activities. This suggested that the loop 2, but not the loops 1 and 3, was essential for the mosquitocidal activity of Cry4Aa. Proteolytic digestion revealed the involvement of loops in the stability of the Cry4Aa structure.

Identification of the loop 2 in domain II as an essential element for mosquitocidal toxicity of Cry4Aa suggest that this loop may be a functional motif for the interaction with its potential receptor(s). Therefore, to characterize a potential receptor-binding site more precisely, I have constructed a series of Cry4Aa mutants in which amino acid residues in the loops 1, 2 and 3 were replaced with alanine. A bioassay using *Culex pipiens* larvae revealed that the replacement of some residues in loop 2 sequence depressed the mosquitocidal activity of Cry4Aa, but the effect was limited. This was inconsistent with the previous results suggesting that the replacement of the Cry4Aa loop 2 causes a significant loss of the mosquitocidal activity. Therefore, I constructed additional mutants in which multiple (5 to 6) residues in the loop 2 were replaced with alanine. Although the replacement of multiple residues also caused some decrease in the mosquitocidal activity, the mutants still showed relatively high activity. On the other hand, the alanine mutants of loop 1 and 3 did not show so much decrease in the toxicity as compared with the wild-type. Since the insecticidal spectrum of Cry4Aa is specific, Cry4Aa must have a specific receptor on the surface of the target tissue and loss of binding to the receptor should cause a complete loss of the mosquitocidal activity. The present results suggested that, unlike the well-characterized Cry1, the receptor-binding site of Cry4Aa is different from the loops 1, 2, and 3 or consists of multiple binding sites that work cooperatively for receptor binding.

論文審査結果の要旨

Howlader 氏は、好気性土壌細菌 *Bacillus thuringiensis* 亜種 *israelensis*(Bti)が産生する蚊の幼虫(ボウフラ)特異的殺虫蛋白質 Cry4Aa の殺虫活性決定に関与する機能構造について詳細な解析を行った。Cry 殺虫蛋白質作用の分子機構については、鱗翅目特異的 Cry1 型殺虫蛋白質の研究がよく進んでいるのに対して Cry4Aa の解明はあまり進んでいない。Cry1 殺虫蛋白質ファミリーの研究成果に基づいて一般的には Cry トキシンのドメイン II、特にループが殺虫特異性に重要であると考えられている。

Cry4Aa の殺虫活性を決定する機能構造を解明するため、ドメイン II のループ１、ループ２、及びループ３を対象としたループ入換え法により作製した変異体の殺虫活性を調査した。その結果、ループ２がその本来の位置に存在することが殺虫活性に必要であることが明らかとなった。また、ループ１とループ３は殺虫活性の決定には必要ではないことも明らかとなった。

さらに Cry4Aa ドメイン II のループ１、２、及び３の機能をより詳細に解析するため、各ループのアラニン置換変異体を作製して殺虫機能の解析を行った。その結果３つのループのアミノ酸をアラニンに置換しても殺虫活性に重大な影響は認められなかった。なお、これらの Cry4Aa 変異体については、ボウフラから調製した BBMV(刷子縁膜小胞)への結合能は野生型と比較して有意な違いは認められなかった。

以上の結果から Howlader 氏はドメイン II のループ２は殺虫活性の決定に必須であるが、それはアミノ酸配列よりはループの長さが重要ではないかと考えている。また３つのループのアミノ酸配列を改変しても殺虫活性に重大な影響が見られなかったことから、これらが Cry4Aa を修飾して人工的に高機能化するための標的部位として利用できると提案している。

したがって Howlader 氏は Cry4Aa の作用機構解明を著しく進展させたと同時に、より高性能な生物殺虫剤開発へ向けた新しい可能性を切り拓いた。よって博士の学位を授与するにふさわしいと判定する。