TOXICITY OF *BACILLUS THURINGIENSIS* SUBSP. *KURSTAKI* STRAIN *HD-1* AGAINST INSECT LARVAE

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ชื่อวิทยานิพนธ์ ความเป็นพิษของเชื้อแบคทีเรีย Bacillus thuringiensis สายพันธุ์ kurstaki HD-1 ต่อหนอนศัตรูพืช

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บทคัดย่อ

เชื้อจุลินทรีย์ B. thuringiensis subsp. kurstaki HD-1 มีผลึกโปรตีนอยู่ 2 ชนิด คือ ชนิดที่มีรูปทรงสี่เหลี่ยมขนมเปียกปูน (bipyramidal) และชนิดที่มีรูปทรงสี่เหลี่ยมจตุรัส (cuboidal) ซึ่งมีน้ำหนักโมเลกุลสัมพันธ์ (M_r) เท่ากับ 135 กิโลดาลตัน (kDa) และ 65 กิโลดาลตัน ตามลำดับ ชนิดแรกมีคุณสมบัติในการฆ่าหนอนศัตรูพืชและชนิดหลังมีคุณสมบัติในการฆ่าลูกน้ำยุง เพื่อที่จะศึกษาประสิทธิภาพในการฆ่าตัวอ่อนของแมลงของผลึกโปรตีนทั้งสองชนิด จึงจำเป็นต้องทำให้ผลึกนี้สะอาดปราศจากสปอร์ (spore) และสิ่งปนเปื้อนอื่นๆ ด้วยการเขย่าเชื้อจุลินทรีย์ที่ได้ทำการล้างแล้วหลังจากเลี้ยงในอาหารเหลวเป็นเวลา 2 วัน อย่างแรงในสารละลาย 0.2 M sodium chloride (NaCl) จะได้ผลึกโปรตีนที่มีสปอร์ปนเปื้อนอยู่ 1.3% เมื่อทำการทดสอบประสิทธิภาพในการฆ่าของผลึกโปรตีนนี้ ต่อหนอนกระทู้หอม (Spodoptera exigua) และลูกน้ำยุงลาย (Aedes aegypti) ควบคู่ไปกับเชื้อจุลินทรีย์ที่ได้จากการล้างด้วยน้ำกลั่น หรือเชื้อจุลินทรีย์ที่ได้จากการล้างด้วย 1 M NaCl ผลปรากฏว่า เชื้อจุลินทรีย์ที่ได้จากการล้างด้วย 1 M NaCl จะมีประสิทธิภาพในการฆ่าหนอนกระทู้หอมดีที่สุด เมื่อเปรียบเทียบกับอีกสองชนิด โดยความเข้มข้นที่ใช้ในการฆ่าหนอนให้ตาย 50% (LC_{50}) ตั้งแต่ 62 ผลึก/ตารางมิลลิเมตรของพื้นที่อาหารในหนอนกระทู้หอมวัยที่หนึ่ง และเท่ากับ 1291 ผลึก/ตารางมิลลิเมตรของพื้นที่อาหารในหนอนวัยที่ห้า แต่ผลึกโปรตีนนี้มีประสิทธิภาพในการฆ่าต่ำที่สุด โดยมีค่า LC_{50} เท่ากับ 207 ผลึก/ตารางมิลลิเมตรของพื้นที่

อาหารในการฆ่าหนอนวัยที่หนึ่ง และ 32,734 ผลึก/ตารางมิลลิเมตรของพื้นที่อาหารในการฆ่าหนอนวัยที่ห้า เมื่อทำการทดสอบในลูกน้ำยุงลาย ผลปรากฏว่า ผลึกโปรตีนมีประสิทธิภาพในการฆ่าสูงที่สุด โดยมีค่า LC_{50} เท่ากับ 3,682 ผลึก/มิลลิลิตรของน้ำในลูกน้ำวัยที่ 1และ 2 และมีค่า LC_{50} เท่ากับ 23,550 ผลึก/มิลลิลิตรของน้ำ ในลูกน้ำวัยที่ 3 และ 4 เมื่อทำการละลายผลึกโปรตีนนี้ใน 2% 2-mercaptoethanol ที่มีpH 10 โปรตีนที่มี M_r = 134 kDa จะถูกละลายออกมาก่อน หลังจากนั้น โปรตีนที่มี M_r = 65 kDa จะถูกละลายออกมาด้วย 0.1 M NaOH เมื่อทำการทดสอบความเป็นพิษของโปรตีนทั้งสองชนิดนี้ โดยใช้หนอนกระทู้หอมวัยที่สามและลูกน้ำยุงลายวัยที่สอง พบว่าโปรตีนชนิดแรกมีความสามารถในการฆ่าหนอนกระทู้หอมได้ดีกว่าผลึกโปรตีน ประมาณ 2 เท่า และโปรตีนชนิดที่สองมีความสามารถในการฆ่าลูกน้ำยุงลายได้ดีกว่าผลึกโปรตีน 10 เท่า อย่างไรก็ตาม โปรตีนชนิดหลังนี้ไม่สามารถฆ่าหนอนกระทู้หอมได้ วิทยานิพนธ์ฉบับนี้แสดงให้เห็นว่า หนึ่ง การปราศจากการปนเปื้อนของสปอร์ในผลึกโปรตีนของเชื้อจุลินทรีย์ชนิดนี้ อาจลดประสิทธิภาพในการฆ่าหนอนของผลึกโปรตีนนี้ และสอง การล้างเอนไซม์ protease ออกจากผลึกโปรตีนโดยใช้ 1 M NaCl จะเพิ่มประสิทธิภาพในการฆ่าหนอนกระทู้หอมของผลึกโปรตีนนี้ และสาม ผลึกโปรตีนรูปทรงสี่เหลี่ยมจตุรัสไม่สามารถฆ่าหนอนกระทู้หอมได้

Thesis Title	Toxicity of *Bacillus thuringiensis* Subsp. *kurstaki* Strain HD-1 against Insect Larvae.
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ABSTRACT

B. thuringiensis subsp. *kurstaki* HD-1 contains two types of proteinaceous crystals, that is a bipyramidal crystal (M_r = 134 kDa), which is toxic to lepidopteran larvae, and a cuboidal crystal (M_r = 65 kDa), which is toxic to dipteran larvae. In order to study the toxicities of these toxin, the purified crytals must be obtained. Vigorous shaking in 0.2 M NaCl was used to produce purified crystals containing impurities of 1.3% spores from cultures of *B. thuringiensis* subsp. *kurstaki* HD-1. Bioassays of these crystals were done against *Spodoptera exigua* and *Aedes aegypti* larvae in parallel with the sample derived from cultures washing in distilled water and/or the sample derived from cultures washing in 1 M. NaCl. When *S. exigua* larvae were used, the sample derived from cultures washing in 1 M NaCl gave highest toxicity than others. This sample gave LC_{50} = 62 crystals/mm^2 diet surface in

first instar larvae to 1291 crystals/mm^2 diet surface in fifth instar larvae. While the purified crystals gave the least toxicity, it gave LC_{50} = 207 crystals/mm^2 diet surface against first instar larvae to 32,734 crystals/mm^2 diet surface in fifth instar larvae. When mosquito larvae were used to test all of these specimens the purified crystals was the most effective one when it was compared with the crude preparations. The LC_{50} was 3,682 crystals/ml against early instar larvae and 23,550 crystals/ml against late instar larvae. When the intact crystals were solubilized in 2% 2-mercaptoethanol at pH 10, the 134-kDa protein was recovered. After this protein was removed, the residue was solubilized in 0.1 M NaOH and the 65-kDa protein was obtained. The toxicity of these proteins were tested against third instar larvae of *S. exigua* and second instar larvae of *Ae. aegypti*. The 134-kDa protein could kill *S. exigua* larvae with 2 folds higher activity than that of the intact crystal. The 65-kDa protein could kill *Ae. aegypti* larvae with 10 folds higher activity than those of the intact crystals. However, this protein could not kill *S. exigua* larvae. This study indicated that : first, the absence of spores may impair the control of *S. exigua* larvae feeding on spore-free products; second, the removal of proteases from the surface of the crystals by using 1 M NaCl could improve the toxicity of the proteinaceous crystal of this strain; and third, the 65-kDa protein alone could not kill *S. exigua* larvae.

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LIST OF ABBREVIATIONS

°C	Degree celcious
ca.	Circa (Latin), approximately
e.g.	Excampli gratia (Latin), for example
et al.	Et alu (Latin), and others
etc.	Et cetera (Latin), other things
g.	gram
hr	hour
i.e.	Id est (Latin), that is
KDa	Kilo-dalton = 10^3 dalton
LC_{50}	50% Lethal concentration end point
M	Molar
2-ME	2-Mercaptoethanol
MW	Molecular weight
min	minute
mM	Millimolar
mg	Milligram
ml	Millilitre
ug	Microgram
ul	Microlitre
N	Normal
NBS	Nutrient broth supplemented with mineral salts
NAS	Nutrient agar supplemented with mineral salts
O.D.	Optical density

LIST OF ABBREVIATIONS (Continued)

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
subsp.	Subspecies
Tris	Tris (hydroxymethyl) aminomethane
TEMED	N,N,N',N'-tetramethylethylenediamine

CHAPTER I

INTRODUCTION

Bacteria are commonly associated with insects, many of which are either pathogenic or produce toxins that kill their hosts. Of the numerous species of bacteria that are reported to kill insects, *Bacillus thuringiensis* is one of them. Following the discovery of HD-1 strain (subsp. *kurstaki*), several companies in U.S.A. and Europe started producing *B. thuringiensis* for commercial scale. *B. thuringiensis* - based products account for nearly all commercial scales of bacterial insecticides today. Several serotypes of *B. thuringiensis* are available in commercial products for the control of a wide variety of insect pests of agricultural crops and forest trees, and of disease vectors, such as mosquitoes and black flies. Worldwide sales, however, are quite limited. Several factors have been responsible for the limited used of *B. thuringiensis* including : (1) high production cost, relative to cheaper and effective synthetic chemicals; (2) narrow host range; and (3) lack of environmental persistance.

B. thuringiensis sales are expected to increase, however, due to several factors including : safety concerns over the use of environmentally disruptive chemical insecticides, increasing incidence of pest resistance to nearly all classes of synthetic chemicals, and the recent push by industry to develop new *B. thuringiensis* products with increased potency and broader host spectra at lower production costs.

The HD-1 strain of *B. thuringiensis* subsp. *kurstaki* is

used in several commercial insecticides for the control of leaf-eating larval Lepidoptera in both agriculture and forestry. Most of the insecticidal activity of the *kurstaki* subspecies is attributed to one or more proteins that make up the characteristic bipyramidal crystal. The production of these proteins is coded by three types of genes classified as *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* (34) also known as the 4.5-, 5.3-, and 6.6-kb class genes (45). Little is known about the role of the corresponding gene products in determining the specificity of the crystal proteins. Considerable differences in specificity of individual *cryIA* proteins have been reported for various Lepidoptera is becoming increasingly relevant for a variety of reasons. Thus there is a need for strain improvement to prevent or delay the development of pest resistance to *B. thuringiensis*, increase host range and persist better in the enviroment. Specificity data can be coupled with existing knowledge of toxin structure to enhance our understanding of the molecular basis of toxin specificity and mode of action. This, in turn, may be useful for tailoring toxin specificity through genetic engineering.

The objective of this study was aimed at determining the specificity of P1 and P2 which were protein products of *cryIA* and *cryII* genes of *B. thuringiensis* subsp. *kurstaki* strain HD-1.

CHAPTER II

BACKGROUND

The molecular basis of δ-endotoxin action and attempt in genetic manipulations with *B. thuringiensis*, have been investigated by many scientists. However, progresses are very slow. Recent advances in these areas are reviewed as follow.

1 Biosynthesis of the parasporal crystal

In general, the parasporal crystal has been produced in cells of *B. thuringiensis* during sporulation cycle. Based on observation revealed by the use of electron microscopy, the development of spore in *B. thuringiensis* can be roughly divided into 7 main stages as shown in figure 2.1. This observation has been described by Bechtel and Bulla since 1976 (9). According to their observation, vegetative growth occured within the first 6 hr after which the cells entered stationary phase. Stage I which occurs at 7th hr is characterized by the formation of axial filament. Stage II , occuring between 7th to 8th hr, begins with the formation of forespore septum. Stage III, occuring between 8th and 9th hr, characterized by the engulfment with mesosome involvement. Stage IV to VI, which begin from 9th to 12th hr, the formation of exosporium, primordial cell wall, cortex and spore coats are accompanied by transformation of the spore nucleoid, respectively. And finally, stage VII, spore maturation and sporangial lysis occur after 12th hr. It has been shown that the crystal components are synthesized before an inclusion can be seen under the electron

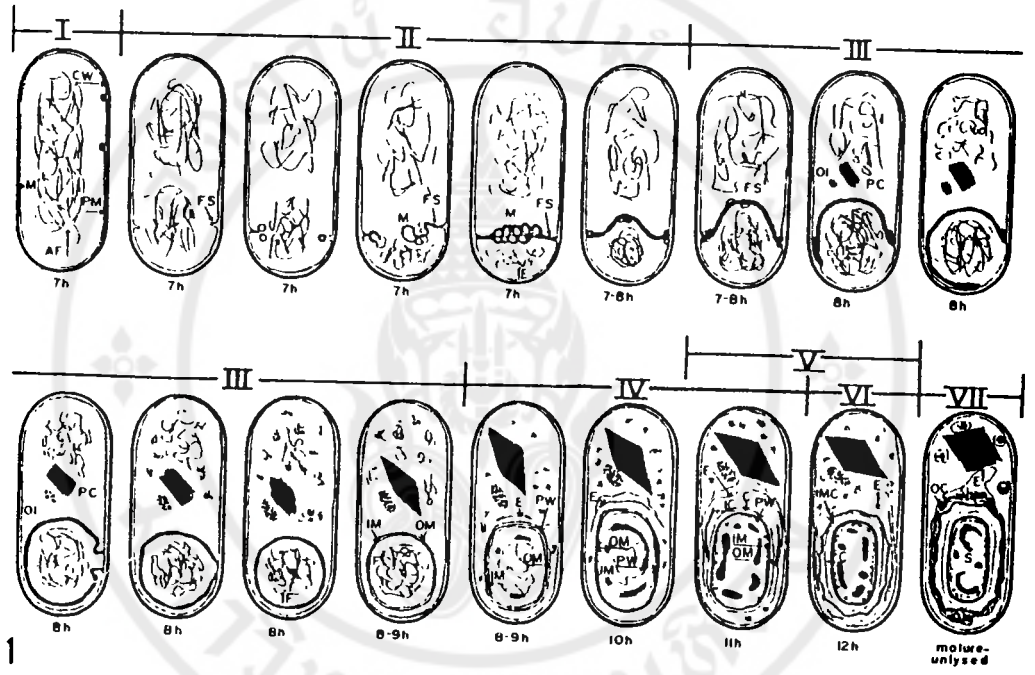

Figure 2.1 Schematic diagram of different stages of sporulation in *B. thuringiensis*. This diagram was based on the observation revealed by the use of electron microscopy reported by Bechtel and Bulla (1976)
Abbreviations : M, mesosome; CW, cell wall; PM, plasma membrane; AF, axial filament; FS, forespore septum; IF, incipient forespore; OI, ovoid inclusion; PC, parasporal crystal; F, forespore; IM, inner membrane; OM outer membrane; PW, primordial cell wall; E, exosporium; LC, lamellar spore coat; OC, outer spore coat; C, cortex; IMC, incorporated mother cell cytoplasm; S, mature spore in an unlysed sporangium.

microscope (47). The observation suggested that the inclusion appears in close proximity to the forespore after the spore septum is completed at the beginning of stage III. The crystal is almost full-sized by the time the exosporium appears (stage IV, 9 hr). It has been suggested that the completion of endotoxin synthesis precedes spore maturation by about 2 hr (69). The cuboidal body develops simultaneously to crystal appearance. Its synthesis is completed before the synthesis of bipyramidal crystal has completed. Some strains of *B. thuringiensis* produce more than one crystal within a cell, however, only one cuboidal body per sporulated cell has been observed in HD-1 strain (37).

The position where crystal formation is initiated has been studied. It has been suggested that the primordial crystal appears near the developing forespore membrane which functions in the initial seeding of the crystal. Then the crystal increases in size and moves to the opposite end of the cell (98). On the other hand, it has been shown that the crystalline inclusion of *B. thuringiensis* subsp. *alesti* which is closely associated with exosporium and crystal subunits are synthesized on this membrane (79, 80). Another suggestion has also been proposed that crystal protein is immunologically and biochemically similar to a protein removed from a spore (19). Later, it has been suggested that crystal develop without any clear association with mesosome, forespore septum, forespore membrane or exosporium (9). The evidence which support the crystal formation is depended upon the formation of forespore and has been demonstrated by Nishimura and Nishiitsutsuji-Uwo (62).

In *B. thuringiensis* subsp. *thuringiensis*, the process of

sporulation and crystal formation is shown to be nutritionally-dependent (67). It has been demonstrated that high concentration of L-cystine (0.25%) inhibits sporulation and crystal formation while moderate concentration (0.15%) only inhibits formation of the crystal but allow heat labile spore to be formed. At low concentration of L-cystine (0.05%), there is no effect on sporulation and crystal formation. Lipid synthesis is also inhibited by high concentration of L-cystine. Mineral salts such as potassium also play an essential role in δ-endotoxin production. It has been shown that *B. thuringiensis* subsp. *kurstaki* and *aizawai* strains produce high yields of δ-endotoxin on the medium containing 330 ug of potassium per ml but not on the medium containing only 11 ug of potassium per ml (87). The non-toxicity-refractile granules (poly-β-hydroxybutyric acid granules) are formed in the latter. The sporulation is also suppressed on the potassium-deficient medium. Other factors involve in crystal formation are optimum temperature ranging from 28 to 32 °C, and pH values between 5.6 to 8.5 for endotoxin production, respectively (69).

2 Genetic of *B. thuringiensis* subsp. *kurstaki*.

2.1 The plasmids of *B.thuringiensis* (Bt).

It has been known since 1981 that crystal formation in various strains of *B. thuringiensis* are involved plasmid(s) (28, 50, 73). The numbers of plasmids contained in these microorganisms varies from 2 to 17 in each strain with sizes ranging from 1.5 to 180 megadalton (MDa) (16, 50). By using plasmid curing experiments and gene-specific probe, the plasmid(s) contained crystal protein

(*cry*) gene(s) have been identified (15, 16, 44, 89). It has been demonstrated that large plasmid(s), ranging in sizes from 33 to ca. 150 MDa, are involved in crystal protein production. For example, crystal protein production is associated with 44- and ca. 150-MDa plasmids in B. thuringiensis subsp. kurstaki strain HD-1; a 150-MDa plasmid in strain HD-1 Dipel; a 50-MDa plasmid in strain HD-73; 44-, 60- and 150-MDa plasmids in strain HD-263. Each plasmid contains single or multiple copies of crystal protein gene as shown in table 2.1.

2.2 The crytal protein genes and their products.

The crystal protein (*cry*) genes have been classified into five major classes by Hofte and Whiteley in 1989 (34). They are listed as follows:

cryI genes are the Lepidoptera-specific crystal protein genes. They are the most common gene types occured in several strains of *B. thuringiensis*.

cryII genes are Lepidoptera- and Diptera-specific crystal protein genes. These genes are contained in *B. thuringiensis* subsp. *kurstaki* strain HD-1 and 14 other strains, subsp. *thuringiensis* Berliner, subsp. *tolworthi* and *kenyae*.

cryIII genes are Coleoptera-specific crystal protein genes. These genes are contained in *B. thuringiensis* subsp. *tenebrionis* and subsp. *san diego*.

cryIV and *cytA* genes are Diptera-specific crystal protein genes present in strains of *B. thuringiensis* subsp. *israelensis*.

Table 2.1 Correlations between specific plasmids and crystal protein production in various strains of *B. thuringiensis* subsp. *kurstaki*.

strain	no. of plasmids	HindIII fragments (kb)		size of plasmids bearing cry genes (Mda)
HD-1	12	4.5	6.6	150 (P1)
		5.3		44 (P1)
		5.0	9.0	150 (P2)
HD-1-Dipel		4.5	6.6	150 (P1, P2)
HD-263	11		6.6	110 (P1, P2)
				60 (P1)
		4.5		44 (P1)
HD-73	6		6.6	50 (P1)

From Carlton and Gonzalez, 1985 (16);
Whiteley et al., 1984 (89); and
Widner and Whiteley, 1989 (90).

The *cry* genes encoded P1 proteins in B. thuringiensis subsp. *kurstaki* are first designated as 4.5-, 5.3-, and 6.6-kilobase (kb) genes on the basis of the size of the Hind III restriction fragment containing 5' end of the genes (45), and they are reclassified later to *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* genes, respectively (34). In an attempt to study expression level of each *cryIA* gene, plasmid curing experiments have been done in *B. thuringiensis* subsp. *kurstaki* strain HD-1 and HD-263 by Yamamoto et al. (95). In strain HD-1 *cryIA(c)* gene is not expressed, the P1 proteins are encoded simultaneously from *cryIA(a)* and *cryIA(b)* genes at the same level. However, in strain HD-263, 70% of the total P1 proteins are encoded by *cryIA(c)* gene and the other 30% are the product of *cryIA(a)* gene. Recently, Masson and co-workers (53) have found that crystals of HD-1 are composed of 13.6% CryIA(a) protein, 54.2% CryIA(b) protein, and 32.2% CryIA(c) protein which are determined by cyanogen bromide cleavage of protoxin.

Many *cryIA* gene in *B. thuringiensis* subsp. *kurstaki* have been studied by isolating the *cry* gene from its plasmid and engineering into a recombinant *Escherichia coli* plasmid for analysis. Many complete nucleotide sequences of the coding regions have been determined (1, 26, 74, 75, 84, 88). It has been shown that the *cryIA(a)* gene contains an open reading frame of 1176 codons, encoding polypeptides of 133.5 kDa. The *cryIA(b)* gene contains an open reading frame of 1155 codons which account for the molecular mass of ca. 130.6 kDa. The *cryIA(c)* gene contains an open reading frame of 1179 codons specify a protein of 133.3 kDa. Therefore the polypeptide compositions of *cryIA(a)* and *cryIA(c)* genes cannot be

cryII gene (*cryIIA* and *cryIIB* genes) have been successfully cloned from *B. thuringiensis* subsp. *kurstaki* strain HD-1 and expressed in *E. coli* (90). DNA sequence of the cloned genes have also been determined. Both genes encoded polypeptides of 633 amino acids having a molecular mass of ca. 71 kDa, slightly larger than that determined for the P2 protein produced in *B. thuringiensis* subsp. *kurstaki* strain HD-1 (97). It has been indicated that *cryIIA* is the distal gene of an operon which is comprised of three open reading frames (designated *orf1, orf2,* and *cryIIA*) by using DNA sequence analysis. The proteins encoded by *cryIIA* and *orf2* are components of small cuboidal crystal found in several subspecies and strains of *B. thuringiensis*. It is not known whether the *orf1* or *cryIIB* gene products are present in cuboidal crystals. The protein encoded by *orf2* has an electrophoretic mobility corresponding to a molecular mass of ca. 50 kDa, although the gene has a coding capacity for a polypeptide of ca. 29 kDa. It has been shown that *E. coli* extracts prepared from the cells expressing only *orf1* and *orf2* are not toxic to either Lepidopteran (*Manduca sexta*) larvae or Dipteran (*Aedes aegypti*) larvae (90).

The proteins encoded from *cryIIA* and *cryIIB* gene display 87% identity in amino acid sequences, however, they exibits different toxin specifities. The *cryIIA* gene product is toxic to both dipteran (*A. aegypti*) and lepidopteran (*M. sexta*) larvae, whereas the *cryIIB* gene product is toxic to the latter only. Recently, hybrids of *cryIIA* and *cryIIB* genes have been generated in *E. coli* and assayed for their resultant gene product toxicity (91). The result demonstrates that a short segment of *CryIIA* corresponding to residue 307 through 382 is able to altering host range speci-

distinguished in polyacrylamide gels. The amino acid sequences deduced from the nucleotide sequences of these three genes had been compared (figure 2.2) (26, 88). These comparisons have demonstrated that the amino acid sequences are nearly identical through residue 280, then diverge until residue 744. A deletion of 26 amino acids is found after amino acid 795 of *cryIA(b)* gene product. C-terminal ends of *cryIA(a)* and *cryIA(c)* are almost identical but in *cryIA(b)* a four amino acid insert is found. The *cryIA(a)* and *cryIA(b)* genes differ in their first half of the variable region while the *cryIA(c)* gene varies in its second half, which is present neither in *cryIA(a)* gene nor *cryIA(b)* gene. Despite their similarities (ca. 80% identity in amino acid sequence), they exibit different, although overlapping, toxicity spectra (34).

It has been shown that the N-terminal 55% of the protoxin are sufficient for toxicity by based on the analysis of peptides produced by recombinant *E. coli* strains bearing deletion of *B. thuringiensis* subsp. *kurstaki* strain HD-1 Dipel *cryIA(a)* gene and also 3'-truncated clones of *B. thuringiensis* subsp. *kurstaki* strain HD-73 *cryIA(c)* gene (1, 74). The N-terminal region (residue 0-599) contains more hydrophobic segments, very few cysteine residues, and more predicted β-structure. The most hydrophobic region, in this part, may be required for the interaction of toxin with the insect gut epithelial cell. The C-terminal region contains more predicted hydrophilic segments, most of the cysteine and lysine residues and more predicted α-helical structure (1, 74, 75).

The gene encoded P2 protein is *cryII* gene. Two kinds of

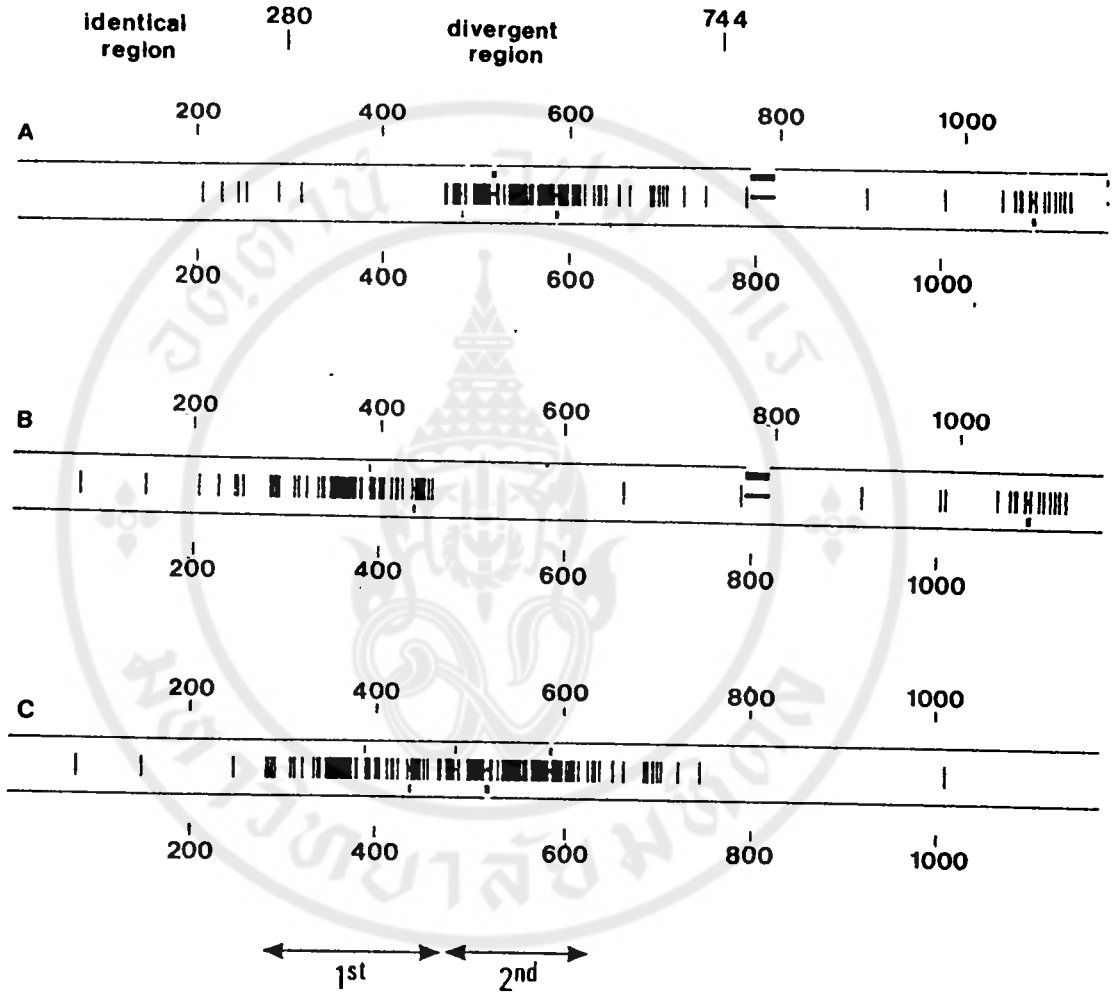

Figure 2.2. Schematic diagram demonstrates the variable regions found between the different *cryIA* gene products. The bars represent amino acid changes and blocks above or below the expanded regions represent gaps. The two horizontal arrows show that the variable region can be divided into two subregions. The sequences are displayed one above the other.
(A) cryIA(b) gene above cryIA(c) gene;
(B) cryIA(b) gene above cryIA(a) gene;
(C) cryIA(c) gene above cryIA(a) gene.

ficity, that is, when this region replaces the corresponding segment of *CryIIB* protein, the resultant hybrid protein acquires toxicity against mosquitoes. The difference of *CryIIA* and *CryIIB* polypeptides by only 18 amino acids in this region indicating that very few amino acid changes can substantially alter the specificities of these toxins. In addition, when the expressing of *cryIIA* and *cryIIB* genes in *B. thuringiensis* subsp. *kurstaki* strain HD-1 has been studied by hybridizing RNA samples preparing from sporulating cell of this strain with either *cryIIA* or *cryIIB* gene-specific probe. It is shown that only *cryIIA* gene can express well. Thus it has been suggested that *cryIIB* gene is transcribed very weakly and/or *cryIIB* mRNA is extremely unstable.

The amino acid sequences of *cryIIA* and *cryIIB* gene products have been compared with that of *cryIA(a)* gene product of *B. thuringiensis* subsp. *kurstaki* strain HD-1 (90). *CryIIA* and *CryIIB* proteins have identical regions which aligned with amino-terminal half of the protein encoded by *cryIA(a)* gene, 19.4% identity is found in a 602-amino acid overlap. When cryIIA and cryIIB genes were aligned, 89% of the bases are identical. Their products show 87% amino acid identity and 66 of 77 changes are conservative. Most of the non-conservative changes are located toward the central region of the polypeptides. The biochemical differences between *cryIA* and *cryIIA* gene products (P1 and P2 proteins, respectively) are summarized in table 2.2.

Table 2.2. Differences between P1 and P2 of subsp. *kurstaki*.

parameter	P1	P2	ref.
Toxicity	Lepidoptera	Lepidoptera and Diptera	97
mol wt (SDS-PAGE)	135,000 (protoxin) 55,000-70,000	65,000 (protoxin) 62,000	97
Shape of inclusion body	Bipyramidal	Cuboidal	37
solubility	pH 10-12 + reducing agent	pH 10-12	96
Time of synthesis		Begins before P1	37
percentage of total crystal protein	70-90	10-30	37
isoelectric point	4.4	10.7	97
Chromatography (Sephacryl S-300)	Eluted before P2		37
Serology (rocket immunoelectrophoresis)	mobility toward (+)	mobility toward (-)	6
Tryptic peptide mapping	complex pattern with resistant core	Relatively resistant	6

From Aronson et al., 1986 (6)

2.3 Expression of cry genes.

The nucleotide sequence of the promoter region of *cryIA(a)* gene from *B. thuringiensis* subsp. *kurstaki* HD-1 Dipel has been determined by analysis of a recombinant plasmid from *E. coli*. The S1 nuclease mapping is used to locate the promotor sites for transcription of the gene in *B. thuringiensis* and in *E. coli* strain carrying the recombinant plasmid (92). It has been demonstrated that there are three different promoter sites, one recognized by *E. coli* and two by *B. thuringiensis* RNA polymerase. In *B. thuringiensis, cryIA(a)* is transcribed from two start sites, located ca. 16 base pairs (bp) apart. *Bt I* is located ca. 85 bp upstream from ATG start codon, which is activated early in sporulation (stage II of sporulation) but decreased at about midsporulation (stage III to IV), at which time *Bt II* (located upstream from Bt I) is activated. In contrast, transcription of the gene in the recombinant *E. coli* strain occurs from a separate start site located between *Bt I* and *II*, and the gene is expressed equally well at all stages of growth. From in vitro studies, the transcription from *Bt I* is catalyzed by a specific *B. thuringiensis* RNA polymerase containing a new sigma subunit of ca. 35 kDa (13), whereas vegetative genes are transcribed by the predominant RNA polymerase which contains a sigma subunit of 61 kDa (34). The transcription from *Bt II* requires a second RNA polymerase containing another new sigma subunit of ca. 28 kDa. In *cryIIA* gene, the transcription requires the sigma-35-containing RNA polymerase. The presumed -10 and -35 regions for the promoter for this operon which are located by S1 nuclease mapping show a strong similarity

to those for the *Bt I* promoter for *cryIA(a)* gene (90).

The "-10 regions" for *Bt I* and *Bt II* have no significant homology to the *B. subtilis* vegetative, σ43, consensus sequence but they are related to promoter regions of certain *B. subtilis* sporulation genes (*spoVC* and *spoVG*). It has been shown that both *spoVC* and *spoVG* are activated early in the sporulation process and are recognized by an alter form of RNA polymerase containing a unique 37-kDa sigma facter (6). The *spoVG* gene also resembles the crystal protein gene in that it contains two overlapping promoters. However, both of these promoters are activated at the same time (1 to 2 hr after the onset of stationary phase) and each is recognized by a differerent polymerase. In contrast, transcription of the "0.3 kb" gene occurs after 4 to 5 hr in stationary phase and apparently requires the other unknown polymerase. The "-10 regions" of *Bt I* has homology with the *spoVG* downstream -10 regions and also with the *spoVC* -10 regions. A further similarity between the *B. thuringiensis* and *spoVG* gene is an A-T rich sequences located about 50 bp upstream from the initiation sites. In *spoVG* gene, this A-T rich region is required for transcription from the downstream promoter. The "-10 regions" of *Bt II* have some homology to the "0.3 kb" gene of *B. subtilis* which is transcribed beginning at stage III or IV of sporulation (89).

It has been shown that the "-35 regions" of the *Bt I* and *Bt II* have no apparent homology to those from genes in *E. coli* or *B. subtilis* (88, 92). Lack of well-defined -35 regions is not a unique feature of δ-endotoxin promoters since the *spoVG* gene of *B. subtilis* is also lack of a well-defined -35 region.

Another feature of the DNA sequence of the upstream region

of the *kurstaki* HD-1 gene is the present of two regions (between nucleotide 428 and 473 and between nucleotide 449 and 475) of hyphenated dyad symmetry. This type of symmetry, which is characteristic of operater sites, is located in regions which include both start sites for transcription of crystal protein gene and therefore, interfere with its expression (92).

The S1 nuclease mapping technique is also used to determined the 3' terminus of transcription of *cryIA(a)* gene and it is found that the terminus is located coincide with an inverted repeat sequence which could assume a very stable stem-loop structure (89). This strong terminator for the crystal protein gene is a relatively G-C rich stem-and-loop terminating in four consecutive uridine residues. It is observed that presence of the terminator significantly enhances the stability of crystal protein mRNA (34). Based on the S1 mapping and the position of this stem-and-loop, the size of the crystal protein mRNA is calculated to be 3730 bases.

The translational start site has been established by comparing the DNA sequence with the amino acid sequence at the amino terminus of the protein. A potential ribosome binding site of 11 nucleotides is found, located three bases upstream from the initiator ATG codon. The sequences have high complementary (9 of 11 bp) to the 3' end of the 16 S *Bacillus* rRNA, implying an efficient translation of crystal protein mRNA (92).

Transcriptional studies have been done with only *cryIA(a)* genes, but there are reported that *cryIA(b)* and *cryIA(c)* genes have the same promoter structures and also transcriptional terminator as *cryIA(a)* gene (1, 26).

3 Purification of parasporal crystals.

A number of methods have been introduced in order to separated the crystalline body from other cell components, in particular, the spore of *B. thuringiensis* (3, 4, 19, 23, 27, 29, 56, 59, 64, 77, 78, 100). Due to the similarity in the size of the endospore and the crystal of this microorganism, the principles of separation, as reviewed by Cooksey (18), are based on the difference in basic properties of the crystal and spore such as relative density, surface properties, solubility and germination of spore. Each property has been used alone or in combination with others to achieve the best separation.

The earlier attempts to obtain purified crystals depend on spontaneous germination and autolysis of spores, followed by differential centrifugation of the crystals (18, 47). Another method which has been employed by Angus (4) is a biphasic separation system using trifluorotrichloroethane as non-polar phase. Basically a paste of spores and crystals is suspended in an aqueous-salt solution in a separating funnel, a non-polar phase is added and the funnel agitated. When the phase separate, spores predominate in the interfacial layer, while crystals remain suspended in the aqueous phase. Retreatment of the interfacial layer yields further quantities of crystals and shaking the aqueous layer with fresh lots of non-polar phase gives purer crystal suspensions. This biphasic system has been investigated further by many workers using different organic solvents such as carbon tetrachloride (64), chloroform (59), and tetrabromomethane (18). Unfortunately, these published methods require many cycles of repeating extrac-

tion in order to achieve acceptable purity. Results of these methods give low yield and destruction of crystals by the organic solvents. Nontheless, it has been shown that the destruction of the crystals by the long exposure of the crystals in the organic solvents could be avoided by the use of a biphasic system containing sodium dextran sulfate 500 and polyethylene glycol 6000 (19, 29).

Besides biphasic systems, the crystals can also be separated by other techniques such as flotation (27, 64, 77) and density gradient centrifugation (3, 23, 56, 78, 100). The procedure has been modified for removing of spores from crystals by air-flotation in a water column (27). It has been suggested that there is no viable spores remaining and this method is quite useful for the preparation of large quantities of crystals. The successful introduction of density gradient centrifugation for purification of the crystals has been led by Fast since 1972 (23), using isopycnic density gradient centrifugation in cesium chloride. This procedure shows a substantial improvement over previous separation techniques because of the higher yield produced and the shorter time required. Later, linear, performed gradient of Renografin-water and sodium diatrizoate-water to separate crystals from spores and other cell components has been modified from Fast's technique (78). Discontinuous gradient centrifugation of Renografin has also been modified by Milne and his coworker (56). The products obtained from gradient centrifugation reveal pure crystal bands but clumping of crystals which caused problems in biphasic and flotation separation system is still the major problem of this separation technique (77). The zonal gradient cen-

trifugation for large-scale production of the crystals has been developed by Ang and Nickerson (3), but this procedure is found to be inconvenient when compared to other density gradient centrifugation because of the difficulties in operating of the instrument. However, this method is recommended because the cost can be reduced significantly by the use of cheaper agent such as sodium bromide. Owing to the proximate densities and hydrophobicities of spores and crystals, gelatin which is known as the foam-producing agent is used to separate spores from the cultures by flotation technique before centrifugation the cultures in a linear gradient of Renografin to increase the purity of the crystals (77). Recently, centrifugation through step gradient of Luox has been developed (100). This technique has produced biochemically pure crystals judging from SDS-polyacrylamide gel electrophoresis, and the clumping of crystals and spores in this gradient is less than those in other types of gradient.

4 Histopathology.

After the Lepidopteran-specific δ-endotoxins are ingested by susceptible insects, they are digested into active toxins by alkalinity in gut juice proteases. The toxicity of this toxin is expressed about 30 min after ingestion. The symptoms observed are as follows : cessation of feeding, peristalsis of the midgut, vomiting, diarrhea, extremely sluggish movement, paralysis, and finally death. Endo and Nishiitsutsuji-Uwo (22) have described the symptoms which could be divided into four stages, as shown in figure 2.3. The brief reviews of this sequence are the following : swelling of columnar cells, disintegration of apical microvilli

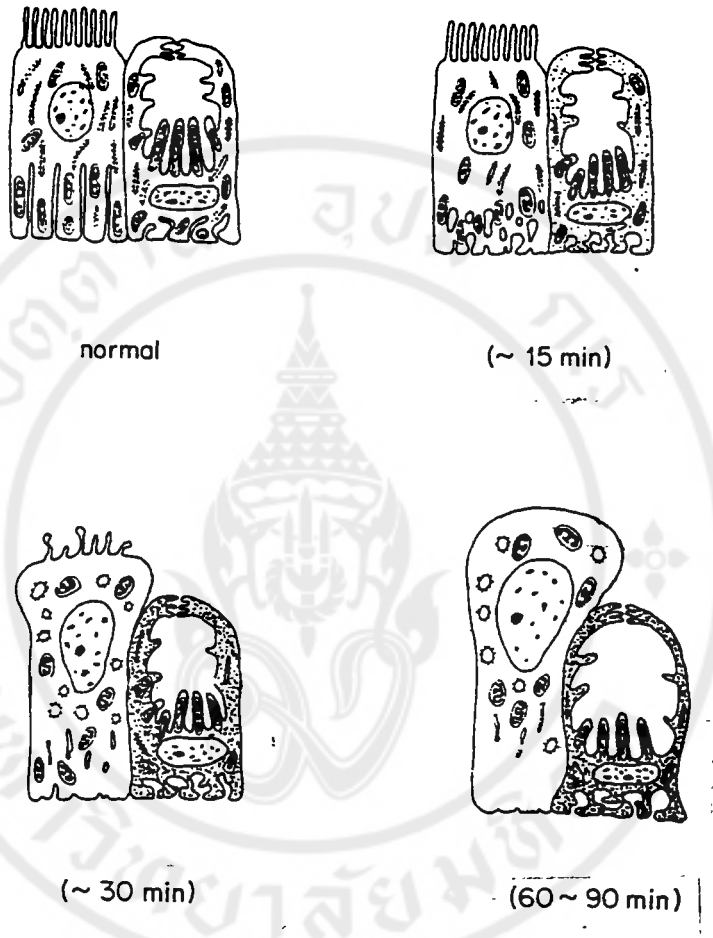

Figure 2.3. A schematic diagram demonstrates the ultrastructural changes in the midgut epithelial cells of *Bombyx mori* induced by *B. thuringiensis* ô-endotoxin. Columnar cells were swollen, followed by disintegration of apical microvilli and deformation of basal infoldings, and finally, goblet cells induced enlargement of the goblet cavity and intraspace of the basal infolding.
Cell on the left side is columnar cell, cell on the right side is goblet cell.

and deformation of basal infoldings, and finally, goblet cell induces enlargement of the goblet cavity and intraspace of the basal infolding. Similar observation is found by Percy and Fast (65).

When δ-endotoxin are ingested by susceptible insects, a number of physiological changes occurred, This has led to several suggestions as to the mechanisms of action of this toxin. Initially, it is believed that the mechanism of action of the toxin is similar to that of valinomysin (5), since the toxin causes an increase in the pH and K^+ concentration of the hemolymph while reducing the pH of the gut juice. However, since oxygen uptake and production of ATP in mitochondria of midgut tissue are inhibited by the toxin (86), it is also suggested that the toxin acts as an uncoupler of oxidative phosphorylation. In another study it is suggested that the toxin does not cause any uncoupling effect and that the target site of the toxin is not the mitochondria but the cell membrane of the susceptible cell. This is supported by the observation that the active transport of K^+ ions from the midgut lumen into the hemolymph is inhibited as early as 1 hr after toxin ingestion (30). Also, it has been demonstrated by Harvey and Wolfersberger (32) that the influx of potassium from the blood to the lumen is inhibited and that the K^+ efflux from the lumen to the blood is stimulated about three-fold by δ-endotoxin in isolated midgut tissue of *Manduca sexta* larvae. It is concluded that δ-endotoxin is not an uncoupler of oxidative phosphorylation but instead an inhibition of K^+ transport. It is believed that the endotoxin acts specifically on the apical plasma membrane of

midgut cells.

Histopathological and biochemical studies of the toxicity of activated toxin on cultured insect cells have provided evidence for a mode of action similar to that described above (21, 58). There is some evidence that the toxin produced by *B. thuringiensis* subsp. *kurstaki* may act as a lectin binding to N-acetylgalactosamine residues expressed on the surface of cells (41). Another evidence is that the δ-endotoxin produced by *B. thuringiensis* subsp. *entomocidus* induces the release of encapsulated [^{14}C] sucrose from reverse phase vesicles composed of phosphatidylcholine and chloresterol (99). The existence of the a polar head group in the phospholipid as well as intermolecular hydrogen bonding at the membrane surface, is found to be of major impor tance in the toxin liposome interaction. It has been investigated that differences in the receptors on brush border membrane of insect midgut are a major determinant of difference in the insect spectrum of the entire lepidopteran-specific toxin family (68). Receptor site heterogeneity in the insect midgut occurs frequently and results in sensitivity to more than one type of δ-endotoxin. Knowles and Ellar (42) have proposed a two-step model in which, after binding a specific plasma membrane receptor, the action of all the δ-endotoxin is to generate small pores in the plasma membrane, either directly by inserting into the membrane, or indirectly by perturbing resident plasma membrane molecules. The creation of these pores (0.5-1.0 nm radious) will lead to colloid-osmotic lysis, that is, an equilibration of ions through the pore resulting in a net inflow of ions, an accompanying influx of water, cell swelling and eventual lysis. Therefore, prior

interaction between the toxin and cell-specific plasma membrane receptors is neccessary before these toxins can insert into, or interact with, the membrane.

CHAPTER III

MATERIALS AND METHODS

1 Chemicals and reagents.

All chemicals used throughout this study were of analytical grade or the purest grade available. Bacteriological media were obtained from Difco. Manganese chloride ($MnCl_2.4H_2O$), sodium chloride (NaCl), glycine, Tris(hydroxymethyl)-aminomethane, sodium dodecyl sulfate (SDS), methanol, glacial acetic acid, glycerol, calcium chloride ($CaCl_2$), hydrochloric acid (HCl), calcium panthothenate, biotin, ascorbic acid and casein were obtained from Merck. Sodium hydroxide (NaOH) and Bromphenol blue were purchased from BDH. Polyacrylamide was bought from Armesco. Coomassie brilliant blue, 2-mercaptoethanol, inositol and N,N,N',N'-tetramethylenediamine (TEMED) were obtained from Fluka. Copper sulfate ($CuSO_4.5H_2O$), zinc sulfate ($ZnSO_4.H_2O$)), ammonium persulfate, all molecular weight standards , N,N'-methylenebis acrylamide (Bis), Triton X-100, niacin, pyridoxine, chloline chloride and sorbic acid were bought from Sigma. Riboflavin was obtained from Eastman. Folic acid was purchased from Calibiochem. Mung-bean and agar for the preparation of medium for rearing Lepidopteran larvae were purchased from groceries.

2 Bacteria and culturing procedure.

2.1 Bacteria.

Bacillus thuringiensis subsp. *kurstaki* strain HD-1 was obtained from Dr Watanalai Panbangred, Department of Biotechnology, Faculty of Science, Mahidol University. Bacterial culture was maintained in nutrient agar (Difco) kept at 4 °C and subcultured every one or two months.

2.2 The preparation of bacterial suspensions.

The bacterial culture of *Bacillus thuringiensis* subsp. *kurstaki* (Btk) was prepared in 500 ml Erylenmyer flasks containing 150 ml of nutrient broth supplemented with mineral salts (NBS) as described by Ponglikitmongkol (66, Appendix 1B).The starting inoculum was made by transferring a single colony of Btk grown on nutrient agar into a 150 ml Erylenmyer flask containing NBS medium and incubated overnight at 28 °C in a gyratory shaker. Inoculum consisting of 2 ml of an exponentially growing culture in NBS medium was transferred into an 500 ml Erylenmyer flask containing 150 ml of NBS medium. The culture were incubated for 48 hr at 28 °C on a gyratory shaker set at 200 rpm. The sporulating culture was harvested and used as a stock for the purification of the protein crystals.

The materials used for bioassay were harvested by centrifugation at 7,000 rpm for 10 min in Kubota KR-20000 T supercentrifuge and then they were divided into two portions. The first portion was washed in distilled water alone but the second portion was washed in 1 M NaCl three times followed by distilled water. The materials were stored at -20 °C for further studies.

3 Purification of protein crystals.

The harvested sporulating cultures were washed in 1 M NaCl containing 0.01% Triton X-100 followed by washing in deionized distilled water. The process of removing spores from the washed pellets were done by flotation technique in which they were shaking them vigorously in 0.02 M NaCl until foam occurred, using a spoon to removed the foam, then the process was repeated until the foam disappeared. Protein crystals were recovered from the mixture by centrifugation at 7,000 rpm for 10 min, then washed three times in deionized distilled water. The purified protein crystals were pooled and stored at -20 °C in deionized distilled water until used. The purity and yield of each preparation of the protein crystals were determined by using direct counting technique with Petroff-Hausser chamber. Under a phase-contrast microscope, the protein crystals had bipyramidal shapes in contrast to ellipsoidal shape of spores. The purity was expressed in term of percent of number of protein crystals per number of spores plus crystals.

4 Preparation of P1 and P2.

The P1 protein fraction was prepared from a stock of purified protein crystals according to the method of Yamamoto and McLaughlin (97) with a slight modification (see figure 3.1). The amount of purified protein crystals which was estimated equivalent to 5.63 mg dry weight in 0.25 ml deionized distilled water containing 2% 2-mercaptoethanol was adjusted to contain a final pH at

10 with 10% NaOH and incubated at 0 °C for 1 hr. After centrifugation, the supernatant was dialysed overnight against distilled water and stored at -20 °C until used. The precipitate (containing P2) was washed and then solubilized in 50 µl 0.1 M NaOH at 0 °C for 10 min. After centrifugation at 10,000 rpm for 15 min, the supernatant was dialysed overnight against distilled water and stored at -20 °C until used.

5 Insects.

5.1 Mosquitoes.

Aedes aegypti larvae were used as a test insect in bioassay of crystal proteins throughout this studies. They were reared by Miss Kanchana Pantuwatana and Mrs Kwansiri Chaimuangmoon. Mosquitoes were reared in an air conditioned insectary maintained at approximately 25 ± 2 °C with photo period of 13 hours of light and 11 hours of darkness.

5.2 Moths.

Spodoptera exigua larvae, or the beet armyworm, were also used as a test insect throughout the experiment. They were started with eggs obtained from Mr. Uthai Ketunuti, Entomology and Zoology Division, Department of Agriculture, Bangkhen, Bangkok 10900. They were reared in an air conditioned insectary maintained at approximately 27 ± 2 °C. The insects were reared as followed :

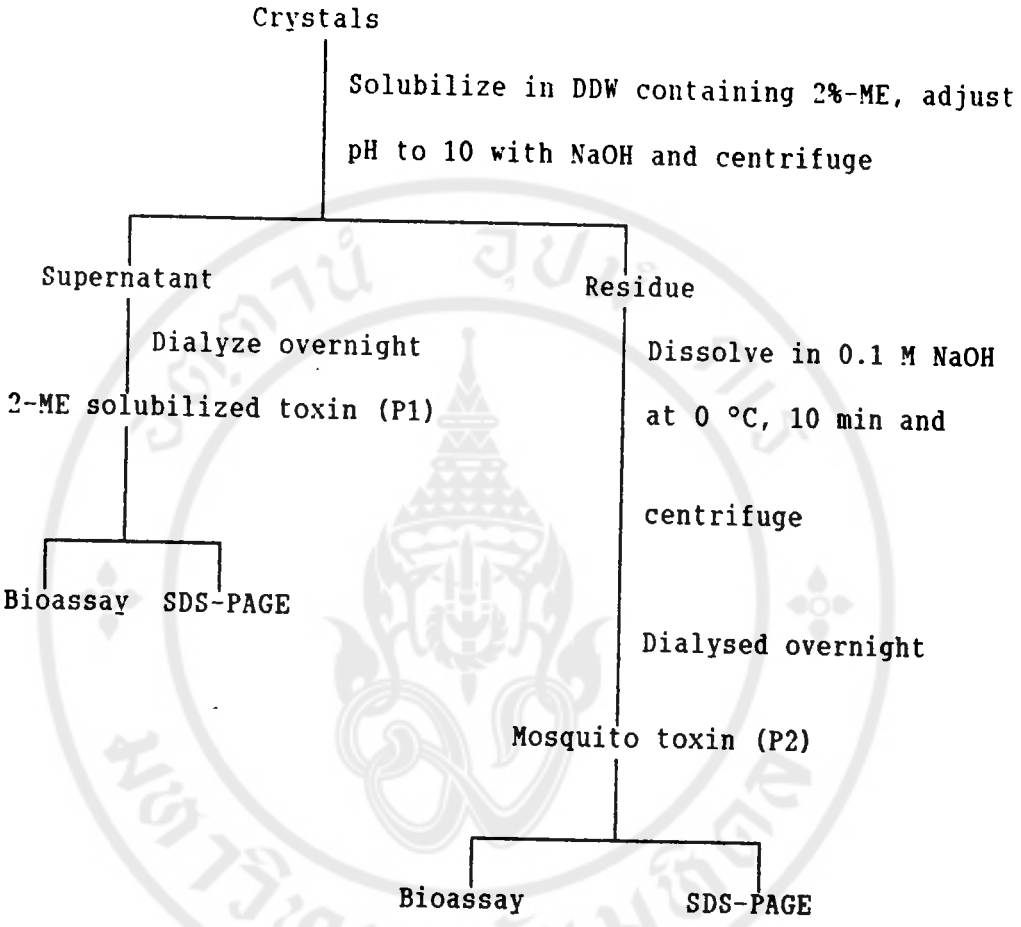

Figure 3.1. Procedure for the solubilization of proteinaceous crystals

Adult males and females were placed in a mating container with a ratio of 1:1. Each container was containing 8 to 12 adults moths. Adults of each sex were separated since they were pupae with an aid of the stereo microscope.

Mating container was made of clear plastic sheet that was made in cylinder shape and placed on a plastic bowl which was covered with a piece of nylon. The size of this plastic cylinder was 11.0 cm in diameter and 12.5 cm in length. The humidity in a mating container was maintained as high as possible by placing water in the plastic bowl about one half of its volume. The top of this container was covered with a tissue paper which used as an oviposition trap. The moths were fed on Panvitamin glucose syrup solution with diluted to make a final concentration of 5-10% glucose in distilled water. Fresh tissue papers were replaced every 2 days after the moths had been emerged. The tissue papers overlaid with eggs were removed from the mating containers and placed in appropriate containers. The eggs were kept in a high humidified condition by adding a piece of wet cotton wool in the bottom of the container.

The eggs were usually hatched in the second day after placed the tissue paper containing eggs in a plastic cup (12.7 cm in diameter and 7 cm high) containing an artificial diet. Each cup contained larvae up to 50 larvae. Two larvae were transferred to a 2-dram vial after day 7. When the larvae developed into pupae they were removed from the vials. Malformed pupae were discarded. Each sex of normal pupae were segregated with an aid of a stereo microscope and transferred into new mating containers.

Figure 3.2. Mating Container

Figure 3.3. Larval Rearing Container

6 Bioassay of toxicities

The beet armyworm (*Spodoptera exigua*) larvae were placed on artificial diets which had been overlaid with the test samples. The samples used were as follow : (a) harvested materials which was washed in distilled water only, (b) harvested materials which was washed in 1 M NaCl followed by distilled water, (c) purified parasporal crystal proteins, (d) protein P1 and P2. The laboratory reared larvae were used throughout the studies. The artificial diets were prepared according to appendix 1 except formalin was not added. The diets were cut into standard core sizes ca. 8 mm in width and 15 mm in length. The size of artificial media was approximately 50x15 mm^2. Each piece of diet was placed in a 13x100 mm sterile test tube. Various concentrations of each sample were made in sterile distilled water. A volume of 30 µl of each test concentration was spread on the diet surface by using micropipette. The final concentrations of samples used to determine the LC_{50} value were transformed into a number of protein crystals/ mm^2 of diet surface or ng of P1 or P2 protein/mm^2 of diet surface. All stages of larvae were used. One larva was placed on a piece of diet tube with a cotton wool plug. Mortality was observed and recorded daily for four days.

Mosquito larvae: The samples used in bioassay against mosquito larvae were the same as used in the toxicity test of *S. exigua* larvae. The first and second instar larvae were tested in one group, and the third and fourth instar larvae were tested in another group. A series of ten fold dilution of each test sample was made and 0.2 ml of each dilution was transferred into the cup containing 10 larvae in 50 ml distilled water. Each test was done

in triplicate manner with a set of control, untreated larvae. The larvae were kept at room temperature and fed with ground rat chaw pellets on the first day. Mortality was observed and recorded daily for 3 days.

Samples of P1 and P2 proteins were tested in different manner by using six-well plate. Each well contained 8 ml distilled water with 10 larvae. The second instar were used and each test were done in duplicate manner. The larvae were kept at room temperature and fed with ground rat chaw pellets on the first day. Mortality was observed and recorded daily for 3 days.

7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The discontinuous buffer system of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done throughout this experiment. This method was modified from methods described by Laemmli (46). The stacking gel (4.6% acrylamide in final concentration) and the separating gel (7.5% acrylamide in final concentration) were prepared according to the method described by Boonsaeng (11). One volume of the sample was mixed with 2 volume of 2x sample buffer containing 0.0625 M Tris-HCl, pH 6.75, 4.6% SDS, 20% glycerol, 10% 2-ME and 0.002% Bromphenol blue. The mixtures were then heated in boiling water for 3 min, then they were loaded onto each well of previously prepared SDS-polyacrylamide gel. Electrophoresis was carried out in a descending direction in Tris-glycine buffer pH 8.3 with the cathode in the top chamber and the anode in the bottom chamber at 150 V. This buffer was consisting of 0.025 M Tris, 0.192 M glycine and 0.1% SDS. By judging from the movement of the dye marker, the electrophoresis was stopped at an appropri-

ate time. The gel was stained with coomassie blue stain for 10 min. The coomassie blue stain solution was composed of 0.2 g coommassie blue and 7% acetic acid in 50% methanol. Subsequently, the gel was destained by repeated washing in destain solution consisting of 7% acetic acid in 30% methanol.

8 Protein concentration.

The amount of protein in samples was determined either by the Bradford's method (12) using bovine serum albumin as standard or by calculation from absorbance measured at 280 nm using tyrosine as standard.

The Bradford's method was done by using Coomassie blue dye reagent. A sample 0.1 ml which had protein ranging from 10 to 200 µg was added with 5 ml of Coomassie blue dye reagent. After 5 min, the optical density at wavelength 595 nm was measured.

Coomassie blue dye reagent was made from 100 mg of Coomassie brilliant blue G-250 dissolved in a mixture of 50 ml 95% ethanol and 100 ml 85% phosphoric acid and the reagent was added with distilled water to make a total volume of 1 litre.

CHAPTER IV

RESULT

1 Purification of the protein crystals.

In crude samples, it contained a mixture of protein crystals, spores, sporulating cells and vegetative cells. It was found that after purification, sporulating cells and vegetative cells were not detected under phase-contrast microscope. The amounts of protein crystals and spores were nearly equal in number in the initial broth prior to purification steps, but were different about ten times after purification. Results of the amount of protein crystals, spores, sporulating cells and vegetative cells observed in crude samples and purified samples were summarized in table 4.1. The purity of the protein crystals was 99% with 25% yield. The purity was expressed in term of percent of number of protein crystals per number of spores plus protein crystals, and the yield of protein crystals was expressed in term of percent of proportion of purified protein crystals compared with protein crystals in crude sample.

2 Characterization of P1 and P2 protein by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Since the evidence of toxic activity for mosquito had been found in protein crystals of *B. thuringiensis* subsp. *kurstaki* (Yamamoto and McLaughlin, 1981), an attempt was made to purify this mosquito toxin. The protein crystals were first solubilized

Table 4.1 Results of purification of parasporal crystal toxins of *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1.

Type of specimen	numbers of material observed/ml				protein crystals	
	protein[a] crystal	spore[a]	sporulated[a] cell	vegetative[a] cell	yield (%)	purity[b] (%)
crude sample	2.63×10^8	1.61×10^8	1.39×10^8	1.17×10^7	100	62
purified sample	1.75×10^{10}	2.24×10^8	0	0	25	99

[a] These were counted by using direct counting technique with Petroff-Hausser chamber and under a phase-contrast microscope.

[b] The purity was expressed in term of percent of number of protein crystals per number of spores plus crystals.

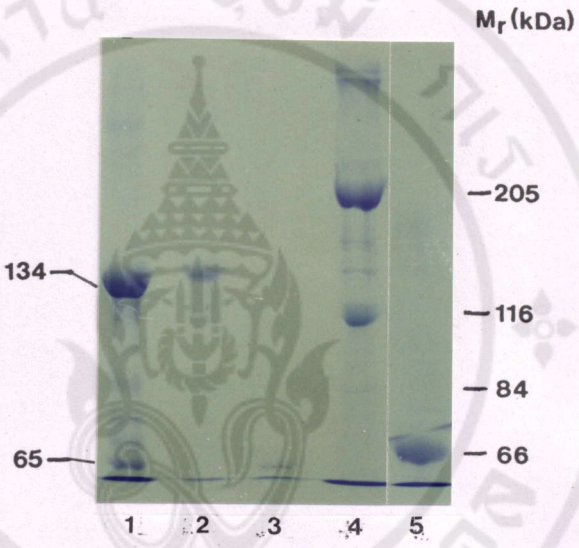

Figure 4.1. Electrophoretic pattern on 7.5% SDS-PAGE of crystal protein of *B. thuringiensis* subsp. *kurstaki* HD-1 (lane 1), solubilized P1 protein (lane 2) and P2 protein (lane 3), and molecular weight markers (lane 4 and 5) : myosin (205 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa) and bovine albumin (66 kDa).

by 2-ME at pH 10 to remove the P1 component and then recovered the mosquito toxin from the residues by dissolving in 0.1 M NaOH. The characteristic of the P1 and P2 components were determined by using SDS-PAGE, in comparison with intact crystals. The intact crystals was solubilized by incubation at 100 °C for 3 min in SDS-sample buffer. An electrogram of these components were shown in figure 4.1 and 4.11. It was found that there was one major band with several minor bands in the P1 protein fraction (lane 2, figure 4.1; lane 4, figure 4.11), whereas there was only one band in P2 protein fraction (lane 3, figure 4.1; lane 5, figure 4.11).

By using myosin (205 Kda), β-galactosidase (116 Kda), fructose-6-phosphatase (84 Kda), bovine albumin (66 Kda) and egg albumin (44 Kda) as molecular weight markers (lane 4 and 5, figure 4.1; lane 6 and 7, figure 4.11), it could be estimated that the relative molecular weight of the major band in P1 protein fraction was equivalent to 134 Kda, and that of the band in P2 protein fraction was 65 Kda.

3 Bioassay of toxicities.

Results of bioassay of certain dosage of samples of *B. thuringiensis* subsp. *kurstaki* strain HD-1 (*Btk HD-1*) protein crystals (that was a sample obtained from either culture washed in distilled water or culture washed in sodium chloride or purified crystals) against *Spodoptera exigua* first instar larvae are summarized in table 4.2. The test was done in two sets, the first set was the comparison between the culture washed in distilled water and the culture washed in sodium chloride, another group was the comparison of culture washed in distilled water and purified

crystals. It was found that in the second set, 3.33% natural mortality was observed in the control group. Thus, percent mortality was corrected by using Abbott's formula (25) to compensate for the mortality in the control group. In the first set the concentration of protein crystals used in the test was varied ranging from 505 to 8 crystals/mm^2 and 501 to 8 crystals/mm^2 of diet surface for a sample washed in distilled water and a sample washed in sodium chloride, respectively. However, the mortality rates were different. The sample washed in distilled water cause percent mortality ranging from 83.33 to 20.00 percent but the sample washed in sodium chloride caused percent mortality ranging from 73.33 to 10 percent according to the concentration used. In the second set the concentrations of protein crystals used in the test were varied ranging from 505 to 8 crystals/mm^2 of diet surface for sample washed in distilled water, and 908 to 14 crystals/mm^2 of diet surface for specimen of purified crystals. The mortality rates obtained were varied from 76.67 to 13.33 percent and 66.67 to 20 percent, respectively, which was correlated to the concentration used. The probit analysis made from data was done and summarized in figure 4.2.

The second instar larvae of *S. exigua* were also used to test against these samples. The test was done in one experiment using specimens obtained from culture washing in distilled water, and from culture washing in sodium chloride in comparison with purified crystals. Results are summarized in table 4.3. Natural mortality did not observed in this experiment. The concentrations of sample which washed in distilled water varied ranging from 4435 to 69 crystals/mm^2 of diet surface and yielded mortality rates

ranging from 86.67 to 13.33 percent which correlated to the concentration used, while the sample which had been washed in sodium chloride yielded mortality rates ranging from 90.00 to 30.00 percent according to the concentration used. The concentrations of purified crystals used were ranging from 4089 to 64 crystals/mm^2 of diet surfaceand yielded mortality rates ranging from 73.33 to 6.67 percent. The probit analysis of data obtained in this experiment are summarized in table 4.3 and figure 4.3.

When the third to the fifth instars of *S. exigua* larvae were used, natural mortalities were not observed. For the third instar larvae, two sets of experiments were done, the first set was tested against the specimen washed in distilled water and that washed in sodium chloride. The second set was tested against specimens washed in distilled water and specimen of purified crystals. The concentration of protein crystals used was varied ranging from 7596 to 168 crystals/mm^2 of diet surface for the sample obtained by washing in distilled water and ranging from 4,478 to 140 crystals/mm^2 of diet surface for the sample obtained by washing in sodium chloride. The mortality rates observed were varied ranging from 86.67 to 40.00 and 96.67 to 33.33 percent, respectively. The concentrations of the specimen obtained by washing in distilled water used in the test was the same as those in the first set while the concentrations of protein crystals made from purified crystals were varied ranging from 9,084 to 142 crystals/mm^2 of diet surface. The mortality rates observed in individual concentration were varied from 93.33 to 10.00 and 86.7 to 13.33 percent, respectively. Results are summarized in table 4.4. The probit analysis of these values were done and summarized

in figure 4.4.

For the assay against fourth instar larvae, the concentrations of protein crystals made from sample washed in distilled water that used in the first set were varied ranging from 33,900 to 1,058 crystals/mm^2 of diet surface. The mortality rates observed in individual concentration were varied from 100 to 16 percent. The concentrations of protein crystals made from sample washed in sodium chloride were varied from 9,950 to 311 crystals/mm^2 of diet surface. The mortality rates observed in individual concentration were varied from 92 to 8 percent. In the second set the concentrations of protein crystals made from specimen washed in distilled watar used were varied ranging from 25,400 to 793 crystals/mm^2 of diet surface while those made from purified crystals were ranging from 61,700 to 1,930 crystals/mm^2 of diet surface. The mortality rates observed in individual concentration were varied from 88 to 12 percent mortalities and 60 to 0 percent, respectively. Results are summarized in table 4.5. The probit analysis of these values were done and summarized in figure 4.5.

For the fifth instar larvae, the concentrations of sample derived from culture washing with distilled water used were varied from 40,600 to 1,270 crystals/mm^2 of diet surface. The mortality rates observed were varied from 87.50 to 8 percent according to the concentration used. The mortality rates were varied from 95.83 to 28.00 percent when tested against sample derived from culture washing with sodium chloride in which the concentrations of protein used varied from 11,900 to 373 crystals/mm^2 of diet surface. In the second set, the mortality rates were varied from 96.00 to 28.00 percent when tested against the protein crystals derived

from sample which prepared by washing the culture with distilled water in which the concentrations of protein crystals used were varied from 57,140 to 1,790 crystals/mm^2 of diet surface, whereas the mortality rates were varied from 72 to 8 percent when tested against the purified crystals at the concentrations ranging from 65,200 to 2,040 crystals/mm^2 of diet surface. The probit analysis of these values were made and summarized in table 4.6 and figure 4.6.

The values of lethal concentration 50 % end point (LC_{50}) against each instar of *S. exigua* larvae obtained by using probit analysis are summarized in tables 4.7. It was shown that the LC_{50} values were increased in parallel with the stage of the larvae except those against the second instar larvae. When the efficacy of each sample was compared between larvae of the same instar, it appeared that the sample derived from culture washing with sodium chloride had the highest larvicidal activity against *S. exigua* larvae and the purified crystals had the lowest larvicidal activity as shown in table 4.7. The activity of the sample derived from culture washing with sodium chloride was 1.6- to 3.4-fold when compared with that of sample derived from culture washing with distilled water

Table 4.2 Percentage mortalities of the first instar larvae *Spodoptera exigua* after fed upon diet containing of various preparations of the protein crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	505	30	25	83.33	5.97
	252	30	19	63.33	5.34
	126	30	12	40.00	4.75
	63	30	10	33.33	4.57
	32	30	11	36.67	4.66
	16	30	11	36.67	4.66
	8	30	6	20.00	4.16
	0	30	0	0.00	-
N	501	30	22	73.33	5.62
	251	30	22	73.33	5.62
	125	30	19	63.33	5.34
	63	30	16	53.33	5.08
	31	30	15	50.00	5.00
	16	30	13	43.33	4.83
	8	30	3	10.00	3.72
	0	30	0	0.00	-
F	505	30	23	76.67	5.70
	252	30	23	76.67	5.70
	126	30	15	50.00	4.95
	63	30	12	40.00	4.68
	32	30	6	20.70	4.06
	16	30	5	16.67	3.88
	8	30	4	13.33	3.70
	0	30	1	3.33	-
C	908	30	20	66.67	5.40
	454	30	12	40.00	4.68
	227	30	21	70.00	5.49
	114	30	15	50.00	4.95
	57	30	7	23.33	4.16
	28	30	7	23.33	4.16
	14	30	6	20.00	4.03
	0	30	1	3.33	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per square millimetre of diet surface.

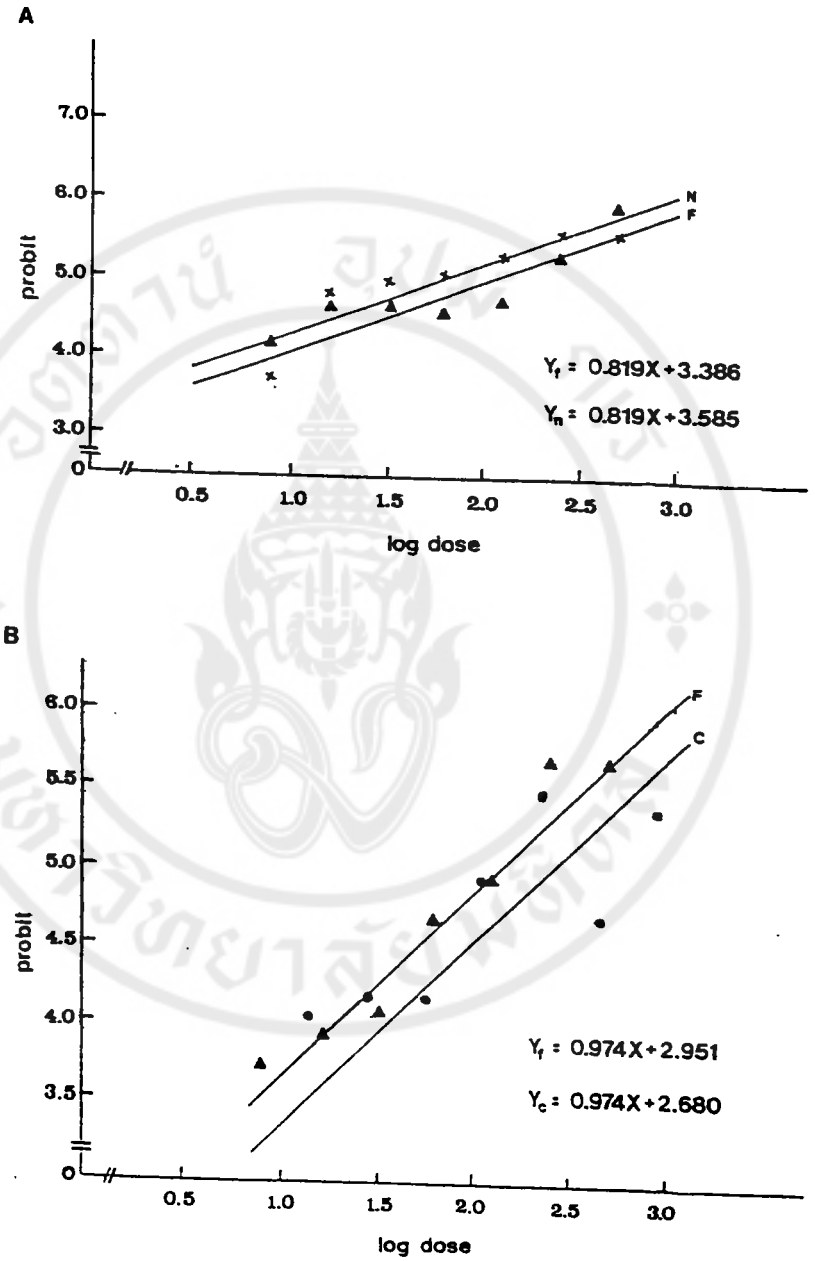

Figure 4.2. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 1st instar larvae of *S.exigua*.
A. sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N)
B. sample derived from washing with distilled water (▲, F) compared with that of purified crystal (•, C).

Table 4.3 Percentage mortalities of the second instar larvae of *Spodoptera exigua* after fed upon diet containing of various preparations of the protein crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	4435	30	26	86.67	6.11
	2217	30	24	82.76	5.95
	1108	30	22	73.33	5.62
	554	30	18	60.00	5.25
	277	30	14	46.67	4.92
	139	30	8	26.67	4.38
	69	30	4	13.33	3.89
	0	30	0	0.00	-
N	4478	30	27	90.00	6.28
	2239	30	25	83.33	5.97
	1120	30	28	93.33	6.50
	560	30	23	76.67	5.73
	280	29	23	79.31	5.82
	140	30	18	60.00	5.25
	70	30	9	30.00	4.48
	0	30	0	0.00	-
C	4089	30	21	70.00	5.52
	2044	30	22	73.33	5.62
	1022	30	13	43.33	4.83
	511	30	8	26.67	4.38
	255	30	6	20.00	4.16
	128	30	3	10.00	3.72
	64	30	2	6.67	3.50
	0	30	0	0.00	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per square millimetre of diet surface.

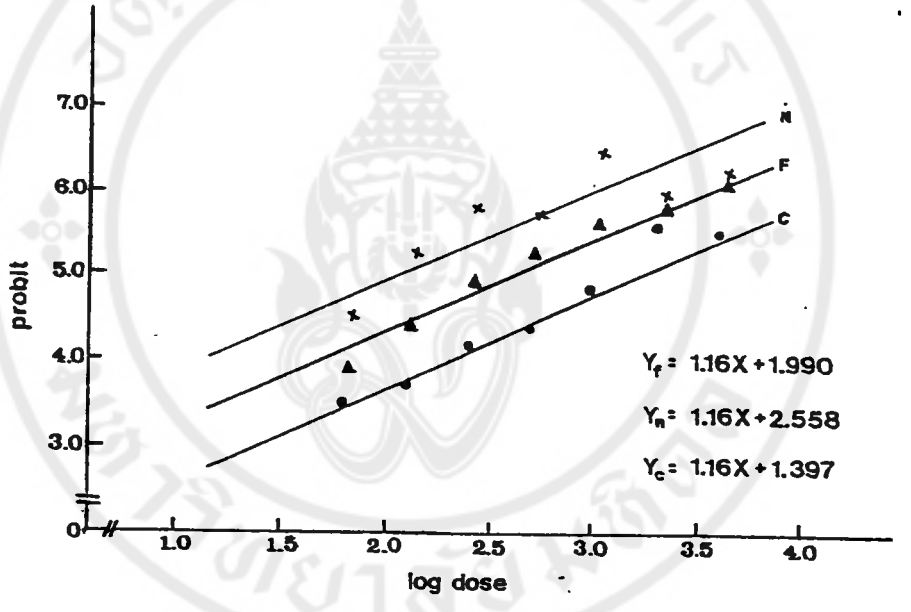

Figure 4.3. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 2nd instar larvae of *S.exigua*. Sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N) and with that of purified crystal (•, C).

Table 4.4 Percentage mortalities of the third instar larvae of *Spodoptera exigua* after fed upon diet containing of various preparations of the protein crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	7596	30	26	86.67	6.11
	3798	30	21	70.00	5.52
	1899	30	20	66.67	5.43
	950	30	19	63.33	5.34
	475	30	16	53.33	5.08
	237	30	12	40.00	4.75
	0	30	0	0.00	-
N	4478	30	29	96.67	6.84
	2239	30	24	80.00	5.84
	1120	30	18	60.00	5.25
	560	30	14	46.67	4.92
	280	30	17	56.67	5.17
	140	30	10	33.33	4.57
	0	30	0	0.00	-
F	7596	29	27	93.10	6.48
	3798	30	28	93.33	6.50
	1899	30	20	66.67	5.43
	950	30	14	46.67	4.92
	475	30	19	63.33	5.34
	237	30	14	46.67	4.92
	168	30	3	10.00	3.72
	0	30	0	0.00	-
C	9084	30	26	86.70	6.11
	4542	30	22	75.86	5.70
	2271	30	19	63.33	5.34
	1136	30	19	63.33	5.34
	568	30	12	40.00	4.75
	284	30	4	13.33	3.89
	142	30	6	20.00	4.16
	0	30	0	0.00	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per square millimetre of diet surface.

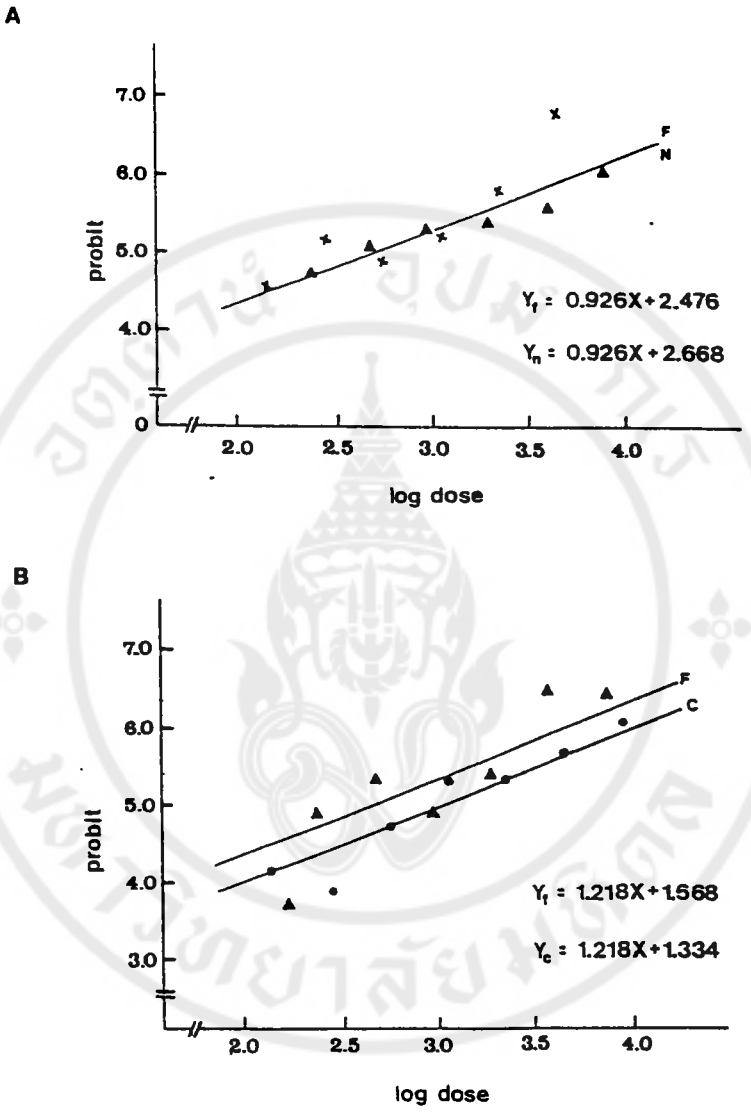

Figure 4.4. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 3rd instar larvae of *S. exigua*.
A. sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N).
B. sample derived from washing with distilled water (▲, F) compared with that of purified crystal (•, C).

Table 4.5 Percentage mortalities of the fourth instar larvae of *Spodoptera exigua* after fed upon diet containing of various preparations of the protein crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	33900	25	25	100.00	+∞
	16900	25	23	92.00	6.41
	8460	25	17	68.00	5.47
	4231	25	12	48.00	4.95
	2115	25	7	28.00	4.42
	1058	25	4	16.00	4.01
	0	25	0	0	-
N	9950	25	23	92.00	6.41
	5000	25	21	84.00	6.00
	2490	25	22	88.00	6.18
	1240	25	10	40.00	4.75
	622	25	11	44.00	4.85
	311	25	2	8.00	3.60
	0	25	0	0	-
F	25400	25	22	88.00	6.18
	12700	25	17	68.00	5.47
	6350	25	17	68.00	5.47
	3170	25	10	40.00	4.75
	1590	25	6	24.00	4.29
	793	25	3	12.00	3.83
	0	25	0	0	-
C	61700	25	15	60.00	5.25
	30900	25	18	72.00	5.58
	15400	25	11	44.00	4.85
	7720	25	5	20.00	4.16
	3860	25	2	8.00	3.60
	1930	25	0	0	-∞
	0	25	0	0	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per square millimetre of diet surface.

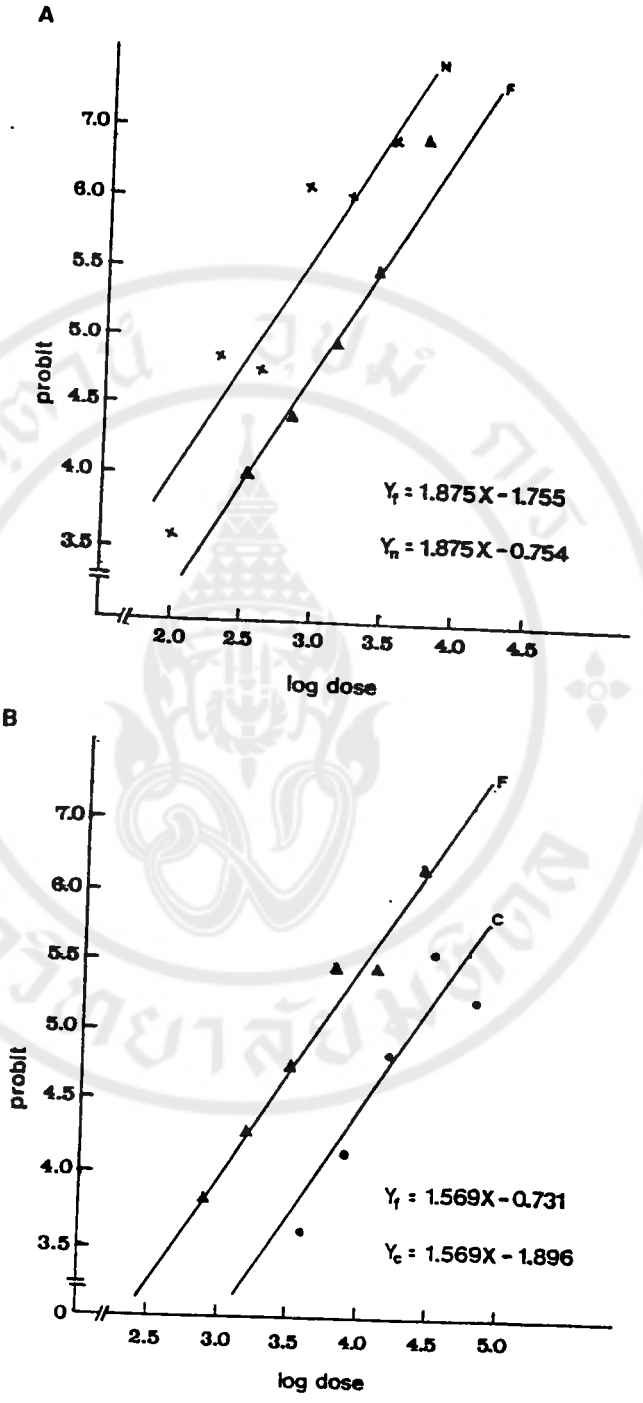

Figure 4.5. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 4th instar larvae of *S.exigua*.
A. sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N).
B. sample derived from washing with distilled water (▲, F) compared with that of purified crystal (•, C).

Table 4.6 Percentage mortalities of the fifth instar larvae of *Spodoptera exigua* after fed upon diet containing of various preparations of the protein crystals of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	40600	24	21	87.50	6.15
	20300	24	21	87.50	6.15
	10200	22	19	86.36	6.10
	5080	23	12	52.17	5.05
	2540	23	4	17.39	4.06
	1270	25	2	8.00	3.60
	0	25	0	0	-
N	11900	22	20	90.91	6.34
	5970	24	23	95.83	6.73
	2990	23	12	52.17	5.05
	1490	22	8	36.36	4.65
	746	21	5	23.81	4.29
	373	25	7	28.00	4.42
	0	25	0	0	-
F	57140	25	24	96.00	6.75
	28600	25	22	88.00	6.18
	14300	25	23	92.00	6.41
	7140	23	16	69.57	5.51
	3570	22	12	54.55	5.11
	1790	25	7	28.00	4.42
	0	25	0	0	-
C	65200	25	18	72.00	5.58
	32600	24	15	62.50	5.32
	16300	23	8	34.78	4.61
	8150	24	4	16.67	4.03
	4080	22	2	9.09	3.66
	2040	25	2	8.00	3.60
	0	25	0	0	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per square millimetre of diet surface.

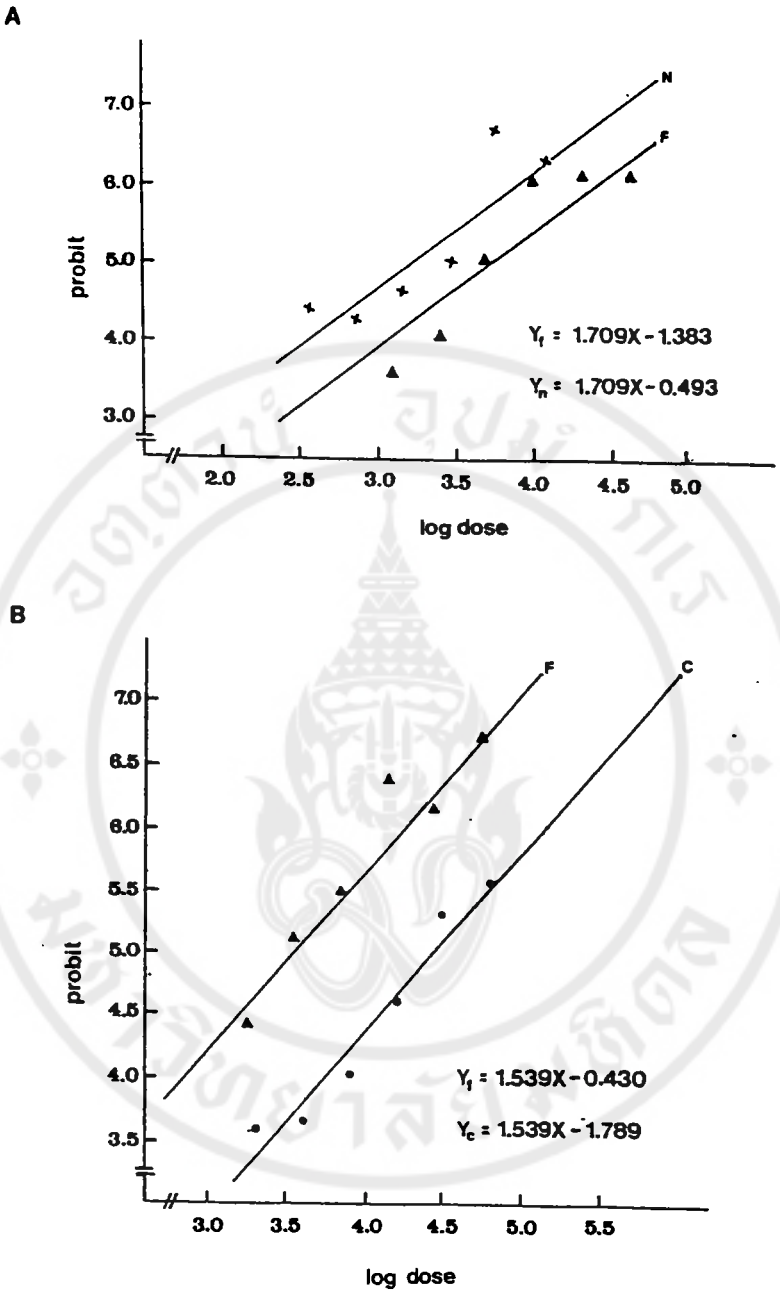

Figure 4.6. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 5th instar larvae of *S.exigua*.

A. sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N).

B. sample derived from washing with distilled water (▲, F) compared with that of purified crystals (•, C)

Table 4.7 Comparison of lethal concentration 50% end point (LC_{50}) values from bioassay of *Spodoptera exigua* at various instars against crude preparation and purified preparation of parasporal crystal toxin of *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1. The mortalities were read after 4 days.

A. The unit was number of crystals/mm^2 diet surface.

instar	LC_{50} (no. of crystals/mm^2)[a/]		
	sporulated culture washed in		purified crystal
	water	NaCl	
1	109	62	207
2	393	127	1276
3	538	333	1011
4	4246	1241	23496
5	4285	1291	32734

B. The unit was expressed as ng protein/mm^2 diet surface.

instar	LC_{50} (ng protein/mm^2)[a/]		
	sporulated culture washed in		purified crystal
	water	NaCl	
1	0.0373	0.0400	0.0947
2	0.1346	0.0818	0.5850
3	0.1845	0.2142	0.4635
4	1.4554	0.7977	4.3640
5	1.4687	0.8295	6.0790

a/ Concentrations of the toxin around the surface of diet.

Results of assay for larvicidal activity of samples derived from *Btk* HD-1 protein crystals against first instar and second instars of *Aedes aegypti* larvae are summarized in table 4.8. The first set was assayed against the sample derived from culture washing with distilled water and that derived from culture washing with sodium chloride. The second set was assay against the sample derived from culture washing with distilled water and that made from purified crystals. It was found that natural mortality occurred at the rate of 6.67 percent in the control group. Thus percent mortality was corrected by using Abbott's formula (25) in order to compensate for the mortality in the control group. The concentrations of protein crystals derived from sample that washed with distilled water were varied from 1.53×10^5 to 49 crystals/ml and the larvicidal activity was found to vary from 100 to 3.33 percent according to the concentration used. The larvicidal activity observed was vary from 68.97 to 3.3 percent when tested against various concentration of protein crystals derived from sample that washed with sodium chloride. In the second set, the larvicidal activity against first and second instar of *Ae. aegypti* larvae was found to varied from 100% to 3.22 percent in response to various concentrations of protein crystals derived from sample that washed with distilled water. The larvicidal activity was found to vary from 100 to 0 percent when tested against various concentrations of purified protein crystals from 9.55×10^4 to 6 crystals/ml as shown in table 4.8. The probit analysis of these data was made and summarized in table 4.8 and figure 4.7.

When the third and fourth instars of *Ae. aegypti* larvae were used as target organism, results of larvicidal activity were

summarized in table 4.9. It was found that the concentrations of the sample used were varied from $1.23x10^6$ to 389 crystals/ml for the sample derived from culture washing with distilled water, and produced mortality rates ranging from 96.67 to 0 percent. The concentrations of sample that obtained from washing with sodium chloride used were varied from $3.63x10^5$ to 114.8 crystals/ml, and the mortality rates were 90.00 to 0 percent. The concentrations of purified protein crystals used were in the range of $9.55x10^5$ to 302 crystals/ml which yielded mortality rates from 96.67 to 0 percent. The probit analysis of these data were done and summarized in table 4.9 and figure 4.8.

The comparison of potency of all specimens was determined using 50% end point (LC_{50}) and results were summarized in table 4.10. Results demonstrated that the LC_{50} values were increased in parallel with the age of the larvae. The purified protein crystals had the highest larvicidal activity against all age group of larvae.

According to the results given in Table 4.2 to 4.10 and figure 4.2-4.8 as the larvae getting older, an inverse relationship between mortality and larval age became apparent. This de crease in susceptibility was measured in a decrease in mortality (Table 4.7 and 4.10).

The larvicidal activities of P1 or P2 fractions against *S. exigua* and *Ae. aegypti* larvae compared with intact crystals were determined using the third instar and the second instar larvae, respectively. Results are summarized in tables 4.11 to 4.13. The P2 fractions were not able to kill *S. exigua* larvae, as shown in Table 4.14, the concentration of P2 fractions which

equivalent to 7.98 ng protein/mm^2 of diet surface can caused mortality rate at only 18.2 percent, therefore the LC_{50} value could not estimate. However, P2 fraction had a significant activity against the mosquito larvae as shown in table 4.14. Its toxicity was 10 folds higher than that of the intact crystals, and 40 fold higher than that of the P1 fractions. The toxicity of P1 fraction against *S. exigua* larvae was 2 fold higher than that of intact crystals. Probit analysis of these data were done and results were summarized in figures 4.9-4.11.

Table 4.8 Percentage mortalities of *Aedes aegypti* larvae at first and second instars after fed upon distilled water containing of culture preparations of the crystal toxins of *B. thuringiensis* subsp. *kurstaki* strain HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	1.53×10^5	29	29	100.00	$+\infty$
	3.09×10^4	28	27	96.43	6.80
	6166	29	12	41.38	4.78
	1230	29	7	24.14	4.30
	245	29	1	3.45	3.18
	49	30	1	3.33	3.16
	0	30	0	0	-
N	4.47×10^4	29	20	68.97	5.50
	8912	29	16	55.17	5.13
	1820	29	4	13.79	3.91
	363	30	3	10.00	3.72
	72	30	2	6.67	3.50
	14	30	1	3.33	3.16
	0	30	0	0	-
F	1.58×10^5	28	28	100.00	$+\infty$
	3.16×10^4	29	19	65.52	5.33
	6310	30	9	30.00	4.32
	1259	31	5	16.13	3.71
	251	30	2	6.66	$-\infty$
	50	29	2	6.90	$-\infty$
	10	31	1	3.22	$-\infty$
	0	30	2	6.66	-
C	9.55×10^4	29	29	100.00	$+\infty$
	1.91×10^4	31	22	70.97	5.49
	3802	30	13	43.33	4.72
	759	30	3	10.00	3.15
	151	31	3	9.68	3.10
	30	29	1	3.45	$-\infty$
	6	27	3	11.11	3.29
	0	30	2	6.66	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per millilitre of distilled water.

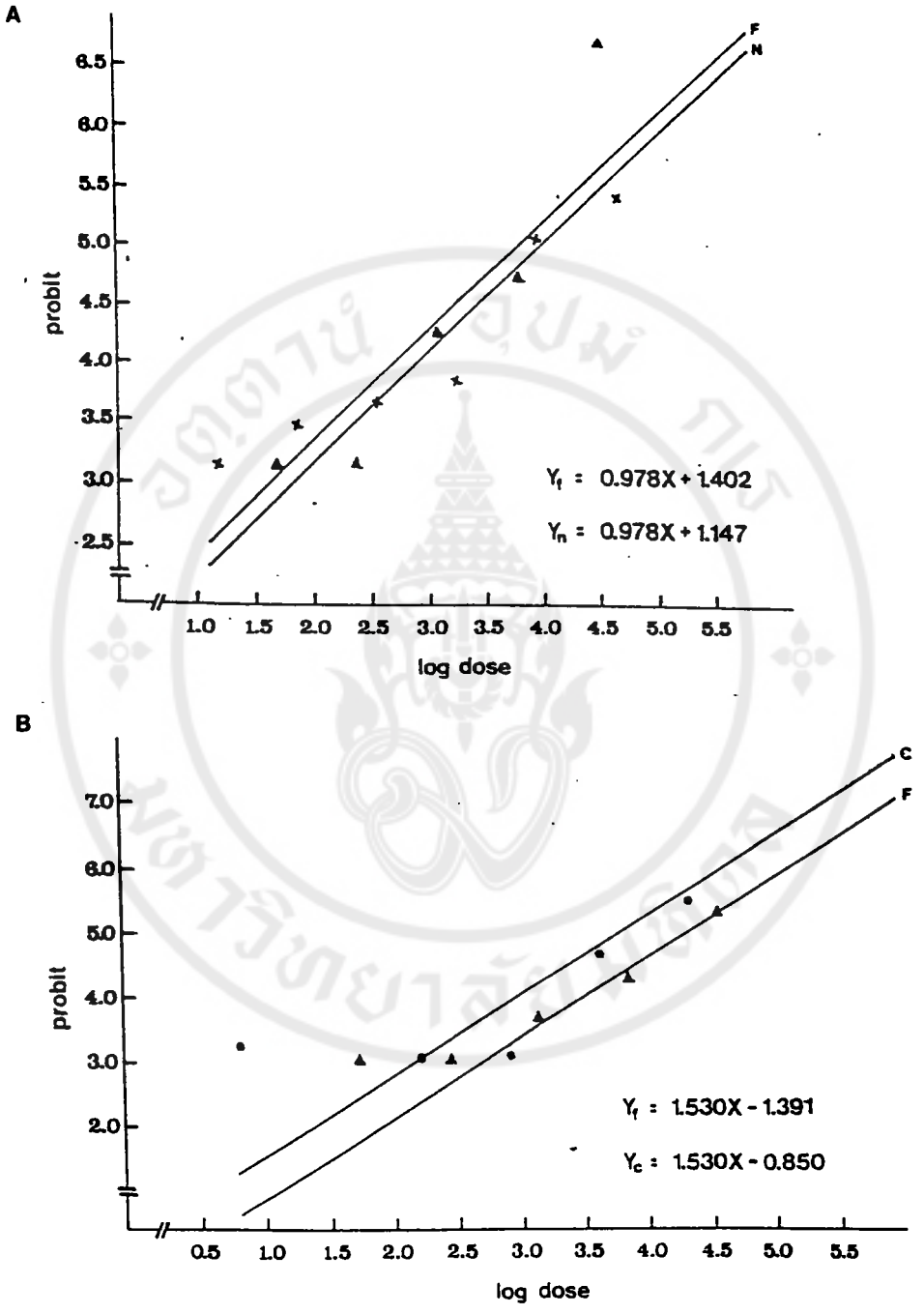

Figure 4.7. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 1st and 2nd instar larvae of *Ae. aegypti*.
A. sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N).
B. sample derived from washing with distilled water (▲, F) compared with that of purified crystal (•, C).

Table 4.9 Percentage mortalities of *Aedes aegypti* larvae at third and fourth instars after fed upon distilled water containing of various preparations of the protein crystals of *B. thuringiensis* subsp. *kurstaki* strain HD-1 at given dosages.

specimen[1]	unit[2]	no. larvae		% mortality	empirical probit
		tested	dead		
F	1.23×10^6	30	29	96.67	6.83
	2.45×10^5	30	27	90.00	6.28
	4.9×10^4	30	20	66.67	5.43
	9772	30	0	0.00	$-\infty$
	1950	30	0	0.00	$-\infty$
	389	30	1	3.33	3.16
	0	30	0	0.00	-
N	3.63×10^5	30	27	90.00	6.28
	7.24×10^4	30	24	80.00	5.84
	1.45×10^4	30	7	23.33	4.27
	2884	30	1	3.33	3.16
	575	30	0	0.00	$-\infty$
	115	30	0	0.00	$-\infty$
	0	30	0	0.00	-
C	9.55×10^5	30	29	96.67	6.83
	1.91×10^5	30	27	90.00	6.28
	3.80×10^4	30	21	70.00	5.52
	7586	30	4	13.33	3.89
	1514	30	3	10.00	3.72
	302	30	0	0.00	$-\infty$
	0	30	0	0.00	-

1. Specimens were sample derived from culture washing with distilled water (F), sample derived from culture washing with 1 M sodium chloride (N) and purified crystals (C).

2. Concentrations of test specimen expressed in term of number of crystals per millilitre of distilled water.

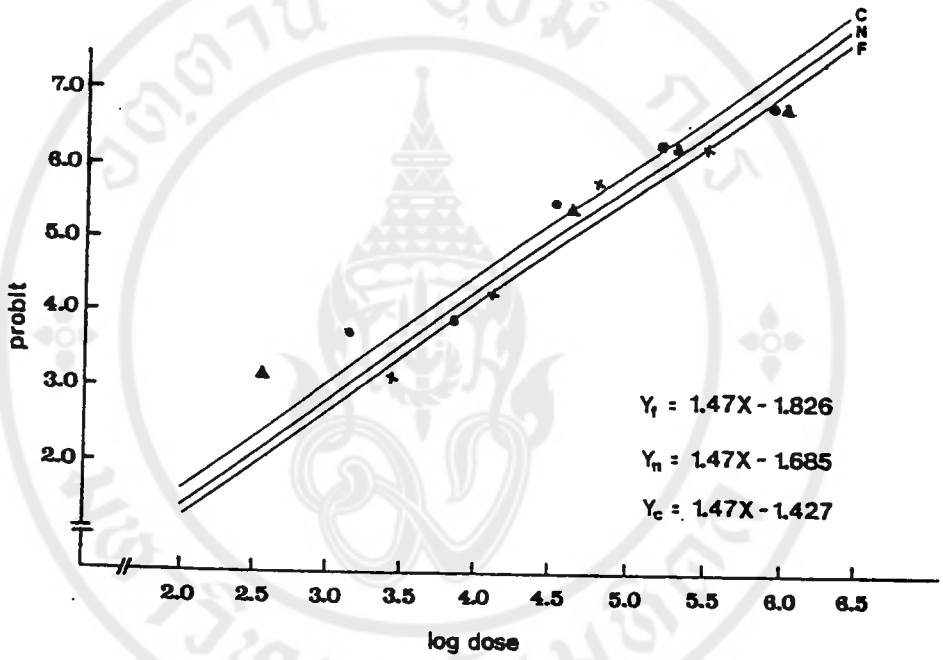

Figure 4.8. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 samples on 3rd and 4th instar larvae of *Ae. aegypti*. Sample derived from washing with distilled water (▲, F) compared with sample derived from washing with sodium chloride (x, N) and with that of purified crystal (•, C).

Table 4.10 Comparison of lethal concentration 50% end point (LC_{50}) values from bioassay of *Aedes aegypti* at various instars against crude preparation and purified preparation of parasporal crystal toxin of *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1. The mortalities were read after 3 days.

A. The unit was expressed as number of crystals/ml

instar	LC_{50} (no. of crystals/ml)		
	sporulated culture washed in		purified crystal
	water	NaCl	
1&2	8,320	14,130	3,682
3&4	44,055	35,318	23,550

B. The unit was expressed as ng protein/ml

instar	LC_{50} (ng protein/ml)		
	sporulated culture washed in		purified crystal
	water	NaCl	
1&2	2.852	9.080	1.690
3&4	15.100	22.692	10.790

Table 4.11 Percentage mortalities of *Spodoptera exigua* 3rd instar larvae after fed upon diet containing of either purified protein crystals or P1 proteins of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

A. protein crystals

unit (ng protein/mm^2)	no. larvae		% mortality
	tested	dead	
2.88	21	19	90.48
1.45	22	16	72.73
0.72	22	12	54.55
0.36	21	7	33.33
0.18	21	6	28.57
0.09	25	9	36.00
0.00	25	2	8.00

B. P1 protein

unit (ng protein/mm^2)	no. larvae		% mortality
	tested	dead	
4.26	22	20	90.91
2.14	24	14	58.33
1.07	21	14	66.66
0.54	22	7	31.82
0.27	22	4	18.18
0.13	25	3	12.00
0.00	25	2	8.00

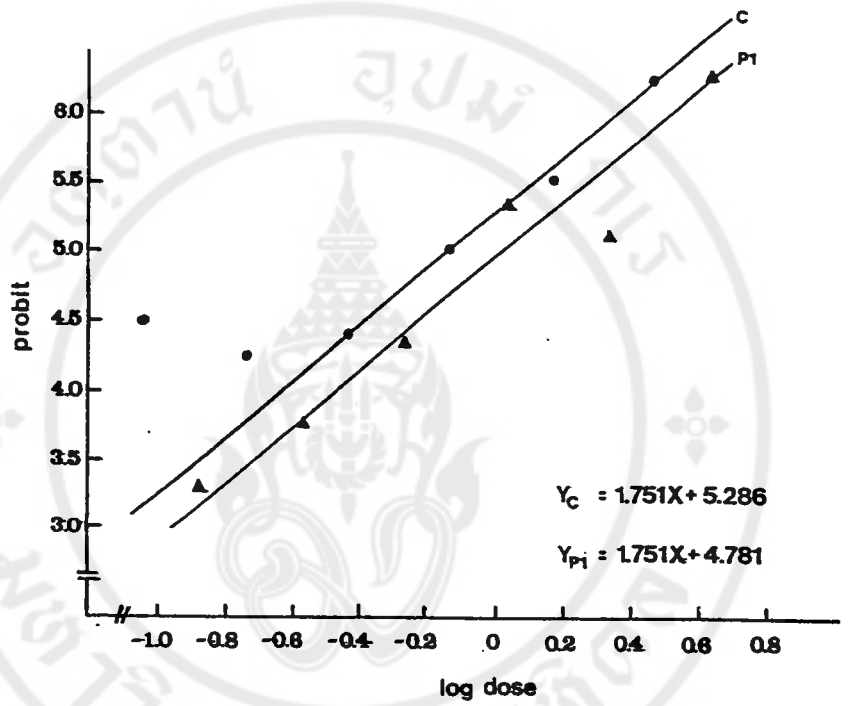

Figure 4.9. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 purified crystal (., C) and P1 protein (▲, P1) on 3rd instar larvae of *S. exigua*.

Table 4.12 Percentage mortalities of *Aedes aegypti* 2nd instar larvae after fed upon diet containing of either purified crystals or P1 protein of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

A. P1 proteins

unit (ng protein/mm^2)	no. larvae		% mortality
	tested	dead	
916.80	20	13	65.00
183.36	21	9	42.86
36.67	20	1	5.00
7.33	21	1	4.76
1.47	20	0	0
0.29	20	0	0
0	20	0	0

B. P2 protein

unit (ng protein/mm^2)	no. larvae		% mortality
	tested	dead	
153.43	20	18	90.00
30.69	20	15	75.00
6.14	19	11	57.90
1.23	20	1	5.00
0.25	20	0	0
0.05	20	0	0
0	20	0	0

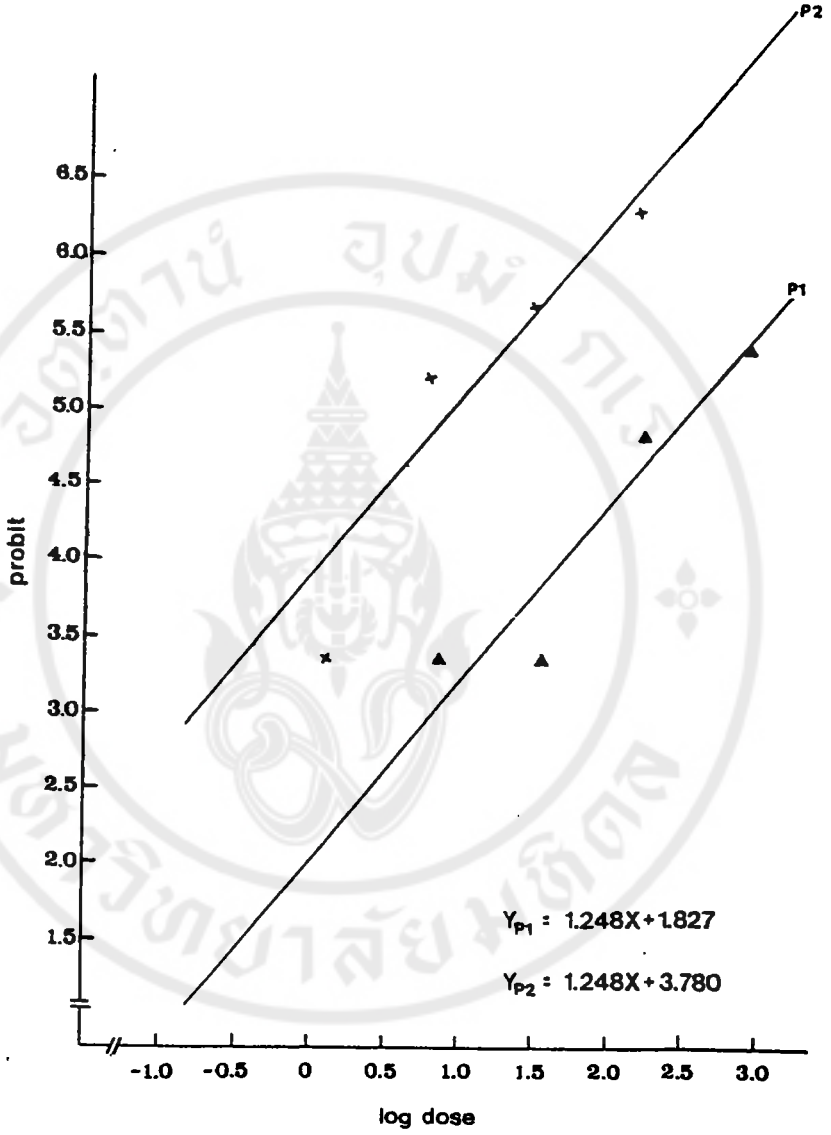

Figure 4.10. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 P1 protein (▲, P1) and P2 protein (x , P2) on 2nd instar larvae of *Ae. aegypti*.

Table 4.13 Percentage mortalities of *Aedes aegypti* 2nd instar larvae after fed upon diet contaminated with either purified protein crystals or P2 protein of *B. thuringiensis* subsp. *kurstaki* HD-1 at given dosages.

A. protein crystals

unit (ng protein/mm^2)	no. larvae		% mortality
	tested	dead	
568.00	20	12	60.00
113.60	20	11	55.00
22.72	20	9	45.00
4.54	20	1	5.00
0.91	20	0	0
0	20	0	0

B. P2 protein

unit (ng protein/mm^2)	no. larvae		% mortality
	tested	dead	
141.40	20	13	65.00
28.28	20	12	60.00
5.66	20	15	75.00
1.13	20	1	5.00
0.23	20	1	5.00
0	20	0	0

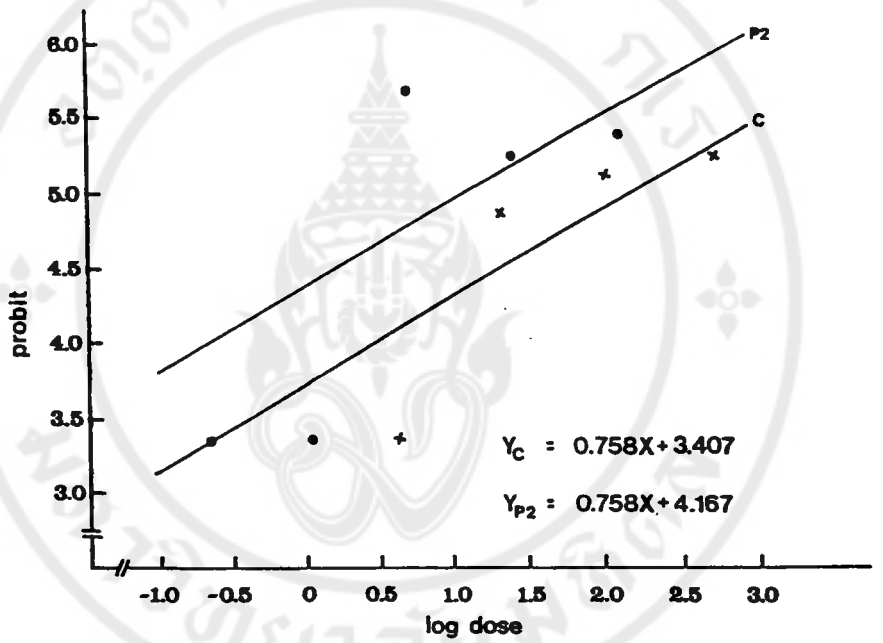

Figure 4.11. Probit diagram, with provisional lines, for effect of *B. thuringiensis* ssp. *kurstaki* strain HD-1 purified crystal (x , C) and P2 protein (. , P2) on 2nd instar larvae of *Ae. aegypti*.

Table 4.14 Toxic activities of protein isolates from the parasporal crystal of *B. thuiringiensis* ssp. *kurstaki*.

Toxins	LC_{50}	
	S. exigua (ng protein/mm^2)	*Ae. aegypti* (ng protein/ml)
crystal	0.686	109.90
P1	1.333	400.87
P2	Not toxic	10.91

The mortality was read at 6 days for *S. exigua* and at 3 days for *Aedes aegypti*.

4 Characterization of the crystal proteins in Btk HD-1 samples by using SDS-PAGE.

The characterization of proteins derived from protein crystals that obtained from various preparations of *Btk HD-1* from cultures was done by using SDS-PAGE. An electrogram of these samples were shown in figure 4.11. The amount of protein crystals that measured in dry weight loaded onto each slot of gel was 240 µg. There were no major difference in the patterns of each sample. The sample derived from culture washing with distilled water (lane 1, figure 4.11), sample derived from culture washing with sodium chloride (lane 2, figure 4.11) and sample of purified crystals (lane 3, figure 4.11), produced one major band similar to the one that found in P1 protein fraction (lane 4, figure 4.11). However, there were other five protein bands near 65 Kda band in the patterns of samples derived from various preparations and purified crystals that could not be seen clearly in P1 or P2 protein fractions.

By using myosin (205 Kda), β-galactosidase (116 Kda), bovine albumin (66 Kda), and egg albumin (44 Kda) as molecular weight markers (lane 6 and 7, figure 4.11), the relative molecular weight of the major band in all samples except that of P2 protein fraction was estimated as 135 Kda. All samples and purified crystals also produced a 65 Kda protein band similar to those found in P2 protein fraction (lane 5, figure 4.11).

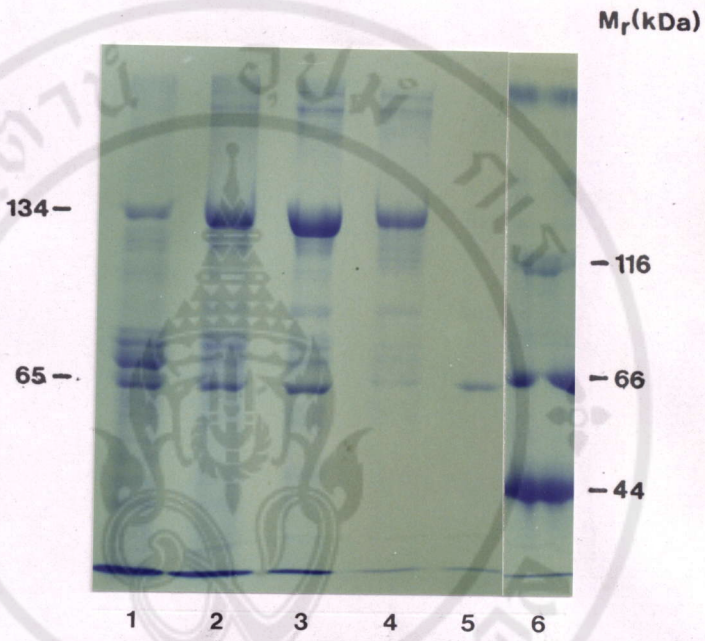

Figure 4.12. Electrophoretic pattern on 7.5% SDS-PAGE of sample derived from culture washed in distilled water (lane 1), sample derived from culture washed in NaCl (lane 2), purified crystal (lane 3), solubilized P1 protein (lane 4), and P2 protein (lane 5). Molecular weight markers (lane 6) are as follows : β-galactosidase (116 kDa), bovine albumin (66 kDa) and egg albumin (44kDa).

CHAPTER V

DISCUSSION

It has been shown that *B. thuringiensis* subsp. *kurstaki* HD-1 contains proteinaceous crystals which can kill lepidopteran and/or dipteran larvae. In order to study the toxicity of these crystals, the purified ones should be obtained. Several methods have been investigated for a long time such as biphasic system using organic solvents and density gradient centrifugation. However, it has been demonstrated that organic solvents can destroy crystals (18). The floatation technique using sodium chloride in this study provided good yield in a reasonably short period of time, without any expensive materials or trained personnel. Results from this experiment showed that the purity of the crystals was always higher than 95%. The yield obtained (25%) was rather low when compared to a biphasic system (35%) which was shown by Cooksey (18). The yield obtained by the flotation technique was the same when compared to that of density gradient centrifugation (20%) which was done by Ponglikitmongkol (66).

To date, two types of the proteinaceous crystals have been found. There are a bipyramidal crystal and a cuboidal crystal (76, 96). Yamamoto and McLaughlin (97) demonstrated that the HD-1 strain contains a 135 kDa protein (P1) which can kill lepidopteran larvae and a cuboidal crystal (P2) which can kill dipteran larvae. The 135 kDa protein is embedded in the bipyramidal crystal and the 65 kDa protein is embedded in the cuboidal crystal. This is demonstrated by Yamamoto and Iizuka (96). They demonstrated that P2 protein does not form the bipyramidal crystal which con-

sists only of P1 by using selective extraction of P2 and electron microscopic observation. Only the cuboidal crystals was solubilized by alkali treatment without a thiol reagent. Antiserum prepared against the only 135 kDa protein which produced by *B. thuringiensis* subsp. *kurstaki* mutant strain HD-73 was tested against proteins derived from protein crystals of *B. thuringiensis* subsp. *kurstaki* HD-1. It was found that antiserum gave a strong reaction against 135 kDa protein but did not gave any reaction against 65 kDa protein (96). Samasanti and his coworker (71) demonstrated that when the crystals was exposed to H_2O_2, the cuboidal body disappeared, they could not detect any 65 kDa protein from H_2O_2 treated crystals by using SDS-polyacrylamide gel electrophoresis and the mosquitocidal activity decreased sharply. In this study, these two proteins were separated by their differential solubility in alkaline reducing buffer. After electrophorysis in SDS-polyacrylamide gel (figure 4.1 and 4.11), it was shown that P1 protein has relative molecular weight of 134 Kda and P2 has relative molecular weight of 65 Kda. This result was similar to those of Yamamoto and McLaughlin (97), Thomas and Ellar (83), and Knowles et al. (43). This crystal preparation was probably contaminated with bacterial proteases even after it was extensively washed in sodium chloride (17, 61, 96). It was suggested that these proteinases would cleaved P1 protein when it was liberated. It should be noted that a large portion of P1 molecule is vulnerable to protease attack (10, 43, 96). Thus there were one or more minor bands migrated in the same lane. It was found that the P2 protein was resistant to protease such as trypsin, chymotrypsin, and *Trichoplusia ni* larvae gut juice. For this reason, they had

not any minor bands appeared with P2 major band when analyzed with SDS-polyacrylamide gel electrophoresis (91, 96).

When P1 protein fraction was tested against *Spodoptera exigua* larvae, it yielded 2-fold lower toxicity than that of intact crystals. This result was similar to that found by Lecadet and Martouret (48). They demonstrated that the dithiothreitol solubilized crystals of subsp. *entomocidus* and *aizawai* had lower toxicity against *Spodoptera littoralis*, in comparison with that of intact crystals. In the same year Jaquet et al. (39) have done toxicity test of both intact crystals and P1 protein of subsp. *kurstaki* HD-1 , *morrisoni* and *kenyae* against *Pieris brassicae* and *Heliothis virescens* larvae. They demonstrated that only minor differences in the ED_{50} (effective dose needed for 50% activity) among crystals and P1 toxin were obtained in the assays with *P. brassicae*. In the case of *H. virescens*, however, predissolution of the crystals greatly improved the activity of these delta-endotoxin. This suggested that the degree of solubility of the crystals in the gut juice of insect larvae may be an important factor influence the potency of Bt delta-endotoxin. However, when P1 protein fraction was tested against mosquito larvae, it could kill mosquito larvae. This might be resulted from contamination of P2 fraction in the preparation which had been demonstrated by Nicholl and his co-workers (60). The toxicity of P1 protein against *Aedes taeniorhynchus* is less than 500-fold when compared with that of P2 protein (97).

The toxicity of P2 protein against *Aedes aegypti* larvae was higher than that of intact crystals. This is similar to that found by Nicholl and his co-workers (60). However, they also found

that the toxicity of this protein against *Anopheles gambiae* was lower than that of intact crystal. Even though P2 toxin could kill many lepidopteran larvae such as cabbage looper (*Trichoplusia ni*), tobacco budworm (*Heliothis virescens*) (96), and tobacco hornworm (*Manduca sexta*) (90), however, it had not any toxicity against beet armyworm (*Spodoptera exigua*) in this study (Table 4.14). It has been shown by Yamamoto and Iizuka (96) that P2 toxin is much less toxic to the tobacco budworm than to the cabbage looper, and the toxicity of this toxin is less against these larvae when compared with P1 toxin. Therefore, the possibility of using P2 toxin in killing lepidopteran larvae may be considered and be improved, either by using genetic engineering or other ways to get better toxicity than P1 toxin.

The insecticidal toxicity is found to depend on the proteinaceous crystals of *B. thuringiensis*. However, many investigators suggest that spore is also involved in the toxicity in some kind of lepidopteran larvae such as *Manduca sexta* (72), *Galleria mellonella*, and *Anagasta kuehniella* (33). In this experiment, the effect of spore against *S. exigua* was studied. The toxicity of purified crystals against *Spodoptera exigua* was less than the crude preparations which contained spores as shown in table 4.7. Therefore, the presence of spores enhanced the toxicity of crystals. When European Corn Borer (*Ostrinia nubilalis*) larvae is tested against four subspecies of *B. thuringiensis* (*kenyae, tolworthi, galleriae,* and *kurstaki* HD-1), it was found that pure crystals were toxic to the larvae, while a combination of spores and crystals are neccessary for maximum larval mortality (57). Li et al. (51) demonstrated that the crystals without live spores

(LC_{50} = 10^{10} crystals/g insect) were virtually inactive against *Galleria mellonella*. But in the presence of 0.001% spores the mortality of larvae increased from 0 to 36%, and when spore concentration was increased to 0.01% it killed 64 % larvae. In contrast to the results with *G. mellonella*, crystals produced larvicidal activity about 30 times as active as spores in *P. brassicae* larvae. Other evidences have been recorded of spore-crystal mixtures being more important in various degrees than the crystals of *B. thuringiensis* only, such as subsp. *alesti* in *Pseudaletia uni puncta* and *Colias eurytheme* (82), subsp. *alesti* and *tolworthi* in *Pieris brassicae* (81), subsp. *kurstaki* in *Choristoneura fumiferana* (24), subsp. *kurstaki* in *Plodia interpunctella* and *Ephestia cautella* (54). According to the study of Heimpel and Angus (33), 3 types of lepidopteran insects have been classified depending upon their susceptibility against *B. thuringiensis* crystal toxins : Type I insect can be killed by the crystals only in 1 to 7 hr, presence or absence of spores has not any effect ; Type II insect can be killed by the crystals in 2 to 4 days, addition of spores enhances the toxic activity of the crystals; and Type III insect is not killed by the ingestion of the crystals in the absence of spores. Thus, it is believed that *S. exigua* larvae do not behave like Type 1 insect. However, the spores of the *B. thuringiensis* HD-1 do not have any effect in enhancing the mosquitocidal activity as shown in table 4.10.

When toxicities of protein crystals produced by *B. thuringiensis* HD-1 were determined against *Ae. aegypti* larvae, it was observed that the larvicidal activity toward *Ae. aegypti* was varied regarding to the preparations and age of larvae. The puri-

fied crystal yielded highest larvicidal activity among the test sample. The protein crystal sample derived from the culture washed in distilled water yield higher larvicidal activity against early instar larvae than that derived from the culture washed in sodium chloride. This observation was different from those found with the late instar larvae. This evidence suggested that spore of the *B. thuringiensis* HD-1 do not involve in the larvicidal activity of P2 protein fraction. It has been demonstrated that the larvicidal activity against *Ae. aegypti* is quite high with the LC_{50} of *B. thuringiensis* HD-1 is approximately $2.6x10^4$ to $1.2x10^5$ bacteria/ml, (63, 70). Thus variations in the LC_{50} values among various studies are probably resulted from the differences in the bacterial strains, the growth conditions of the bacteria, the variations in the mosquito strains used in the bioassay, the differences in the bioassay sytem, or the procedures used in the preparation of the proteinaceous crystals.

To determine whether the decrease in susceptibility was due to natural resistance increasing with age, the probit mortality lines for each larval instar which showed in figure 4.2 to 4.8 were analyzed. The calculated slopes of the log concentration-probit regression lines for test against early instars were lower than those for tests against late instars (figure 4.2-4.8). This is not similar to that found by Mohd-Salleh and Lewis (57) and Wraight et al. (94). These investigators found that the calculated slopes of the probit regression lines for test against early instars were steeper than those calculated for later instar larvae. The slopes of the probit regression lines of the purified preparations were different from the slope of the crude prepara-

tion probit regression and may reflect the contribution of spores to toxicity (40, 55).

Bibilos and Andrew (10) demonstrated that proteases were produced during the late exponential phase of growth in the strains of *B. thuringiensis* and their activities increased dramatically at the onset of sporulation. Chestukhina et al. (17, 61) have found that the surface of crystal contained a mixture of both serine proteases and metalloproteases. They reported that high salt wash (1 M NaCl) was sufficient to remove 87% of the absorbed proteolytic activity. In this study, the crude preparation derived from culture washing in distilled water had less larvicidal activity against *S. exigua* larvae than that derived from culture washing in sodium chloride as shown in table 4.7. Therefore, the removal of proteases from *B. thuringiensis* subp. *kurstaki* HD-1 protein crystals increased the toxicity of protein crystals against *S. exigua* by 1.6 to 3.4 fold. This finding was comparable to the observation found by MacIntosh et al. (52). They found that serine protease inhibitor could potentiate the insecticidal activity of *B. thuringiensis* subsp. *kurstaki* protein crystals against *Heliothis virescens* by 2 to 4 folds. None of the protease inhibitors had insecticidal activity when tested alone. Tojo and Aizawai (85) reported that the toxicity of crystals decreased markedly after long incubation with gut juice protease of *Bombyx mori*. Bulla et al (14) suggested that any further breakdown probably was detrimental to toxic activity of the protein crystals. Thus the crude preparation derived from culture washing in NaCl, , the proteinceous crystals were not breakdown due to its proteases were removed. which resulted in high toxicity when compared with the

crude preparation derived from the culture washing in distilled water. However, the observation was different when tested against mosquito larvae (table 4.10), in which similar results were found only in later instars but not in early instars. MacIntosh (52) suggested that the toxicity of *B. thuringiensis* subsp. *israelensis* against *Ae. aegypti* could be increased by using protease inhibitor. But it was shown that P2 protein in *B. thuringiensis* HD-1 was resistant to proteases (96), therefore, the presence or absence of protease might not affect mosquitocidal of this protein crystals.

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APPENDIX 1

A. Preparation of an artificial diet for rearing of the beet armyworm.

Ingredient	Quantity.
Mung bean	375 g
Dried baker's yeast	25 g
Ascorbic acid	7.5 g
Sorbic acid	5 g
Methyl parabenzoate	7.5 g
Casein	7.5 g
Vitamin mixtures	15 ml
Formalin (40%)	6 ml
Agar	32 g
Distilled water	1800 ml

Three hundred and seventy five grams of raw mung bean was blended in 800 ml of water in a 3-litre beaker. Formalin, dried baker's yeast, methyl parabenzoate, sorbic acid, casein and ascorbic acid at the above mentioned amount were added and homogeneously mixed using magnetic stirrer. The dissolved warm agar was made from 32 grams of agar in 1 litre of distilled water. Vitamin mixtures were added and the whole mixture was further mixed using magnetic stirrer until homogeneous color was obtained. The medium was poured into rearing jars and let it cool down in a horizontal position. After cooling, the jars were stored for further studies.

Vitamin mixtures were composed of 600 mg niacin, 500 mg inositol, 600 mg calcium panthothenate, 150 mg thiamine, 300 mg riboflavin, 150 mg pyridoxine, 150 mg folic acid, 12 mg biotin, 2

mg vitamin B12 and 5 g choline chloride in 100 ml distilled water.

B. The nutrient broth supplemented with mineral salts (NBS) was consisting of 8 g nutrient broth, 0.08 g $CaCl_2$, 0.05 g $MnCl_2.4H_2O$, 0.005 g $ZnSO_4.7H_2O$, and 0.005 g $CuSO_4$ $.5H_2O$ in 1 litre of distilled water. Whenever solid media are required, 20 g of agar is added into the medium before sterilization by autoclave.

APPENDIX 2

Probit analysis (25) is the method of analysis that use of probit transformation in the statistical analysis of biological data resulting in linear regression of scatter and high variation data. Probit tranformation is only a convenient trick for represent the sigmoid response curve by a straight line, then the probit regression line is a very satisfactory representation of the results of the experiment. The probit regression line is subsequently useful for estimating the mediun lethal concentration, LC_{50}. The confidence interval can also be calculated by computing the 50th percentile of the distribution, m, and standard deviation, s_m.

The relative potency of two stimuli can be calculated by drawing parallel probit regression lines of two series of the response against the logarithm of the dose. The expression in relative potency is more trustworthy than the LD_{50} and can be used in comparison where as this stimuli has compared with a standard prepapation before.

The steps in computing the estimation of probit regression line and the relative potency are as follows :

1. Column x, the logarithm base 10 of concentrations which were selected.

2. Column n, the number of tested objects corresponse to x and column r, the number of objects responding corresponse to x. These data were recorded during the experiment.

3. Column p, compute the proportion of objects responding, $p = 100r/n$.

4. Read the probit of each p from Table I in reference , and enter as the 'empirical probit' to 2 decimal places. If p = 0 or p = 100, enter $-\infty$ or $+\infty$ respectively.

5. Plot empirical probits against x. Draw a provisional straight line to fit the points, judging its position by eye.

6. Read the expected probit, Y on the Y-axis at each x used in the experiment.

7. Column w, the weighting coefficient for each of Y which can be obtained from Table II in reference 25.

8. The next two columns, nw and nwx are the result of multiplication of previous corresponding column n, w, and x.

9. For each of expected probit Y, and p, the working probit, y, is read from Table IV (25).

10. For each value of y, calculated the value nwy and put in the column nwy.

11. Compute :

Snw = the sum of column nw

Snwx = the sum of column nwx

Snwy = the sum of column nwy

$Snwx^2$ = the sum of the products of elements in columns x and nwx.

Snwxy = the sum of the products of elements in columns y and nwx.

$Snwy^2$ = the sum of the products of elements in columns y and nwy.

12. Compute :

$b = S_{xy}/S_{xx}$

$\bar{x} = Snwx/Snw$

$\bar{y} = Snwy/Snw$

$S_{xx} = Snwx^2 - (Snw)^2/Snw$

$S_{xy} = Snwxy - (Snwx)(Snwy)/Snw$

$S_{yy} = Snwy^2 - (Snwy)^2/Snw$

The relationship between probit and concentration may be written :

$$Y = \bar{y} + b(x-\bar{x}) \quad \text{----------(I)}$$

In order to test the goodness of fit of the linear to the data. The X^2-test is used. The method of calculating X^2 is the weighted sum of the squares of the differences between the empirical and weighted probits. This may be written :

$$X^2 = Snw(y-Y)^2$$

$$= S_{yy} - S_{xy}^2/S_{xx}$$

with (k-2) degree of freedom (k is the number of doses)

If X^2 calculated $<$ or = $X^2(1-\alpha)$, k-2, the line adequately represent the data.

If X^2 calculated $>$ $X^2(1-\alpha)$, k-2, then the straight line does not adequately describe the relationship between stimulus and response.

After the statistical prove of the fitted line, the estimation of LC_{50} is

$$m = \bar{x} + (5 - \bar{y})/b$$

The additional steps in calculating relative potency are :

13. Summation of S_{XX}, S_{XY} and S_{YY} of every groups that have compared the relative potency. Hence an improved estimate of the regression coefficient is obtained by equation :

$$b = \Sigma S_{XY}/\Sigma S_{XX}$$

This regression coefficient is used in equation I to give improved estimates of the lines :

$$Y = \bar{y} + b(x-\bar{x})$$

$$X^2 = \Sigma S_{YY} - \Sigma S^2_{XY}/\Sigma S_{XX}.$$

with (k-2) + (n-1) degrees of freedom (n is the number of stimuli used.

An example of data of the *S. exigua* larvae (4th instar) to illustrate the whole procedure of calculating relative potency by probit analysis method.

Table 1 First stage of calculations for estimation of potencies of purified crystals relative to sample washed in distilled water

x	n	r	p(%)	Emp.	Y	nw	y	nwx	nwy	Y(cal)
					sample washed in distilled water (F)					
4.40	25	22	88.00	6.18	6.1	10.12	6.17	44.629	62.440	6.18
4.10	25	17	68.00	5.47	5.7	13.29	5.45	54.489	72.431	5.71
3.80	25	17	68.00	5.47	5.2	15.69	5.46	59.622	85.667	5.23
3.50	25	10	40.00	4.75	4.7	15.40	4.75	53.900	73.150	4.76
3.20	25	6	24.00	4.29	4.3	13.29	4.29	42.528	57.014	4.28
2.90	25	3	12.00	3.83	3.8	9.26	3.83	26.854	35.466	3.81
					purified crystals (C)					
4.79	25	15	60.00	5.25	5.7	13.29	5.19	63.659	68.975	5.63
4.49	25	18	72.00	5.58	5.2	15.69	5.56	70.448	87.236	5.15
4.19	25	11	44.00	4.85	4.8	15.69	4.85	65.741	76.097	4.68
3.89	25	5	20.00	4.16	4.3	13.29	4.17	51.698	55.419	4.20
3.59	25	2	8.00	3.60	3.9	10.12	3.65	36.331	36.938	3.73
3.29	25	0	0	$-\infty$	3.4	5.94	2.91	19.543	17.285	3.26

Emp. = Empirical probit,

Y(cal) = Y values calculated from eqation (1.1) and (1.2) for F and C, respectively.

F : Snw = 77.05 , Snwx = 282.022 , Snwy = 386.168

$Snwx^2$ = 1048.9534 , Snwxy = 1438.7378 , $Snwy^2$ = 1975.2801

S_{xx} = 16.6833 , S_{xy} = 25.2670 , S_{yy} = 39.8372

C : Snw = 74.02 , Snwx = 307.420 , Snwy = 341.951

$Snwx^2$ = 1292.3273 , Snwxy = 1445.8975 , $Snwy^2$ = 1628.3509

S_{xx} = 15.5500 , S_{xy} = 25.7072 , S_{yy} = 48.5939

Table 2 Second stage of calculations for relative potencies of toxins

	$\bar{x}$	$\bar{y}$	
F	3.6602	5.0119	
C	4.1532	4.6197	
	$Snwx^2$	Snwxy	$Snwy^2$ substrate S^2_{xy}/S_{xx}
F	1048.9534	1438.7378	1975.2801
	1032.2701	1413.4708	1935.4429
	16.6833	25.2670	39.8372 - 38.2671 = 1.570
C	1292.3273	1445.8975	1628.3059
	1276.7773	1420.1903	1579.7120
	15.5500	25.7072	48.5939 - 42.4990 = 6.095
Total	32.2333	50.9742	88.4311 - 80.6113 = 7.820

$b = 50.9742/32.2333$

$= 1.581$

$Y = \bar{y} + b(x-\bar{x})$

F : $Y = 5.0119 + 1.581(x-3.6602)$

$= 1.581x - 0.775$ ———————— (1.1)

C : $Y = 4.6197 + 1.581(x-4.1532)$

$= 1.581x - 1.947$ ———— (1.2)

$X^2 = S_{yy} - S^2_{xy}/S_{xx}$

F : $X^2 = 39.8372 - (25.2670)^2/16.6833$

$= 1.570$

C : $X^2 = 48.5939 - (25.7072)^2/15.5500$

$= 6.095$

Total : $X^2 = 88.4311 - (50.9742)^2/32.2333$

$= 7.820$

Table 3 Analysis of X^2

	d.f.	Σ squares
Parallel of regression	1	0.155
Heterogeneity	8	7.665
Total	9	7.820

If $\alpha = 0.05$, we find that $X^2_{.95}$, for 9 d.f. (degree of freedom) = 16.9, the calculated $X^2 = 7.820$. Since this is not larger than $X^2_{.95}$, we accepted the fitted lines.

The estimate of LC_{50} are : $m = \bar{x} + (5-\bar{y})/b$

F : $m = 3.6602 + (5-5.0119)/1.581$

$= 3.653$

C : $m = 4.1532 + (5-4.6197)/1.581$

$= 4.394$

Equation (1.1) and (1.2) have been used to calculate a new set of values of Y in a final column of table 1. Agreement is good and further calculation is not neccessary.