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Degradation of the Parasporal Crystal Proteins of *Bacillus thuringiensis* and Induction of Stress Protein Synthesis in *Bt* δ-endotoxin Ingested Larvae of Fall Webworm, *Hyphantria cunea*

*Bacillus thuringiensis*의 내독소 단백질의 분해와 흰불나방 섭취유충에서 스트레스 단백질합성의 유발

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ABSTRACT The protein components of each crystal toxin of *Bacillus thuringiensis kurstaki* and *aizawai* were separated by SDS-PAGE. The major crystal proteins from both strains were composed of polypeptides having molecular weights of 130 kd and 64 kd. Digestive mixture of both toxins with trypsin and gut juices shared 62 kd polypeptide which may be major active toxin. However, aizawai produced much less amount of 62 kd polypeptide than *kurstaki* did. On ingestion with Bt δ-endotoxin, larvae of *Hyphantria cunea* developed 45 kd stress protein in the several tissues including fat body, which was induced by heat and cold shock.

KEY WORDS *Bacillus thuringiensis kurstaki, aizawai, Hyphantria cunea,* δ-endotoxin, stress protein

초 록 *Bacillus thuringiensis kurstaki*와 *aizawai*의 내독소단백질을 알칼리용액 또는 트립신, 곤충소화액 등을 처리하여 전기영동한 후 단백질패턴을 비교하였다. 두 균주의 주요 결정단백질은 130 kd과 64 kd의 단백질이었으며, 소화액이나 효소로 처리한 경우 공통적으로 62 kd의 활성독소가 생성되었다. 그러나, *aizawai*는 *kurstaki*에 비해 현저히 적은 양의 62 kd 단백질을 생성하였다. 흰불나방의 유충이 *Bacillus thuringiensis* 독소를 섭식하였을 때 지방체를 비롯한 몇 가지 조직에서 45 kd의 스트레스 단백질이 유발되었는데, 이 단백질은 열충격이나 저온충격시에도 마찬가지로 생성되었다

검색어 Bacillus thuringiensis *kurstaki, aizawai, Hyphantria cunea,* δ-내독소, 스트레스 단백질

Bacillus thuringiensis produces a crystal protein (δ-endotoxin) in the sporulating cell, which is specifically toxic to the larvae of certain insects. These protein crystals are composed of one or more polypeptides in the form of inactive protoxins which are highly toxic only when ingested (Angus 1956). The effective proteolytic conditions at an alkaline pH of gut juice of the susceptible insects are thought to be responsible for the activation and the selective action of this toxin (Lecadet and Lereclus 1984). Although lots of information on the investigations concerned to the enzymatic degradation of the crystal protein is found in the literature (Lecadet and Dedonder 1966, Faust *et al.* 1974, Kim *et al.* 1992), a well-defined analysis of the digested products is required to elucidate the molecular mechanism of action-the activation of a specific insecticidal function. In this paper we have compared the digested products of the crystal δ-endotoxins of *B. thuringiensis* var. *kurstaki* and *aizawai* by gut juices and trypsin, in addition to the induction of stress protein by δ-endotoxin-ingested larvae of fall webworm, *H.*

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cunea.

MATERIALS AND METHODS

Bacterial Strains and Insects

B. thuringiensis var. *kurstaki* HD-1 and *B thuringiensis* var. *aizawai* were obtained from Dr. Faust (USDA, Maryland). Further, *Bombyx mori* and *H cunea* required for the experiments were obtained from a colony maintained in the laboratory of Insect Pathology, Seoul National University, and Insects Resource Laboratory of Genetic Engineering Research Institute, KIST, respectively. Larvae were reared on respective artificial diet at 26± 1°C and 75± 5% relative humidity with a photoperiod of 16h-light and 8h-dark.

Growth and Toxin Preparation

The growth and crystal purification were performed as described by Thomas and Ellar (1983). The δ-endotoxin inclusions from *B. thuringiensis* were separated from spores and cell debris by ultracentrifugation on discontinuous sucrose density gradients.

Purified crystals were dissolved in pH 9.5, 50 mM sodium bicarbonate/NaOH containing 10 mM dithiothreitol for 90 min at 37°C. The soluble fraction was activated by incubation with gut juices from *B. mori* and *H. cunea*, or 0.05% trypsin (Sigma Co.) at 37°C for various times. Crude gut juices were prepared as vomits by electric shock (Nishiitsutsuji-Uwo *et al.* 1979).

Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) using a 12.5% separating slab gel unless otherwise noted. All samples were heated at 90°C for 9 min in the presence of 2% SDS and 5% 2-mercaptoethanol. Gels were Coomassie-stained following completion of the run.

Induction of Stress Proteins

For heat and cold shock, last instar larvae of *H. cunea* were kept at 42°C for 2h or at 4°C for 6h, respectively. To induce the stress proteins by *B. thuringiensis* δ-endotoxin, larvae were fed with toxin-mixed diets(100 μg/g, w/w). Fat body, Malpighian tubule, and gut were isolated 12h after toxin ingestion. To compare the stress proteins by parasitism with those by *B. thuringiensis* toxication, nematode-infected brown planthoppers were obtained from laboratory of nematode (Gyeongsang National University) and body proteins were electrophoresed.

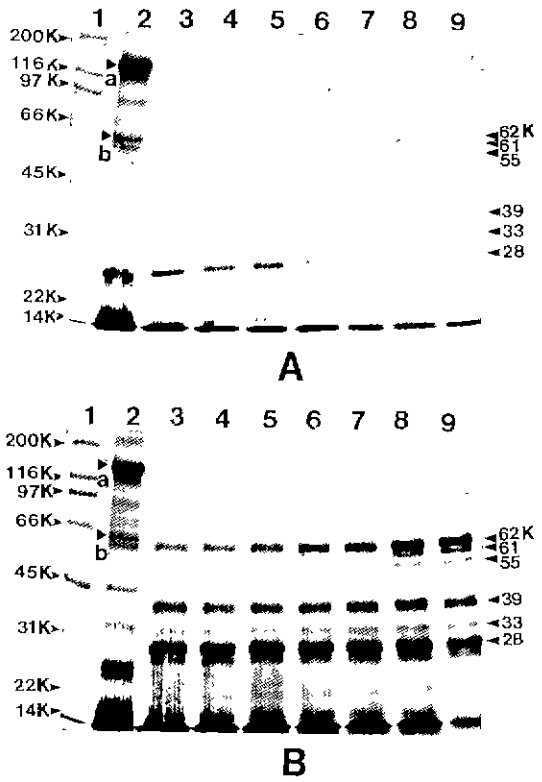

Fig. 1. Activation of *B. thuringiensis* var *kurstaki* (Btk) crystal δ-endotoxin by gut extracts of *Hyphantria cunea* (A) and *Bombyx mori* (B) SDS-PAGE of crystal δ-endotoxin. Coomassie-blue-stained track 1, molecular mass standard; track 2, native crystal protein; tracks 3-9, gut extracts-activated crystal proteins for 0.25, 0.5, 1, 3, 6, 9, and 24h: a, 130 kd (P_1): b, 64 kd (P_2).

RESULTS AND DISCUSSION

The crystal toxins of *B thuringiensis* var. *kurstaki* HD-1 were dissolved in alkaline solution and separated by SDS-PAGE The SDS-PAGE pattern of the products showed one predominant band of apparent molecular weight 130 kd, together with 64 kd, 62 kd, and several other bands (Fig. 1A, B). Yama-

moto & McLaughlin (1981) reported that *B. thuringiensis* var. kurstaki HD-1 contains one or more protoxins (P_1) in 130-140 kd size range, but in addition native crystals contain an extra polypeptide(P_2) of 65 kd. They showed P_1 and P_2 were almost equally toxic to the cabbage looper, *Trichoplusiani*, larvae. In our experiment, 130 kd polypeptide corresponds to the P_1 spectrum and 62 kd polypeptide corresponds to the P_2 spectrum, respectively. We also observed the crystal proteins in the presence of an alkaline buffer showing the 62 kd active toxin without futher enzyme treatment. Bulla *et al.* (1981) showed the generation of toxic peptide from the crystal protein of subsp. *kurstaki* by spontaneous hydrolysis at alkaline pH. In our result, several other minor polypeptides might be the residual spore contaminants and degraded intermediates. The number and size of the polypeptides constituting the crystal protein from *B. thuringiensis* were strikingly divergent (Calabrese *et al* 1980, Huber *et al.* 1981, Huber & Lüthy 1981, Kim *et al.* 1991). The major protoxins are, however, involved in P_1 and P_2 categories. Bulla *et al.* (1981) demonstrated degradation of the crystal protein during solubilization and pointed out the presence of a protease associated with the crystal.

When protoxin was incubated with protease from *H. cunea* and *B. mori* gut juice, 130 kd polypeptide disappeared and fragmented proteins appeared as bands representing molecular weights of 62 kd, 61 kd, 55 kd, 39 kd, 33 kd, and 28 kd on SDS-PAGE. In our results, three polypeptides having molecular weight of 62 kd, 61 kd and 55 kd are active toxin which might have been originated from P_1 and P_2 (Yamamoto & McLaughlin 1981). These results are consistent and are similar to the results from several laboratories indicating that protoxin activation yields an active toxin of molecular mass 55-72 kd (Haider *et al* 1986, Schnepf & Whiteley 1981). Temporal gut juice treatment indicated that 62 kd toxin increased with incubation time (Fig. 1).

Tryptic digestion of *B. thuringiensis* var *kurstaki* crystal protein produced a protein pattern similar to *H. cunea* gut juice-activated samples after 24hincubation (Fig. 2A). Further proteolysis (8 days) yielded polypeptides of 62 kd and 61 kd (Fig. 2B). Tryptic digestions of crystal proteins from four serotypes (Lilly *et al.* 1980) obtained toxic polypeptides approximately of molecular weight 70 kd. This divergent result might be due to the difference in the method of crystal dissolution and the procedure of analysis employed (Tanada 1993). In the present study, proteolytic processing of var. *krustaki* protoxin with lepidoptera gut juices and with trypsin, yielded same molecular mass of toxic peptides.

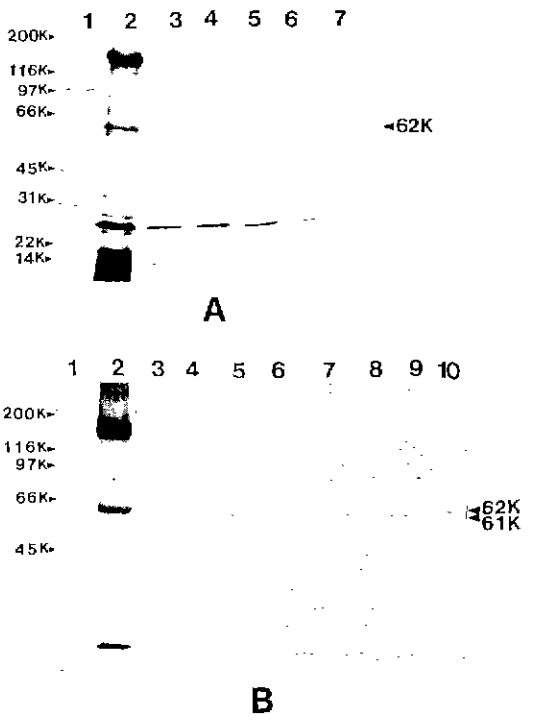

Fig. 2. Coomassie blue stained SDS-PAGE of crystal δ-endotoxin after trypsin treatments. A, molecular mass standard (track 1); *B. thuringiensis* var. *kurstaki* native crystal protein(track 2). Crystal proteins incubated at 37 °C for 0.25, 1, 6, 9, and 24h (tracks 3-7); *B. molecular* mass standard (track 1); *B. thuringiensis* var. *kurstaki* native crystal protein (track 2) Crystal proteins incubated at room temperature for 1, 2, 3, 4, 5, 6, 7 and 8 days (tracks 3-10).

Crystal proteins of *B. thuringiensis* var. *kurstaki* and *aizawai* had approximately equal quantitites of 130 kd and 64 kd bands (Fig. 3A, B). Var. *aizawai*, however, showed more minor bands having low masses than var. *kurstaki*. When solubilized proteins were treated with trypsin or with gut juice, the pro-

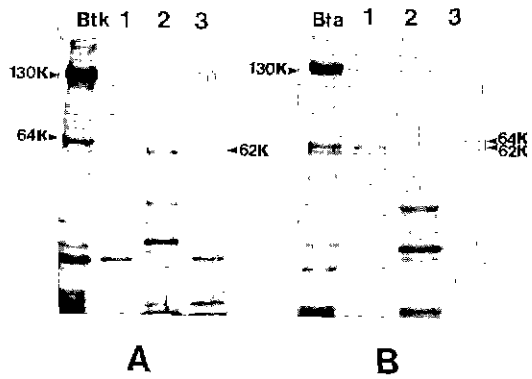

Fig. 3. SDS-PAGE of protoxin activation profiles from *B. thuringiensis* using gut extracts and trypsin. A, *B thuringiensis* var. *kurstaki*; B, *B. thuringiensis* var. *aizawai*; 1, *Hyphantria cunea* gut extract ; 2, *Bombyx mori* gut extract ; 3, trypsin; track 1, protoxin of Btk (A) and Bta (B), respectively.

tein pattern of digestive mixture was similar in both toxins. Whereas active toxin having molecular weight of 62 kd showed difference in quantities. After digestion, var. *kurstaki* developed distinct band of 62 kd product, while digestive mixture of var. *aizawai* produced a trace amount of 62 kd peptide. This study suggests that although *B. thuringiensis* var. *aizawai* shows the dual host specificity for dipteran and for lepidopteran cells (Tanada 1993), its toxicity to lepidopteran may be weaker than that of var. *kurstaki*. In our experiments, trypsin and proteinases in gut juices had no effect on the protein patterns (data not shown).

The δ-endotoxin from *B. thuringiensis* var. *kurstaki* was fed to last instar larvae of *H. cunea* to determine the effect on the protein patterns of several tissues including fat body. After toxin ingestion, larval tissues were dissected and electrophoresed under denaturing condition. We also compared the protein patterns induced by heat and cold shock with those by Bt toxin

On ingestion with *B. thuringiensis* toxin, larvae developed 45 kd stress protein in three tissues: fat body, Malpighian tubules, and gut (Fig. 4A). By heat and cold shock, the larvae developed same stress protein with molecular weight of 45 kd, together with 24 kd protein (Fig. 4B). Unexpectedly, nematode-infected brown planthopper showed the 45 kd stress protein, which increased in amount upon parasitism (Fig. 4C).

The heat shock response is a general homeostatic mechanism that protects cells and the entire organism from the deleterious effects of environmental stress (Nover 1991). It has been shown that heat shock proteins play major role in many cellular processes and have a unique role in several area. Heat shock response is a common phenomenon with that induced by bacteria or the unicellular ciliate Tetrahymena, or fungi, or insects, or parasites, or vertebrates (Schlesinger *et al.* 1982, Nover 1991, Maresca & Carratu 1992)

The present study suggests that *B. thuringiensis* infection on insect larvae induces the stress protein like heat shock protein. Several authors reported that upon heat shock *E. coli* and *Bacillus subtilis* produced heat shock protein known to enzymes involving Lys-tRNA synthetase (Van Bogelen *et al.* 1983), Lon protease (Goff *et al.* 1984. Amosti *et al.* 1986), and Serine protease (Lipinska *et al.* 19 90) Yoo *et al.* (1994) reported that 46 kd polypeptide, which occurs in the hemolymph of Bt-injected larvae of *Heliothis assulta*, is a kind of antibacterial protein. The 45 kd stress protein found in our study is likely to be antibacterial protein. The studies on the *B. thuringiensis*-induced stress protein and the purification of active toxins are already in progress in our laboratory.

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REFERENCES

Angus, T. A. 1956. Association of toxicity with protein crystaline inclusions of *Bacillus thuringiensis* Ishiwata. *Can. J. Microbiol* **2**: 122-131.

Amosti, D. N., V. L. Singer & M. J. Chamberlin. 1986. Characterization of heat shock in *Bacillus thuringiensis*, *J. Bacteriol.* **168**: 1243-1249.

Bulla, L. A., K. J. Kramer, D. J. Cox, B. I Jones, I I Davidson & G I. Lookhart 1981. Purification and characterization of entomocided protein of *Bacillus thuringiensis*. *J. Biol. Chem.* **256**: 3000-3004.

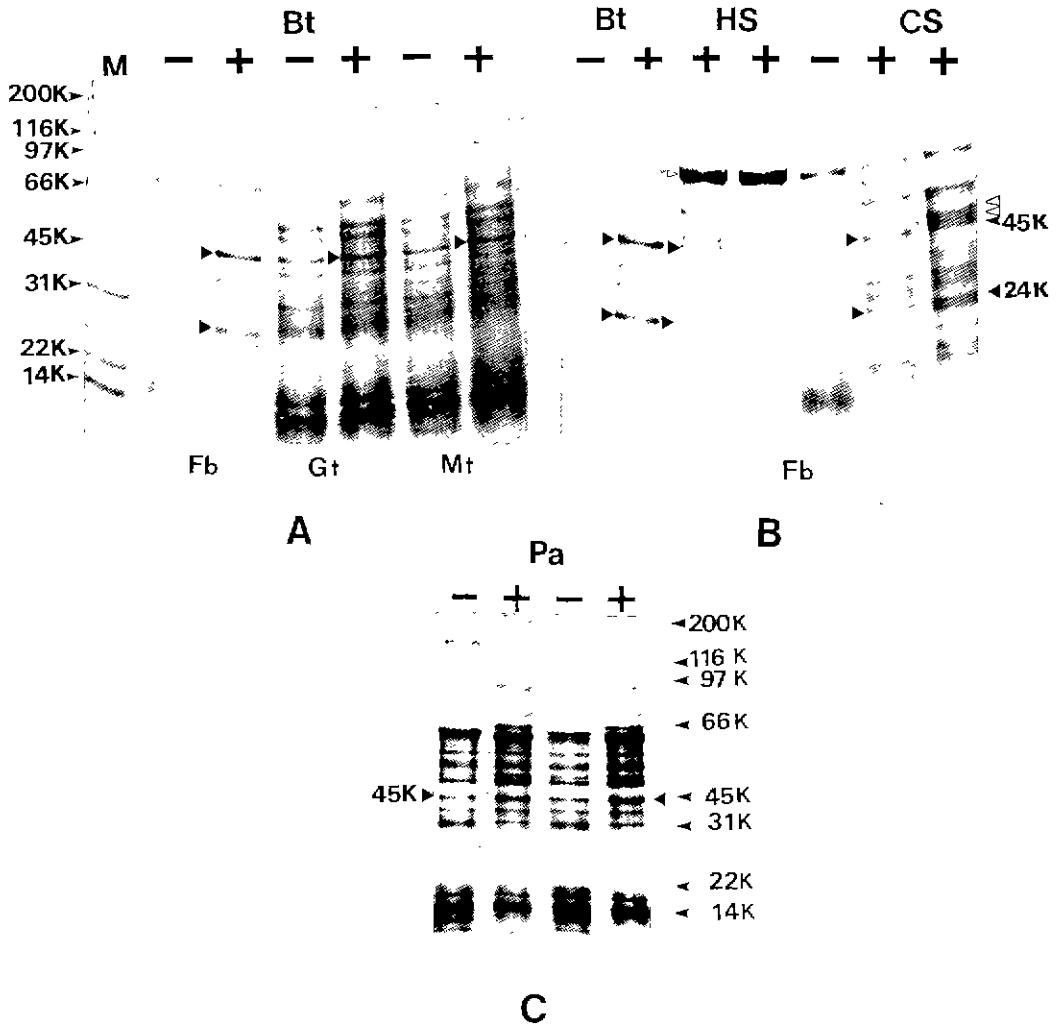

Fig. 4. Induction of stress proteins by *B thuringiensis* var. *kurstaki* δ-endotoxin (Bt), heat shock (HS), cold shock (CS), and parasitism (Pa). A and B. *Hyphantria cunea*; Fb, fat body; Mt, Malpighian tubule; Gt, gut; −, control; +, Bt-toxin ingestion; +, heat and cold shock. C. brown planthopper. −, control; +, parasitism . M, molecular mass standard. (▶; 45 kd stress protein; ▷, heat and cold shock specific protein).

Calabrese. D. M., K W. Nickerson & L. C. Lane. 1980. A comparison of protein crystal subunit sizes in *Bacillus thuringiensis*. *Can. J. Microbiol.* **26**: 1006-1010.

Faust, R. M., R. S. Travers & G. M. Hallam. 1974. Preliminary investigations of the molecular mode of action of the δ-endotoxin produced by *Bacillus thuringiensis* var *alesti*. *J. Invertebr. Pathol.* **23**. 259-261

Goff. S A., L. P. Casson & A L Goldberg. 1984 Heat shock regulatory gene htpR influences rates of protein degradation and expression of the lon gene in *E coli*. *Proc. Natl. Acad. Sci.* **81**: 6647-6651

Haider, M. Z., B. H. Knowles & D. J. Ellar. 1986 Specificity of *Bacillus thuringiensis* var. colmeri δ-endotoxin is determined by differential proteolytic processing by larval gut proteases *Eur. J. Biochem.* **156**: 531-540.

Huber, H. E. & P Luthy. 1981. *Bacillus thuringiensis* delta-endotoxin · Composition and activation In "Pathogenesis of Invertebrate Microbial Diseases" (E.W. Davidson, ed), pp. 209-234. Allanheld, Osmun & Co. Totowa, New Jersey.

Huber, H. E., P Luthy, H. R. Ebersold & J L. Cordier. 1981 The subunits of the parasporal crystal of *Bacil-*

lus thuringiensis: Size, linkage and toxicity. *Arch. Microbiol.* **129**: 14-18.

Kim, Y. H., S. H. Kim & S. K. Kang. 1991. Purification of parasporal protein crystals of *Bacillus thuringiensis*. *Korean. J. Seric. Sci.* **33**: 32-36

Kim, Y. H, S. H. Kim & S. K. Kang. 1992 Degradation of the parasporal crystal proteins of *Bacillus thuringiensis* in gut juice of some insects. *Korean J Seric. Sci.* **34**: 41-48.

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

Lecadet, M M. & R. Dedonder. 1966. Les protease de *Pieris brassicae* I. Purification et proprites. *Bull. Soc. Chim. Biol.* **48**: 631-660.

Lecadet, M. M. & D. Lereclus 1984. Structure and activity of the *B thuringiensis* δ-endotoxin. In "Bacterial Protein Toxins". (J.E. Alouf, F J Fehrenbach. J. H. Freer & J. Jeljaszewicz, eds.), pp. 147-154. Academic Press, London.

Lilly, M, R. M. Ruffeh & H. J. Somerville. 1980. Purification of the insecticidal toxin in the crystal of *Bacillus thuringiensis*. *J. Gen. Microbiol* pp 118· 1-11.

Lipinska, B., M. Zylicz & C. Georgopoulos. 1990. The HtrA(DegP) protein, essential for *Escherichia coli* survival at high temperatures is an endopeptidase. *J. Bacteriol.* **172**: 1791-1797.

Maresca, B. & L. Carrau. 1992. The Biology of the heat shock response in parasites. *Parasitology Today,* **8**: 260-266.

Nishiitsutsuji-Uwo, J., Y. Endo & M. Himeno. 1979 Mode of action of *Bacillus thuringiensis* δ-endotoxin: Effect on TN-368 cells. *J. Invertebr. Pathol.* **34**: 267-275.

Nover, L. 1991. Inducers of HSP synthesis : heat shock and chemical stress. In "Heat shock response". pp. 6-25. CRC Press, New York

Schlesinger, M. J., M. Ashburner & A. Tissieres. 1982. Heat Shock from Bacteria to Man. Cold Spring harbor. New York· Cold Spring Harbor Lab pp 440.

Schnepf, H E & H. R. Whiteley. 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *E coli. Proc. Natl. Acad Sci. U.S.A* **78**: 2893-2897.

Tanada, Y 1993. *Bacterial infection: Bacillaceae.* In "Insect Pathology" (Tanada T. and Kaya H K, eds.) Academic Press, New York pp. 90-120.

Thomas, W. E & D J. Ellar. 1983. *Bacillus thuringiensis* var. *israelensis* crystal δ-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *J. Cell. Sci* **60**: 181-197.

Van Bogelen, R. A, V Vaugh & F C. Neidhardt 1983. Gene for heat-inducible Lysyl tRNA synthetase maps near cad A in *E. coli. J. Bacteriol.* **153**: 1066-1068.

Yamamoto, T. & R. E. McLaughlin 1981. Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taemiorhynchus. Biochem Biophys. Res. Commun* **103**: 414-421

Yoo, C M, S E Jeong & H. C. Lee. 1994 Purification of antibacterial proteins in *Heliothis assulta* injected with *Bacillus thuringiensis. Korean J. Zool.* **2**: 274-280.

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