Akhmad Rizali

Bacillus thuringiensis from Indonesia as a Unique Insecticidal

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Most strains of B. thuringiensis produce delta-endotoxin crystals toxic to lepidopteran and dipteran insects. Recently, however several researches have shown that B. thuringiensis is also widely distributed in natural soils of various area and Mulberry leaves. In the present study I found the unique strains, Bacillus thuringiensis serovar entomocidus INA288 and serovar aizawai Bun 1-14 as Mosquitocidal insecticide, both of which were isolated in Indonesia.

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978-613-8-18292-4

Bacillus *thuringiensis* from Indonesia as a Unique Insecticidal

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ACKNOWLEDGMENT

The author wishes to express his sincere gratitude to his adviser, Dr. TOSHIHIKO Iizuka, Professor of Bioscience at the Faculty of Agriculture, Hokkaido University for his excellent guidance, suggestion, constant encouragement, and critical review in the preparation of this manuscript.

Dr. Hisanori Bando, Professor of Bioscience and Dr. Shin- Ichiro Asano for their valuable suggestions in the performance of experiments and preparation of the manuscript.

Professor Dr.H. Sutarto Hadi, M.Si., M.Sc, Rector of Lambung Mangkurat University for his assistance and support to finish this project.

Professor Ir. H. Luthfi Fatah, MS., PhD. Dean of Faculty Agriculture, Lambung Mangkurat University for his suggestion, assistance and support this work.

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Chapter I. GENERAL INTRODUCTION

Numerous chemical insecticides have been used in order to control pests, which damage for agriculture. While chemical insecticides have knock down effect to the insect pests, they are too expensive in the developing countries and harmful to both human and the environment. In addition, target insect pests rapidly develop biological resistance especially at higher rates of application. The chemical insecticides are still contributing to human life enormously, but they have been distributed in ecological system of organisms including human beings because of their low specific toxicity to any organism and their low specific toxicity to any organism and their slight decomposition in nature (Shorey and Hall, 1962). Therefore, many biological controls of insects have been investigated. Currently, researches on the use of pathogenic microorganisms to control insect pests are increasing. Microbial peat control is practiced in different parts of the world trough utilization of pathogens like fungi, bacteria, viruses and nematodes. Bacterials research causing disease in insect began in the late nineteenth century. It was a study of flacherrie of the silkworm, *Bombyx mori* (Burges and Hussey, 1971 and Burges, 1981). Ishiwata (1901) in his report on the discovery of sotto bacillus, referred briefly to occurrence of sotto bacillus-like organism, which causes the diseases to silkworm larvae.

Berliner (1991) proposed the name of *Bacillus thuringiensis* for a species of bacillus which was isolated from the diseased larvae of the Mediterranean flour moth Anagasta (Ephestia) *kuhniella* Zell. Later, Berliner (1915) noted infection of the larvae after the ingestion of the bacillus or its spores, described and named it *Bacillus thuringiensis* (1927) isolated the same bacillus from the same insect host, which Berliner had found earlier. This strain is now maintained as *B. thuringiensis* serovar *thuringiensis* (serotype H-1). Berliner (1915) and Mattes (1927) noticed that the vegetative remains of sporulating cells assumed a rhomboid shape. Hannay (1953) described this crystalline inclusion in the sporangium of the organism and made further interpretations of the data being accumulated on this bacillus at that time. Neither Berliner nor mattes attributed those parasporal bodies any role in the disease process caused by ingestion of sporulating *B. thuringiensis*.

B. thuringiensis is a gram positive soil bacterium, and produces a crystalline inclusion body during sporulation (Bulla et al, 1980). This parasporal body is composed of proteins termed “delta-endotoxin”, and specifically toxic to insects. In addition,*B. thuringiensis* produces another toxin namely alpha-exotoxins, beta-exotoxin, and gamma-exotoxin. All of the toxic substances may not be presented in the bacterium (Heimpel, 1967).. In another hand, krieg (1961) has defined various toxic substance produced by *B. thuringiensis* as follow: (a) thermolabile endotoxin; (b) thermostable exotoxin; (c) bacillogenic antibiotic; (d) lecithinase; (e) proteinase.

B.thuringiensis has been studied world wide over the past decades, mainly because this gram positive bacterium producer significant amounts of crystal proteins with toxic activity against economically important insect larvae (DeSouza et al, 1993).

The most attractive characteristics of the *B. thuringiensis* proteins for insect control are their specificity an high unit activity. Members of non-target insect orders are not susceptible to the potent effects of the lepidopteran-specific and dipteran-specific insecticidal proteins (Bulla, 1985).

Intensive screening programs have identified strains of *B. thuringiensis* from soil samples, plant surfaces, dead insects, and stored grains from all over the world. The isolated strains show a wide range of specificity against different insect orders (Lepidotera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phithiraptera or Mallophaga, and Acari) and other invertebrates (Nemathelminthes, Platyhelminthes, and Sarcomastigophora) (Feitelson, 1993). Currently 45 different serotypes have been classified (Table 1) by Lecadet et al, (1994). The first classification of insecticidal crystal protein genes (cry gene) (Tabel 2) was based on insecticidal activity (Hofte and Whiteley, 1989)., with different cry proteins denoting their gnes tixic to various insect and vertebrate groups as follow: *cryI* toxic to lepidopterans, *cryII* toxic to lepidopterans and dipteran, *cryIII* toxic to coleopterans, *cryIV* toxic to dipterans, and *cryV* and *cryVI* toxic to nematodes (Feitelson et al., 1992).

Most strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to lepidopteran insects such as moth (Dulmage, 1981). But some strains of *B. thuringiensis* produce delta-endotoxin crystal toxic to dipteran insects such as mosquitoes (de Barjac, 1978; Golberg and Margalit, 1977) and blackflies (Undeen and Negel, 1978). Lepidopteran-specific delta-endotoxins are composed of 130 kDa proteins (Calabrese et al., 1980; Bulla et al., 1977) while dipterans-specific delta-endotoxin are composed of several proteins (Pfannenstiel et al.,1984; Lee et al., 1985). Yamamoto and McLaughlin (1981) has reported P1 (135 kDa) and P2 (65 kDa) proteins from serovar kurstaki HD-1 toxic to Lepidoptera and Diptera. Molecularweight of major components of dipteran-specific delta-endotoxin are 28 kDa, 38 kDa, 65 kDa, and 130 kDa. This unfavorable property as commercial insecticides is due to 28 kDa protein of dipteran-specific delta-endotoxin (Ward and Ellar, 1987; Pfannenstiel., 1986). The insect specificity of the *B. thurigiensis* insecticidal proteins has, however, limited their usefulness in many important agricultural situations. None of the proteins encoded by the previously characterized *B. thuringiensis* genes exhibit insecticidal activity against coleopterans (beetles). Molecular weights of major components of coleopteran specific delta-endotoxin are 68 kDa and 50 kDa (McPherson., 1988).

Since Golberg and Margalit (1977) isolated *Bacillus* strains possessing a high larvicidal activity, specificity for mosquitoes, from the soil of mosquito-breeding site in Israel. On the basis of this strain, de Barjac (1978) established *Bacillus thuringiensis* serovar *israelensis* (H antigen 14).

Thereafter, many workers have reported the occurrence of highly mosquitocidal *B. thuringiensis* starins, belonging to serovar israelensis (Balaraman et al., 1981; Zhang et al., 1984; Shim et al., 1990; Abdel-hameed et al., 1990), and serovar medellin (Orduz et al., 1992) in various regions of the world. However, it is generally accepted that the mosquito-specific *B. thuringiensis*, with moderate to low toxicity, is also disseminated in natural environments. Recenly, however, several workers have shown that *B. thuringiensis* is also widely distributed in natural soils of various area. Delucca et al., (1981) reported that *B. thuringiensis* made up less than 0.5% of more than 46,000 bacteril isolates recovered from various soils in the United States. Travers et al., (1987) described a technique which increased the frequency at which *B. thuringiensis* could be recovered from soils. However, Martin and Travers (1989) have recovered *B. thuringiensis* from numerous soils obtained from around the world. While Ohba and Aizawa (1989) have reported recovery of over 300 *B. thuringiensis* isolates from Japanese soils and sericulture areas. It has also been found in soils of the Philippines (Padua et al., 1980), Vietnam (Martins and Travers, 1994), Indonesia (Hastowo et al. 1992). Thailand (Attathom et al., 1994) and Malaysia (Kawalek et al., 1995). However, very little is known about its distribution in other regions of Southeast Asia.

When author started this study, no mosquitocidal activity of *B. thuringiensis* has been isolated yet in Indonesia. However, Sasaki et al. (1996) isolated *B. thuringiensis* serovar *kurstaki* INA-02 from soil in Indonesia, which was toxic to lepidoteran insects, so I aimed at screening of mosquitocidal *B. thuringiensis* strains from soil and mulberry leaves in Indonesia, and to increase our knowledge of the distribution of *B. thuringiensis* in the tropic Asian condition.

Table 1. Classifiction of *Bacillusthuringiensis*

SEROTYPE (H-ANTIGEN)		ESTERASE TYPE REFERENCE	SUBSPECIES	PRODN. OF B-EXOTOXIN IN
	1	*thuringiensis*	*thuringeinsis*	+
Subsp.				
	2	*finitimus*	*finitimus*	-
3a	3b	*alesti*	*alesti*	-
4a	4b	*kurstaki*	*kurstaki*	-
4a	4c	*sotto*	*sotto*	-
5a	5b	*kenyae*	*kenyae*	-
5a	5c	*galleriae*	*galleriae*	-
	6	*canadiensis*	*canadiensis*	-
	7	*entomocidus*	*entomocidus*	-
8a	8b	*morrisoni*	*morrisoni*	+
8a	8c	*ostriniae*	*ostriniae*	-
8b	8d	*nigeriensis*	*nigeriensis*	-
	9	*tolworthi*	*tolworthi*	+
	10	*darmstadiensis*	*darmstadiensis*	-
11a	11b	*toumanoffi*	*toumanoffi*	+
11a	12c	*kyushuensis*	*kyushuensis*	-
	12	*thompsoni*	*thompsoni*	-

Table 1.continued…….

SEROTYPE (H-ANTIGEN)		ESTERASE TYPE REFERENCE	SUBSPECIES	PRODN. OF B-EXOTOXIN IN
	13	*pakistani*	*Pakistani-*	
	14	*israelensis*	*israelensis-*	
	15	*dakota*	*Dakota*	-
	16	*indiana*	*Indiana-*	
17		*tohokuensis*	*tohokuensis*	-
18		*kumamotoensis*	*kumamotoensis*	+
19		*tochigiensis*	*tochigiensis*	-
20a	20b	*yunnanensis*	*yunnanensis*	-
20a	20c	pondicheriensis	pondicheriensis	-
	21	*colmeri*	*colmeri*	-
	22	*shadongiensis*	*shadongiensis*	-
	23	*japonensis*	*japonensis*	-
	24	*neoleonensis*	*neoleonensis*	-
	25	*coreanensis*	*coreanensis*	-
	26	*silo*	*silo*	-
	27	*mexicanensis*	*mexcanensis*	-

Source : Barjac, D.H. and E. Frachon., 1990.

Table 2. Mosquitocidal *Bacillus thuringiensis* delta-endotoxin genes

Name	Original	Accession	Reference	Year	Coding region
Cry2AaI	*cryIIA*	M31738	Donovan et al	1989	156-2054
Cry4AaI	*cryIVA*	Yoo423	Ward & Ellar	1987	1-3540
Cry4BaI	*cryIVB*	XO7423	Chungjatporn Chai et al	1988	157-3564
Cry10AaI	*cryIVC*	M12662	Thorne et al	1986	941-2965
Cry11AaI	*cryIVD*	M31737	Donovan et al	1988	41-1969
	Cry288		Iizuka et al	1996	
	Cry19A		Rosso et al	1997	

Chapter II. Occurrence of mosquitocidal *Bacillus thuringiensis* serovar *entomocidus* in the soil of Indonesia

INTRODUCTION

Bacillus thuringiensis is gram-positive, spore-forming bacterium that produces parasporal crystal during the sporultaion stage. The crystal is made of one or more proteins toxic to some insect species. Golberg and Margalit (1977) isolated a *bacillus* strains possessing a high larvicidal activity, specific for mosquitoes, from the soil of a mosquito-breeding site in Israel. However. De Barjac (1978) established *B. thuringiensis* serovar israelensis (H antigen 14) as highly mosquitocidal activity. Another investigators have reported the occurrence of highly mosqutocidal *B. thuringiensis* strains belonging t serovar israelensis (Balarman et al.,1981; Zhang et al., 1984; Shim et al., 1990; Abdel-Hameed et al., 1990). To date, most strains of *B. thuringiensis* produce inclusions toxic to dipteran insects, though three are subspecies such as serovar *morrisoni* PG-14 (Padua et al., 1984), *fukoukaensis* (Yu et al., 1991), *darmstadiensi*s (Kim et al., 1984), *kyushuensis* (Held et al., 1990), *jegathes*an (Kawalek et al., 1995), *hi*go (Ohba et al., 1995), and *canadiensis* (Ishii and Ohba, 1997), which produce toxins that are predominantly toxic to dipteran insects.

In this chapter, I describe the identification, characterization and profiles of the crystal protein peptides between mosquitocidal serovar *entomocidus* INA288 and other mosquitocidal strains. I found that *entomocidus* INA288 had mosquitocidal activity and similar genes between *B. thuringiensis* serovar *israelensis* and serovar *fukoukaensi*s.

MATERIALS AND METHODS

Bacterial stains.

The strain of *B. thuringiensis* used in the present study were*B. thuringiensis*serovar*israelensis* ONR60A and *entomocidus* (orginal strain) and *B. thuringiensis* serovar *entomocidus*INA288. The *B. thuringiensis* serovarf *fukuokaensis*, and *kyhusuensis* were provided and cultured in our laboratory.

Isolation and identification.

B. thuringiensis serovar INA288 which had been isolated from Indonesia soil, was prepared according to the method. One gram of soil samples was suspended in 9 ml of sterile distilled water and shaken for 5 min. the upper layer of the soil suspension was transferred to a test tube and heated at 80°C for 5 min in water bath to kill non-spore-forming organism and vegetative cells.to prepare the sporulated culture, bacteria were grown on nutrient agar pH 7.2, at 30°C for 4 days. Formation of spores and parasporal inclusion were monitored with a phase-contrast microscope. The culture was scratched on the agar slant as a stock.

Identification by H-serotyping

The isolate of *B. thuringiensis* INA288 was identified by H-serotype. In order to make antibody, H-antisera to the reference strain of *B. thuringiensis*serovar*entomocidus* (original starin) were prepared according to the method. For H-serotyping of the strains, actively motile bacteria were selected by passing trhoughcraigie's tubes at 37^{0}C for 24 hr. Slide agglutination was performed by mixing one drop of 3 to 4hr-old flagellated broth culture (N-broth and Agar 0.8%) of the bacteria on a glass slide with one drop of 20 to 50-fold dilution of H antiserum. Specific H agglutination was determined 3 to 5 min after mixing.

Morphology of parasporal inclusion.

Isolates were examined with a HITACHI S-800 Scanning Electron Microscope (SEM) at a magnification of 10,000x, according to the method *B. turingiensis* serovar *entomocidus* INA288 were cultured on N-broth agar at 30^{0}C until almost all cells lysed (overnight).The crystal and spores (about 100 mg wet weight) were washed in 10 ml of 50 mMTris-HCL (pH 8.0). The final precipitate was resuspended in 1 ml of distilled water, and 20 ul of the suspension was air-dried on a glass disk (O 10 mm). After the sample was coated with carbon and gold, it was observed and photographed with SEM.

Biological activity.

The strain were examined for their larvicidal against the larvae of the silkworm, *Plutella xylostella* and *Spodoptera litura*. The insect cultures were maintained in this laboratory. Toxicity

test with the Lepidopteran insect, *B. mori*, *P. xylostella* and *S. litura*, were done by introducing ten 3rd-intar larvae were fed on an articial diet dropped with 0.3 ml of the bacterial suspension and rear at 25°C for 48 hr to determine mortality. The *B. thuringiensis* isolates were examined for oral insecticidal activity against the insects were prepared by the following procedures. Overnight culture of serovaINA288, and *israelensis*ONR60A were grown on 2 ml of nutrient broth at 30°C using tube glass. Then, 200 ul of the overnight culture was plating on nutrient agar, reincubated for 4 days at 30°C. Sporulated cultures were harvested by centrifugation at 10,000 g for min at 4°C. The pellet was washed three times by centrifugation in mMTris-HCL and 1 M NaCL at 4°C, the bacterial suspensions were finally suspended in 500 ul of sterile distilled water. The bacteria were also tested against larvae of the mosquitoes, *Aedes aegypti*, *Aedes japonicus* and *Culexquinque fasciatus*. Ten 2nd-instar larvae were placed in a test tube containing 10 ml of the spore-paraspora inclusion suspension, respectively, under levels 1 ul/ml. The tubes were kept at 22°C for 24 hr without feeding.

SDS-PAGE and Western blotting

Parasporal inclusions were separated from spores and cell debris using Percoll (Pharmasia) as the following: Percoll solution were added 1 M NaCL solution and the *B. thuringiensis* sample was added for separating of crystal proteins from spores by centrifugation at 15.000 rpm at 4^0C for 20 min. In the first step, Percoll solution (Percoll: 0.15 M NaCL = 8:2) was prepared, and then 1 ml of crystals and spores mixture (10 mg [dry weight]/ml) pored carefully the top of 30 ml of percoll solution, and centrifuged at 15.000 rpm (30,000 g) for 30 min. After recovering the crystal protein, crystal protein was immediately washed three times by distilled water. Finally, the pellet was suspended with 500 ul sterile distilled water. The purity of parasporal inclusions was monitored with a phase-contrast microscope. SDS-PAGE of parasporal inclusion proteins was done by the method[26] using a 10% running gel with a 3% stacking gel. After electrophoresis, gel was stained with 0.04% Coomasie brilliant blue (Sigma Chem.Co.). The following prestained reference proteins (Bio-Rad Laboratories) were used as molecular markers: myosin (200 kDa), B-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

Production of inclusion protein antibodies and immunobloting.

Antisera were raised to guinea pig against parasporal inclusion proteins of the *B. thuringiensis* serovar entomocidus INA288. Guinea pig was immunized by five subcutaneous injections, at weekly intervals. The solubilized parasporal inclusions were mixed with an equal volume of Freund's complete adjuvant (Difco). One week later, these were followed by intravenous booster injections of adjuvant-free solubilized inclusion proteins. The guinea pigs were bled one week after injection of 2 qnd 9 mg of serovar entomocidus INA288, respectively. The antiserum was centrifuged at 3000 rpm for 10 min, supernatant was collected and added 0.1% NaN_3 till storing of it at-20°C. Three days later, antiserum was cheked with SDS-PAGE and Western bloting.

For immunoblot analysis (Towbin et al., 1979), the separated proteins were transferred electrohoretical from SDS-Polyacrylamide gel to a ECL nitrocellulose membrane (Amersham) using an electrophoreticaly tran-blott (Bio-Rad) according to the manufacturer's instructions. The membrane was blocked with 3% skim milk in TBST buffer (100 mM Tris-HCL {pH 7.5}, 150 mM NaCl, and 0.1% {v/w) Tween 20) for 3 hr at 4°C, than the membrane was incubated in TBST buffer with 1% of plyclonal antibodies of *B. thuringiensis* serovar *entomocidus* INA288 for 1 hr. the nitrocellulose membrane was washed three times with TBST, and then incubated with perooxidase IgG guinea pig in 1.5% TBST for 1 hr. The membrane was washed three times with TBS buffer and visualized with hydrogen peroxide (H_2O_2) and 4-chloro-1naphthol as describe in the instructions from BIO-Rad.

DNA preparation

Overnight cultures of the *B. thuringiensis* strains were grown in 2 ml of nutrient broth at 30°C using glass tube. One ml of the overnight culture was inoculated into 50 ml of typtose phosphate broth (Difco) using 200 ml culture flask and reincubated for 4-5 hr until the synchronized culture had reached 10-15% optical transmission. The cells are finally harvested by centrifugation at 500 g for 10 min. Cells were suspended in approximately 1 ml of lysozyme (10 mg/ml; Wako pure chemical Industries, Ltd.), preincubated for 60 min at 37°C. Lysis was brought to incubate for 15 min at 60°C by the addition of 8% SDS (Sodium dodecyl sulfate) buffer in TES and an equal volume of 5 M NaCl to the cleared lysate, and the whole mixture was left overnight at 4°C. The mixture was centrifuged for 10 min at 14,000 rpm (Himac CF 15D), the supernatant fluid was carefully decanted to new tube, was added immediately by the addition of RNase (1 ug/ml), and

preincubated for 30 min at 37°C. The cells were thoroughly washed by Phenol/Chloroform, the supernatant fluid was carefully transferred to another new tube, and then 100% ethanol (ETOH) was added. After mixture suspension was stored at 80°C for 20 min, it was centrifuged at 15,000 rpm for 15 min and the supernatant fluid was discarded. Finally, the pellet was dissolved in 500 ul of electrophoresis buffer.

Polymerase chain reaction (PCR) procedure

The template DNA is first denatured in the presence of a large molar excess of the two oligonucleotides and the four dNTPs. The reaction mixture is cooled to temperature by allowing the oligonucleotide primers to anneal to their target sequence, after which the annealed primers are extended with DNA polymerase (LIFE TECH, Elongase). The cycle of denaturation is at 30 times, at 92^{0}C for 1 min, annealing is at 48^{0}C for 1 min and polymerization is at 72^{0}C for 2 min. The PCR technique was used to identify the contents of *B. thuringiensis*serovar*entomocidu*s INA288, using oligonucleotide primers specific to *Cry1*(Kalman et al., 1993), *CryII*(Asano et al., 1993) and *CryIV*(Asano, 1996). Identification of cry genes in genomic DNA sample extracted from *B. thuringiensis*serovar*entomocidus*INA288 strains was based on unique-size DNA fragments amplified by PCR for each cry gene (Asano, 1996).

Cloning and sequencing of the cry gene amplified from serovar *entomocidus* INA288

The amplified gene of *B. thuringiensis* seovar *entomocidu*s INA288 was ligated into pGEM-T (Promega Co.). DNA sequences were obtained by dideoxy chain termination method (Sanger et al., 1977) with { alpha -^{32}P} dATP (Amersham and a sequence version II kit from United State. Biochemicals.

RESULTS

Isolation and Identification

The *B. thuringiensis* strains have been isolated from soil samples of West Java in Indonesia. *B. thuringiensis* serovar *entomocidus* INA288 was screened from that sample.

Identfication by H-serotyping

In order to identifyserovar INA288 strains by H-serotype cell with broth, they were dropped to glass slide and the motility of cels was observed under phase-contrast microscope. Since isolate of *serovar* INA288 gave positive reaction in the H 6 serum agglutination test, it was identified as *B. thuringiensis* serovar *entomocidus.*

Morphology of INA288 by Scanning Electron Microscope (SEM)

Morphology of the crystals from serovar *entomocidus* (original starin) has been reported and the crystals are bipyramidal-formed (Fig.1). On the other hand, interestingly, *entomocidus* INA288 produced large cuboidal-form crystals (Fig.2).

Quantitative toxicity test of isolates

The serovar *entomocidus strain and israelensis ONR60A were bioassayed* against 4th-instar larvae of *B. mori, P. xylostella, S. litura, A. japonicas, A. aegypti and C. quiquefasciatu*s. Respectively, serovar *entomocidus (original strain) showed toxic activity* against latter 3 former lepidoteran species, while *entomocidus* INA288 had also the, toxicity against later 3 dipteran species (*A. aegypti, A. japonicas* and *C. quinque fasciatus*) (Table 3). The 50% lethal concentration of crystal inclusions for each dipteran species was higher than of serovar *israelensis* ONR60A but lower than *fukuokaensi*

SDS-PAGE analysis

The purity of *B. thuringiensis* serovar *entomocidus* INA288 parasporal inclusion by percoll showed more than 90% depending on monitoring with a phase-contrast microscope. SDS-PAGE

analysis of parasporal inclusion purified from *B. thuringiensis* serovar *entomocidus* INA288 and three other serovar with mosquitocidal activity, *israelensis* ONR60A, *kyushuensis*, and *fukuokaensis*, demonstrated that serovar *entomocidus* INA288 has significant differences in protein composition, compared with serovar *israelensis* ONR60A (mainly 130 kDa, 70 kDa, and 27 kDa) *kyushuensis* (mainly 140-25 kDa) (Held et al., 1990) and *fukuokaensis* (mainly 90-27 kDa) (Fig.3A). The crystals of serovar *entomocidus* INA288 indicated that the polypeptide 70 kDa was dominant, while *entomocidus* (original strain) consisted mainly of 130-65 kDa.

Immunoblot analysis

B. thuringiensis serovar *israelensis* ONR60A and *fukoukaensis* peptides little croos-reacted with monoclonal antibodies formed against *B. thruringeinsis* serovar *entomocidus* INA288 (Fig.3B). An anti-70 kDa-peptide monoclonal antibody revealed little cross-reactivity with a monoclonal antibody directed against the 130 kDa peptide of *B. thuringiensis*serovar*israelensis* ONR60A. The other two serovar, *B. thuringiensis* serovar *entomocidus* (original strain) and *kyushuensis* did not demonstrate cross-reactivity with monoclonal antibody of *B. thuringiensis*serovar*entomocidus*INA288. The immunoblot analysis showed that 70 kDa peptides of *B. thuringiensis* serovar *entomocidus* INA288 had similar cry gene with *B. thuringiensis*serovar *israelensis* ONR60A and *fukoukaensis*.

Identification of Cry genes in *B. thuringiensis*serovar*entomocidus* INA288

The PCR was used to survey a number of *B. thuringiensis* strains for their crystal protein gene content. One set of primer pairs was used to detect, in one reaction. In the identification of cry genes of *B. thuringiensis* serovar *entomocidus* INA288, *cryI*, *cryII*, and *cryIV* primers were used. PCR with these primers and DNA template generated a uniquely size fragment for each cry type gene. Therefore, the size of PCR product indicated the presence of particular crystal protein genes. Previous reports concerning the occurrence of crystal protein genes were confirmed by this technique. Serovar *entomocidus* INA288 did not amplify *cryIC*, *cryIIA*, *cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD* genes. However, their cry genes had mosquitocidal activities. This fact shows that serovar *enomocidus* INA288 contains novel cry genes for mosquitocidal activities.

Cloning and sequence of *entomocidus* INA288

When the total DNA of *entomocidus* INA288 was screened with the PCR-amplified pGEM-T, demonstrated that the sequence of the insert was located in a large plasmid DNA. In addition, the low-sequence homologies were demonstrated between the insert DNA and cry4A or cry4B gene from serovar *israelensis*. The predicted dipteracidal polypeptide of *entomocidus* INA288 (cryINA288) consisted of 703 amino acids with conserved blocks and amino acid sequence was aligned to that of cry4Aa (Fig. 4). Furthermore, an alignment of conserved block sequence among the cryINA288 and other mosquitocidal proteins was performed (Fig. 5).

DICUSSION

In recent year.The discoveryof bacteria like *B. sphericus* and *B. thuringiensis*serovar*israelensis* which are highly toxic to dipteran larvae, and theseformulation have been used on a large scale in the field and in the various countries. This initial success has been tempered by the result obtained for resistance to *B. thurngiensis*serovar*sphaericus*. In the search for potential alternatives to the application of *B. thuringiensis s*erovar*israelensis*, isolation of novel mosquitocidal strains is very important. In present research, it is found that *B. thuringiensis*serovar*entomocidus* INA288 (belonging to serotype H 6) isolated from soil in Indonesia, prossess novel mosquitocidal activity toxin. It was observed that *B. thuringiensis*serovar*entomocidus* INA288 has cuboidal shaped crystal protein, while serovar*entomocidus* (original strain) has bipyramidal and irregular shaped one. However, *B. thuringiensis*serovar*israelensis* ONR60A has irregular crystal protein. In this study, the parasporal inclusion from *B. thuringiensis*serovar*entomocidus*INA288 were separated and compared its insecticidal activity to those species from two orders (Lepidoptera and Diptera). Another hand, cuboidal inclusion composed of a 66 kDa subunit proteins toxic to both lepidopteran and dipteran (mosquito and black fly) insects. the isolates with bipyramidal and cuboidal-shape predominated on the soil and dead insect. In spite of 70 kDa peptide in *B. thuringiensis*serova*entomocidus* INA288, this protein has no activity against the three species of lepidoteran insects and only toxic against the three species of dipteran insects. Interestingly, *B. thuringiensis*serovar*entomocidus* (original strain) is only toxic to the *B. mori*, *P. xylo*stella and *S. litura*. Parasporalinclusion of serovar*entomocidus* INA288 are slightly more toxic than

serovar*israelensis*ONR60A to all mosquito species, such as *A. japonicas*, *A. aegypti*, and *C. quinquefasciatus*.

The molecular bands of these proteins suggest that there is no major overlap among any these proteins with those of *B. thuringiensis* serovar *israelensis*, *fukoukaensis* and *entomocidus* INA288.The antibodies of *B. thuringiensis*serovar *entomocidus* INA288 showed immunoblot reactivity to protein inclusions of *B. thuringiensis*serovar *israelensis*ONR60A, and serovar *fukoukaensis*.The results suggests that there are similarity gene between serovar *entomocidus* INA288, serovar *israelnsis*, and serovar *fukoukaensis*. Ishii and Ohba (1993) demonstrated that antibodies of *kyushuensis* showed immunoblot ractivity to inclusion proteins of serovar *darmstadiensis* 73-E-10_2 and weak reactivity to those of serovar *canadensis*.

The PCR screening results suggest that *cry4Aa* had similarity with cryINA288. Therefore, cry INA288 seems a novel mosquitocidal cry gene. However, serovar*entomocidus*(original strain) encodes only *cry1Aa*, *cry1Ab*, *cry1B* and *cryIC*, which have not been thought to be dipteracidal activity.

Squencing analysis of the insert DNA revealed the amino acid sequence of the polypeptide encoded. The predicted dipteracidal polypeptide of INA288 (cryINA288) consist of 703 amino acids with conserve blocks and the amino acid sequence is aligned to that of *cry4Aa*. This strain contains a novel crystal protein gene, cryINA288 was 38%. Therefore, cryINA288 seems a novel mosquitocidal cry gene. However, after analyzing all amino acid sequences of this gene, amino acid comparison should be done for *cryINA28*8, *cry4Aa*, and *cry4A*b.

Fig. 1.Scanning electron microscopy showing spores and paraspporal crystal of *Bacillus thuringiensis* serovar *entomocidus* (original strain). Bar indicates 3 um.

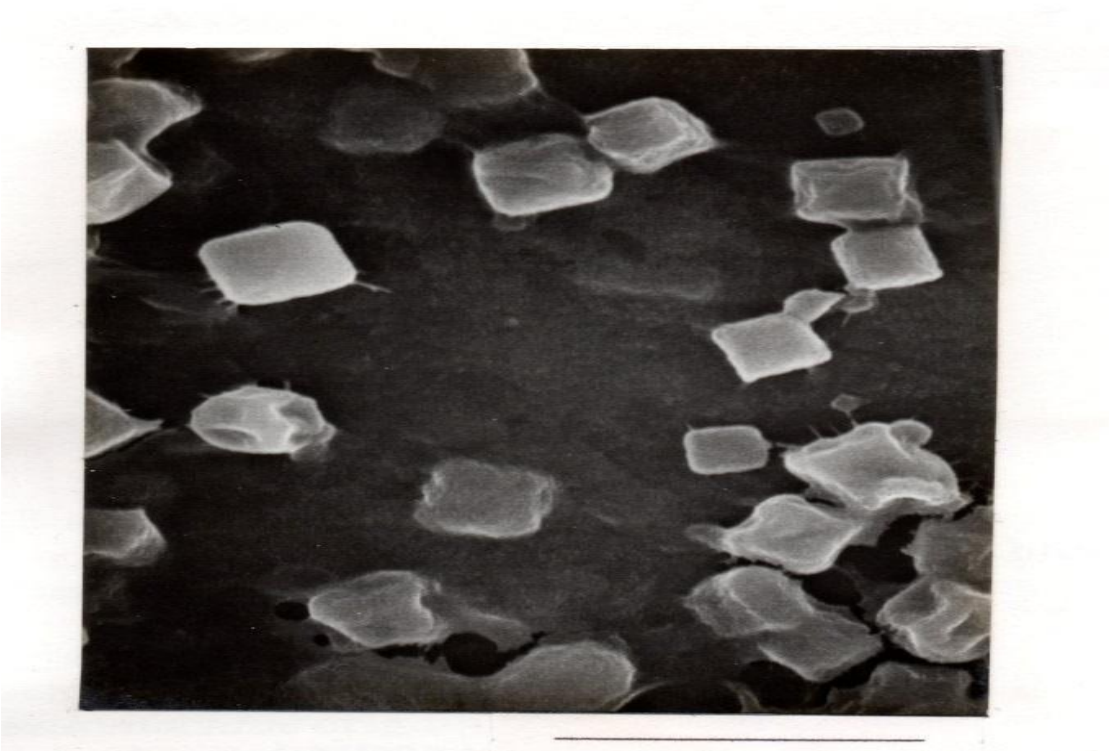

Fig. 2.Scanning electron microscopy showing spores and parasporal crystal of *Bacillus thuringiensis* serovar *entomocidus* INA288. Bar indicates 3 um.

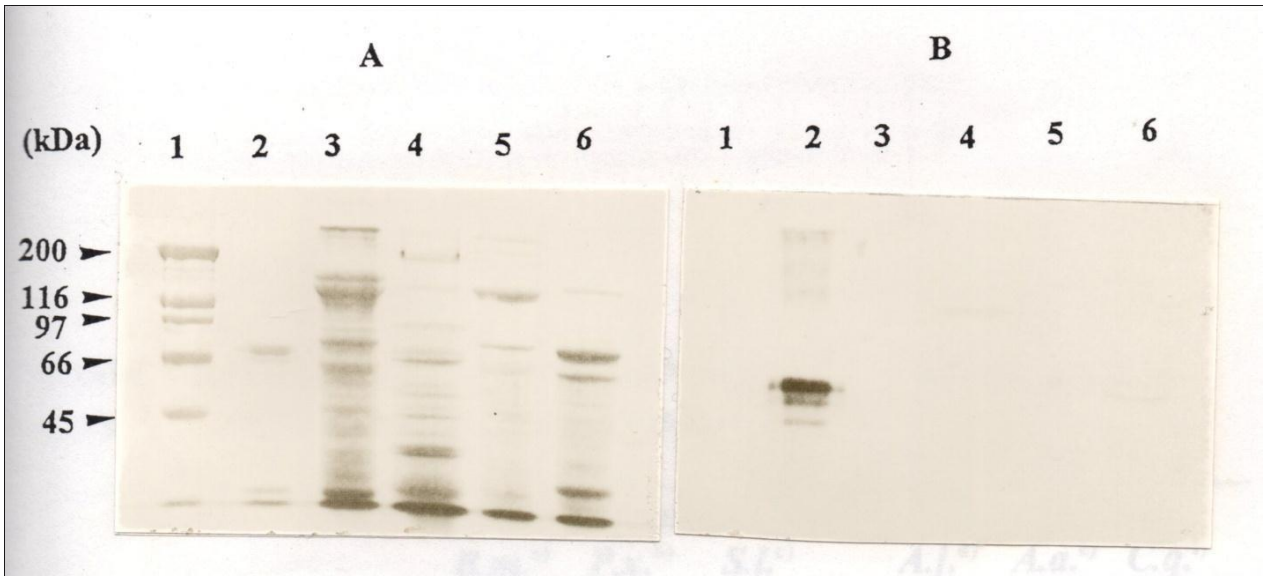

Fig. 3. SDS-PAGE analysis and western blotting analysis

(A) SDS-PAGE gel (10%) stained with Coomassie brilliant blue

(B) Western blotting and EIA analysis

Lane 1: molecular weight marker

Lane 2: *serovar entomocidus* INA288

Lane 3: *serovar entomocidus* original strain

Lane 4: *serovar israelensis* ONR60

Lane 5: *serovar kyushuensis*

Lane 6: *serovar fukoukaensis*

Table 3. Toxic activity of three *B.thuringiensis* strains against some insect species

Strain	Lepidopteracidal			Dipteracidal		
	B, m,.	***P.x.***	***S.l.***	***A.j.***	***A.a.***	***C.q.***
***Entomocidus*original**	+	+	+	-	-	-
***Entomocidus* INA288**	-	-	-	+	+	+
***israelensis* ONR60A**	-	-	-	+	+	+

B.m: Bombyxmori, P.x: Plutellaxylostella, S.l: Spodopteralitura
A.j:Aedes japonicas, A.a: Aedesaegypty, C.q: culexquinquefasciatus

```
a   46   DWLNMCQQNQQYGGDF-E----TFID-S-GE-L-SAYTIVVGTVLTGFGF   95
         |||  |  |  |     |    |    | |  | |  ||  | || | |
8    1   DWLDIC--NPEYRYSNPEANRNTKAAMSFGVGLVS--TIL-G-VL-G-GP   50
    96   T--TPLGLALIGFGTLIPVL-F-PAQDQS-NT---WSDFITQTKN-I---  145
            | || | ||  |   || | || |   ||   |   |   |  |
    51   ISVT-LG-AIIGVVTA--VLEFIPA-DEYDNTKETWGVLIAAIKELIYEE  100
   146   IKKEIASTYISNANKI-LNRSFNVISTYHNHLKTWENNPN-P--QNT-QD  195
         || | |     || |  |     |   | | |  | |    |  ||  |
   101   IKGE-AM----NAAKAKLDGLYKVMKNYDNKLNVWKNGDKSPVEQNEIQR  150
   196   VR--TQ------I-QLVHYHFQNVIPELVNSCPPNPSDCDYYNILVLSSY  245
         |   |       | |     ||    | | |  |          |
   151   VFADTNNSFLLLISQ-----FQQLGHE-V-SFLP----------LF----  200
   246   AQAANLHLTVLNQAV----KFEA-YLKNNRQFDYLEP-LPTAIDYYPVLT  295
         | ||| ||  |   |    | |  |  ||    |    |    |     |
   201   AVAANFHLLLLRD-VSIYGK-EWGYT-NNIIEGYHSDQL----DM----T  250
   296   KAIEDYTNYCVTTYKKGLNL---IKTTPDSNLDGNINWNTYNTYRTKMT-  345
            |||||| | ||| |||    ||   |  ||    |  || ||  ||
   251   Q---DYTNYAVDTYNKGLEEAKKIKNS-DK-LD----WDFYNQYRRDMTL  300
   346   TAVLDVVALFPNYDVGKYPIG--VQSELTREIYQVL-N-----FEESPYK  395
         | |||| |||| ||| ||||   |  |||||||    |    |   |
   301   T-VLDVIALFPTYDVRKYPISTKV--ELTREIYTDMINYINNPFMTNPVE  350
   396   YYDFQ-Y---QEDS----LTRRPHLFTWLDSLN--FYEKAQT------TP  445
            |  |   |  |    ||| |||||||      ||   ||      |
   351   GQRFAGYTVAQFNSIENALTREPHLFTWLKEVTGYFY--AQYGQQSFMTG  400
   446   N-NFFTSHY--NM----F----H---Y---TL---DNISQK---S--SV-  495
          |  || |  |    |    |   |   |    ||   |   |  |
   401   IQN--TS-YRTNYEDYPFSGPLHGVRYAGDTARSVDNNG-KDVYSIYSTM  450
   496   FG---NHN-VTDKLKSLGLATNIYIFLLNVISL--DNKYLNDYN--NIS-  545
         |    | | |   |  | |   | |   |     |     |     |
   451   FPLETN-NHVRE-LRP-GTAY--Y-FG--VKGHRHDAT---DRRTGNSSQ  500
   546   KMDFFITNGTRLLE--KELT---A-G-SGQIT---Y-DVNK--NIFGLPI  595
         |    |  |    |  |  |   | | |  |    | |  |  |     |
   501   K----IL-G----EDSK--TGRIATGPSYFISEIPYYD--KETNET---I  550
   596   LKRRENQGNPTLFPT-YDNYSHILSFIKSLSIPATYKTQ------VYT--  645
          |      ||  |  | |  | | | |  | |  |
   551   --R------PT--PEKY-N--HRLSYI-S----A-YATDCGRISGVRGDG  600
   646   -F------AWTHSSVDPKNTIYTHLT--TQIPAVKANSLG-TASK-VVQG  695
          |      |||| | || |||  |    ||| ||||     |    || |
   601   CFRTPQMCAWTHVSADPYNTI--HPDKITQISAVKAFYIWDTGEGQVVSG  650
   696   PGHTGGD
         |||||||
   651   PGHTGGD
```

Fig. 4. Amino acid comparison between cry4Aa and cryINA288

```
Block 1
INA288      ---LF----AVAANFHLLLLRD-VSIYGK-EWGYT
4Aa         YNILVLSSYAQAANLHLTVLNQAV----KFEA-YL
4Ba         YELLLLPIYAQVANFNLLLIRDG-----------L
10A         YRIPTLPAYAQIATWHLNLLKHAA----TYYN-IW
11A         YEGVSIALFTQMCTLHLTLLKDGI----LAGS-AW
11B         YEGVSISLFTQMCTFHLGLLKDGI----LAGSD-W
Consensus   -di--la-fAQ-a--HL-LLkDA-------a---W

Block 2
INA288      WDFYNQYRRDMTLT-VLDVIALFPTYDVRKYPISTKV-------ELTREIYTD
4Aa         WNTYNTYRTKMTT-AVLDVVALFPNYDVGKYPIG-------VQSELTREIYQV
4Ba         WITFNDYKREMTI-QVLDILALFASYDPRRYPADKIDNTKLSKTEFTREIYTA
10A         WNMYNTYRLEMTL-TVLDLIAIFPNYDPEKYPIG-------VKSELIREVYTN
11A                FRNMCNLYVFPFAEAWS
11B                FRNMCSLYVFPFSEAWS
Consensus   W--f--fR-EMTL--VLDiiAiFa--D---YPI----------adLaRdiYTd

Block 3
INA288      CAWTHVSADPYNTI--HPDKITQISAVKAFYIWDTGEGQWSGPGHTGGD
4Aa         FAWTHSSVDPKNTIYTHL-TTQIPAVKANSLG-TASKVVQ-GPGHTGGD
4Ba         FAWTHKIVDPNNQIYTDA-ITQVPAVKSNFLN-ATAKVIK-GPGHTGGD
10A         FSWTHTSVDFQNTIDLDN-ITQIHALKALKVS-SDSKIVK-GPGHTGGD
Consensus   faWTH-S-d-N-I-a----ITQIP--K---i---aa-iV--GPG-TGGD
```

Fig. 5. Conserved blocks 1, 2, and 3 among mosquitocidal cry proteins.

(a=AGPST; d=DENQ; f=ILMV; k=KR)

Chapter III. Novel *Bacillus thuringiensis* serovar aizawai strains isolated from mulberry leaves in Indonesia

INTRODUCTION

There are many thousands of isolates of *Bacillus thuringiensis* protein parasporal bodies. Some of them belong to the *Bacillus thuringiensis* species *B. thuringiensis* spores and/or inclusion bodies usually express insecticidal activities. Several *B. thuringiensis* strains have been used for control agents of agricultural important insect pests on global basis. The insecticidal activity of parasporal inclusions makes this bacterium a promising agent for microbial control of insect pests of agricultural and medical importance (Luthy et al., 1982). In recent years, the need for environmentally safe pesticides has encouraged the search for new strains of *B. thuringiensis* with unique insecticidal spectra. These novel isolates will augment the current commercial *B. thuringiensis* strains that exhibit various degrees of activity toward larvae of insect orders of Lepidoptera, Diptera, and Coleoptera.

The crystal proteins produced from *B. thuringiensis* serovar israelensis are toxic to the larvae of mosquitoes (Golberg and Margalit, 1977; de Barjac, 1978) and blackflies (Undeen and Nagel, 1978; Undeen and Berl, 1979). From both health and economic standpoints, control of these dipteran species is important, because they are vectors for human diseases such as malaria and onchocerciacis (Margalit and Dean, 1985).

B. thuringiensis isolates have been recovered from numerous sources, including soils, grain dust, plant leaves, diseased insect larvae from insectaries, and sericultural environments (Filosa and Dengler, 1972; Saga and Yanagisawa, 1982). The principal source of novel *B. thuringiensis* isolates has been soil. Hastowo et al.(1992) reported that 135 strains of *B. thuringiensis* were isolated from soils of sericultural insectaries and environments of various regions in Indonesia. Distribution of *B. thuringiensis* in the soil of Indonesia is wider than in the soil of Japan (Iizuka et al., 1995), because the tropical regions, as Indonesia, offered a higher chance of isolating *B. thuringensis.* Smith and Couche (1991) isolated*B. thuringiensis* strains from the phylloplane. In recent years, Ohba (1996) has reported the *B. thuringiensis* strains were isolated from the mulberry leaves in Japan.

During joint research of Iizuka and Lay (Institut Pertanian Bogor), they isolated hundreds of *B. thuringiensis* strains from soil sample and mulberry leaves in Indonesia, and these strains have been maintained in Laboratory of Apllied Molecular Entomology, Faculty of Agriculture, Hokkaido University. In this study, I report that a novel isolate (serovar aizawai Bun 1-14) with highly mosquitocidal activity was found in the collection of this laboratory. A comparative study of its characterization and identification is also described.

MATERIALS AND METHODS

Bacterial stains.

The strain of *B. thuringiensis* serovar aizawai IPL and serovar israelensis ONR60A (Iizuka et al., 1982) and *entomocidus* INA288 (Iizuka et al., 1996) have been maintained and cultured in this laboratory.*B. thuringiensis* serovar *aizawai* Bun 1-14 was originally isolated from mulberry leaves in West Java, Indonesia by Iizuka and Lay (not published).

Identification by H-serotypeing

The isolate of *B. thuringiensis*Bun 1-14 was identified by H-serotype. In order to make antibody, H-antisera to the reference strain of *B. thuringiensis*serovar*aizawai* IPL (Iizuka et al., 1982) were prepared according to the method of Ohba and Aizawa (1978). For H-serotyping of the strains, actively motile bacteria were selected by passing throughcraigie's tubes at 37^0C for 24 hr. Slide agglutination was performed by mixing one drop of 3 to 4hr-old flagellated broth culture (N-broth and Agar 0.8%) of the bacteria on a glass slide with one drop of 20 to 50-fold dilution of H antiserum. Specific H agglutination was determined 3 to 5 min after mixing.

Morphology of parasporal inclusion.

Isolates were examine with a HITACHI S-800 Scanning Electron Microscope (SEM) at a magnification of 10,000x, according to the method *B. turingiensis* serovar *entomocidus* INA288 were cultured on N-broth agar at 30^0C until almost all cells lysed (overnight).The crystal and spores (about 100 mg wet weight) were washed in 10 ml of 50 mMTris-HCL (pH 8.0). The final precipitate was resuspended in 1 ml of distilled water, and 20 ul of the suspension was air-dried on a glass disk (O 10 mm). After the sample was coated with carbon and gold, it was observed and photographed with SEM.

Biological activityfor *A. stephensi*

The eggs of *Anopheles stephensi* (strain BEECH) were kindly supplied by Dr. H. Saitoh (Fukouka Industrial Technology Center, Japan). The mosquito cultures maintained at 22°C (16 L8D) in our laboratory. Toxicity test with the mosquito, *A. stephensi*, was done by introducing ten 2nd-instar larvae into a test tube containing 10 ml bacterial suspension.The *B. thuringiensis* isolates were examined for oral insecticidal activity against the A. stephensi, which were prepared by the following procedures. Overnight culture of serovar aizawai Bun 1-14, and *israelensis* ONR60A were grown on 2 ml of nutrient broth at 30°C using tube glass. Then, 200 ul of the overnight culture was plating on nutrient agar, reincubated for 4 days at 30°C. Sporulated cultures were harvested by centrifugation at 10,000 g for min at 4°C. The pellet was washed three times by centrifugation in mMTris-HCL and 1 M NaCL at 4°C, the bacterial suspensions were finally suspended in 500 ul of sterile distilled water. The bacterial suspension were finally suspended in 500 ul of sterile distilled water. In bioassays, 5 fold serial dilutions of the suspension were prepared in distilled water. Larvae were kept unfed and the mortalities were counted after 24 hr of incubation at 22°C. Assays were done in triplicate and the 50% lethal concentration values (LC_{50S}) were determined by probit analysis (Finney, 1971).

SDS-PAGE and Western blotting

Parasporal inclusions were separated from spores and cell debris using Percoll (Pharmasia) (Baba et al., 1990) as the following: Percoll solution were added 1 M NaCL solution and the *B. thuringiensis* sample was added for separating of crystal proteins from spores by centrifugation at 15.000 rpm at 4^{0}C for 20 min. In the first step, Percoll solution (Percoll: 0.15 M NaCL = 8:2) was prepared, and then 1 ml of crystals and spores mixture (10 mg [dry weight]/ml) pored carefully the top of 30 ml of percoll solution, and centrifuged at 15.000 rpm (30,000 g) for 30 min. After recovering the crystal protein, crystal protein was immediately washed three times by distilled water. Finally, the pellet was suspended with 500 ul sterile distilled water. The purity of parasporal inclusions was monitored with a phase-contrast microscope. SDS-PAGE of parasporal inclusion proteins was done by the method[26] using a 10% running gel with a 3% stacking gel. After electrophoresis, gel was steined with 0.04% Coomasie brilliant blue (Sigma Chem.Co.). The following prestained reference proteins (Bio-Rad Laboratories) were used as

moleculer markers: myosin (200 kDa), B-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

For immunoblot analysis (Towbin et al., 1979), the separated proteins were transferred electrophoretically from SDS-Polyacrylamide gel to an ECL nitrocellulose membrane (Amersham) using an electrophoretical tran-blott (Bio-Rad) according to the manufacture's instructions. The membrane was blocked with 3% skim milk in TBST buffer (100 nM Tris-HCL {pH 7.5}, 150 mM NaCL, and 0.1% {v/w} Tween 20) for 3 hr at 4°C, then the membrane was incubated in TBST buffer with 1% of polyclonal antibodies of B. thuringiensis serovar entomocidus INA288 for 1 hr. The nitrocellulose membrane was washed three times with TBST, and ten incubated with peroxidase IgG guinea pig in 1.5% TBST for 1 hr. The membrane was washed three times with TBS buffer and it was visualized with hydrogen peroxide (H_2O_2) and 4-Chloro-1-naphthol as described in the instructions from Bio-Rad.

Production of inclusion protein antibodies and immunobloting.

Antisera were raised to guinea pig against parasporal inclusion proteins of the *B. thuringiensis* serovar *entomocidus* INA288. Guinea pig was immunized by five subcutaneous injections, at weekly intervals. The solubilized parasporal inclusions were mixed with an equal volume of Freund's complete adjuvant (Difco). One week later, these were followed by intravenous booster injections of adjuvant-free solubilized inclusion proteins. The guinea pigs were bled one week after injection of 2 qnd 9 mg of serovar *entomocidus* INA288, respectively. The antiserum was centrifuged at 3000 rpm for 10 min, supernatant was collected and added 0.1% NaN_3 till storing of it at-20°C. Three days later, antiserum was cheked with SDS-PAGE and Western blotting.

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with TBS buffer and visualized with hydrogen peroxide (H_2O_2) and 4-chloro-1naphthol as describe in the instructions from BIO-Rad.

DNA preparation

Overnight cultures of the *B. thuringiensis* strains were grown in 2 ml of nutrient broth at 30°C using glass tube. One ml of the overnight culture was inoculated into 50 ml of typtose phosphate broth (Difco) using 200 ml culture flask and incubated for 4-5 hr until the synchronized culture had reached 10-15% optical transmission. The cells are finally harvested by centrifugation at 500 g for 10 min. Cells were suspended in approximately 1 ml of lysozyme (10 mg/ml; Wako pure chemical Industries, Ltd.), preincubated for 60 min at 37°C. Lysis was brought to incubate for 15 min at 60°C by the addition of 8% SDS (Sodium dodecyl sulfate) buffer in TES and an equal volume of 5 M NaCl to the cleared lysate, and the whole mixture was left overnight at 4°C. The mixture was centrifuged for 10 min at 14,000 rpm (Himac CF 15D), the supernatant fluid was carefully decanted to new tube, was added immediately by the addition of RNase (1 ug/ml), and preincubated for 30 min at 37°C. The cells were thoroughly washed by Phenol/Chloroform, the supernatant fluid was carefully transferred to another new tube, and then 100% ethanol (ETOH) was added. After mixture suspension was stored at -80°C for 20 min, it was centrifuged at 15,000 rpm for 15 min and the supernatant fluid was discarded. Finally, the pellet was dissolved in 500 ul of electrophoresis buffer.

Polymerase chain reaction (PCR) procedure

The template DNA is first denatured in the presence of a large molar excess of the two oligonucleotides and the four dNTPs. The reaction mixture is cooled to temperature by allowing the oligonucleotide primers to anneal to their target sequence, after which the annealed primers are extended with DNA polymerase (LIFE TECH, Elongase). The cycle of denaturation is at 30 times, at 92^{0}C for 1 min, annealing is at 48^{0}C for 1 min and polymerization is at 72^{0}C for 2 min. The PCR technique was used to identify the contents of *B. thuringiensis* serovar *entomocidu*s INA288, using oligonucleotide primers specific to *Cry1* (Kalman et al., 1993), *CryII* (Asano et al., 1993) and *CryIV* (Asano, 1996). Identification of cry genes in genomic DNA sample extracted from *B. thuringiensis* serovar *entomocidus* INA288 strains was based on unique-size DNA fragments amplified by PCR for each cry gene (Asano, 1996).

Southern blot hybridization

DNA extraction from *aizawai* Bun 1-14 was digested with *Bam*HI, then incubated at 30°C for 1 hr. The digest solution was electrophoresed using Agarose 0.8%, applied trans blotting on to a nitrocellulose membrane (Hybond-N^{+}) to the surface of the gel and trans blotting onto a Nylon membrane (Hybond-N^{+}) with using Electropdoresis Apparatus (BRL) (Life Technology, Inc.) for 1 hr, 100V. The membrane was dried, fixed with UV transilluminator for 3 min and was transferred to prehybridize and 3 mg denatured (Salmon sperm DNA). After incubation of the membrane at 42°C overnight, a denatured INA288B probe labeled with {^{32}P-dATP} by Megaprime labeling kit (Amersham) was added in prehybridization solution, and incubated at 42°C overnight. Then, the membrane was washed twice with 30 ml of the first solution (2x SSC, 0.1% SDS at 50°C for 1 hr). The hybridization profile was visualized by using Bas 1000 system (Fuji Film Co.).

RESULTS

H-serotyping of Bacillus thuringiensis isolates

In order to identify Bun 1-14 and Bun 2-1 strains by H-serotype cells with broth, they were dropped to glass slide and the motility of cells was observed under phase-contrast microscope. Since isolates of Bun 1-14 and Bun 2-1 gave a positive reaction in the H 7 serum agglutination test, both of tem were identified as *Bacillus thuringiensis* serovar *aizawai*.

Morphology of Bun 1-14 and Bun 2-1 by scanning electron microscopy(SEM)

Morphology of the crystal from serovar *aizawai* IPL has been reported by Iizuka et al. (1982). They reported that serovar *aizawai* IPL produced bipyramidal and irregular shaped crystal proteins. However, observation conducted using high resolution SEM revealed that *B. thuringiensis* serovar *aizawai* Bun 1-14 and serovar *aizawai* 2-1 produced only irregular-shaped proteins (Fig.6). some*B. thuringiensis* strains produced irregular shaped crystal proteins.

Quantitative toxicity test of isolates

Toxicity of inclusion of *B. thuringiensis* serovar IPL, Bun 1-14 and Bun 2-1 was shown in Table 4, against the *B. mori, P. xylostella, A. aegypti, C. quinquefasciatus*, and *S. litura*. By the result, *B. thuringiensis* serovar *aizawai* IPL showed toxic activity to *B. mori, P. xylostella*, and *S. litura*, even though was not toxic to *A. aegypti*, and *C.quinquefasciatus*. on the other hand, aizawai Bun 1-14 was toxic to the dipteran insects, *B. mori, P. xylostella*, and *S. litura*, while *aizawai* Bun 2-1 was not toxic to any insects of both groups.

As the strain of *aizawai* Bun 1-14 had a mosquitocidal activity, differences in the toxic activity to mosquito larvae between Bun 1-14 and another mosquitocidal strains, serovar *aizawai* strains and serovar *israelensis* ONR60, were demonstrated using 2nd-intar larvae of *Anopheles stephensi*. Sporulated cultures were washed three times for removing the B-exotoxin activity by 1 M NaCL and dried at room temperature for one week. Serovar *aizawai* Bun 1-14 and israelensis ONR60 showed highly toxic activities against *A. stephensi*, while both serovar

aizawai IPL and *aizawai* Bun 2-1 were not toxic to this insect. Ohba (1996) reported that the serovar aizawai strains, which were isolated from mulberry leaves in Japan, had a moderate toxicity against mosquito larvae. The lethal concentration (LC_{50}) value of *B. thuringiensis* serovar aizawai Bun 1-14 was 0.0057 ug/ml, and an almost similar activity was seen in the case of *B. thuringiensis* serovar *israelensis* ONR60A (0.027 ug/ml) (Table 5). This fact showed that serovar *aizawai* Bun 1-14 was a novel isolate from mulberry leaves.

SDS-PAGE analysis

The purity of *B. thuringiensis* serovar *aizawai*Bun 1-14 and *aizawai* Bun 2-1 parasporal inclusion by percoll showed more than 90%, depending on monitoring with a phase-contrast microscope.

SDS-PAGE analysis of parasporal inclusions purified from serovar *aizawai* Bun 1-14 demonstrated that the protein composition of its srain is significantly different from that of *B. thuringiensis* serovar *israelensis* (mainly 130 kDa, 70 kDa, and 27 kDa) and serovar *entomocidus* INA288 (70 kDa). The crystal of serovar *aizawai* Bun 1-14 consisted mainly of 69 kDa peptides, while serovar *aizawai* Bun 2-1 consisted mainly of 130-65 kDa peptides (Fig. 7A).

Immonublot analysis

To investigate the antigenic relationship among the *B. thuringiensis* serovar *aizawai* Bun 1-14 and serovar *aizawai* Bun 2-1 crystal toxins, and two other serovar *entomocidus* INA288 and *israelensis* ONR60A with known mosquitocidal activity, western blotting and enzyme immunoassay (immunoblotted) were demonstrated (Fig. 7B). the antibody of entomocidus INA288 had cross-reactivities against *B. thuringiensis* serovar *aizawai* Bun 1-14 and serovar *israelensis* ONR60A. The immunoblot analysis showed that the 69 kDa peptides of *B. thuringiensis* serovar *aizawai* Bun 1-14 had encoded in similar cry genes with *B. thuringiensis* serovar *israelensis* ONR60A (130 kDa peptides). However, immunoblot profiles of the serovar *aizawai* bun 2-1 was quite different from those strains.

Identification of Cry genes in *B. thuringiensis* serovar *aizawai*

The PCR method was used to survey a number of *B. thuringiensis* strains for their crystal protein gene content. One set of primer pairs was used to detect, in one reaction. In the identification of cry genes of *B. thuringiensis* serovar *aizawai IPL*, *aizawai* Bun 1-14 and *aizawai* Bun 2-1, *cryI*, *cryII*, and *cryIV* primers were used. PCR with these primers and DNA template generated a uniquely size fragment for each cry type gene. Therefore, the DNA size of PCR product indicated the presence of particular crystal protein genes. Both of serovar *aizawai* Bun 1-14 and *aizawai* Bun 2-1 did not amplify*cryIC*, *cryIIA*, *cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD* genes (Table 6). Since their cry genes had mosquitocidal activities it seemed that these

strains contained novel cry genes that are responsible for the unique insecticidal activities, because B. thuringiensis serovar *aizawai* Bun 1-14 had a highly toxicity against *A. stephensi*.

Southern blot hybridization

Southern hybridization signal was detected from plasmid DNA of *aizawai* Bun 1-14 and its DNA was digested with *Bam*HI (Fig. 8), which were probed of *entomocidus* INA288B. The result showed that the toxin genes from *aizawai* Bun 1-14 had a little homology to the toxin genes from *B. thuringiensis* serovar *entomocidus* INA288B. The INA288B probe was hybridization with a large plasmid and *Bam*HI 20 kb fragment of *aizawai* Bun 1-14.

DISCUSSION

While *B. thuringiensis* strains used to be isolated from soil, dust (grain dust) and sericultural areas, *B. thuringiensis* serovar *aizawai* Bun 1-14 and *aizawai* bun 2-1 were isolated from mulberry leaves in Indonesia. Indeed, Smith and Couche (1991) have reported that many *B. thuringiensis* strains were isolated from the phylloplane of deciduous and conifer trees. In addition, Ohba (1996) examined the mulberry leaves for the occurrence of *B. thuringiensis* in Japan. In the present study, the source of *B. thuringiensis* serovar aizawai Bun 1-14 and *aizawai* Bun 2-1 was mulberry leaves in Indonesia and *aizawai* Bun 1-14 showed mosquitocidal activity. This fact means serovar *aizawai* is a unique strain.

In the morphological study by using SEM, *B. thuringiensis* serovar *aizawai* IPL as the type *aizawai* strain has bipyramidal and irregular shaped crystal proteins (Iizuka et al., 1982). Interestingly, *aizawai* Bun 1-14 and aizawai Bun 2-1 produced only irregular shaped crystal proteins, which were similar to the forms produced by serovar *israelensis* ONR60A (Iizuka et al., 1982), serovar *morrisoni* PG-14 (Padua et al., 1984) and serovar *kyushuensis* (Held et al., 1990). In generally, *B. thrurinegiensis*, which produces irregular shaped crystal proteins, has a mosquitocidal activity.

The insecticidal activity of *B.thurngiensis* serovar aizawai IPL showed only on toxicity to the leidopteran insects (*Bombix mori, Plutella xyloetella*, and *Spodoptera litura*). However, *aizawai* Bun 1-14 was toxic to the dipteran insects (*Aedes Aegypti, Anopheles stephensi* and *Culex quinquefasciatus*), while *aizawai* Bun 2-1 was not toxic to lepidopteran insect and dipteran insect species. The LC50 of *aizawai* Bun 1-14 against *A. stephensi* was calculated to compare the

toxicities of *B. thuringiensis* serovar *israelensis* ONR60A and serovar *entomocidus* INA288. The mosquitocidal activity of this isolate was almost similar to those of *B. thuringensis* serovar *israelensis* ONR60A and serovar *entomocidus* INA288. The results indicated that this strain has highly toxic activity against *A. stephensi*. Iizuka et al. (1996) reported that serovar *entomocidus* INA288 as toxic *to A. japonicas, A. aegypti* and *C. quinquefasciatus*. Ohba et al. (1995) reported that *B. thuringiensis* serovar *higo* (flgellar serotype 44) as also toxic to *A. stephensi* and *C. pipiens molestus*. A new mosquitocidal *B. thuringiensis* serovar *jegathesan*, which as composed of polypeptides of 77 kDa had toxic to *C. quinquefasciatus, A. aegypti, A.togoi. A. albopictus, A. maculates,* and *Mansonia uniformis* (Kawalek et al., 1995). On the other hand, *B. thuringiensis* serovar *medellin* (flagellar serotype 30) isolated in Columbia includes peptides of 100 to 30 kDa and showed a toxicity to *C. quinquefasciatus, C. pipiens, A. aegypti*, and *A. stephensi. B. thuringiensis* serovar *fukoukaensis*, which consisted of polypeptides of 90 to 27 kDa, was toxic to A. aegypti, and C. quinquefasciatus (Yu et al., 1991). In addition, Ishii and Ohba (1997) reported that *B. thuringiensis* serovar *canadensis* composed of major proteins of 65 kDa had toxic to *A. aegypti*. Moreover, *B. thuringiensis* serovar *israelensis* ONR60A had constituted of mosquitocidal 130 kDa, 70 kDa, and 27 kDa proteins, and serovar *entomocidus* INA288 constituted of mosquitocidal 70 kDa proteins, respectively. *B. thuringiensis* serovar*darmstadiensis* 73-E-10-2, which were composed of polypeptides of 125 to 28 kDa, had mosquitocidal activity to *A. aegypti* (Kim et al., 1984; Drobniewski et al., 1989). In the present study, it was cleared that the crystal protein of serovar *aizawai* Bun 1-14 was constituted of 69 kDa peptides and this was not the same peptide wit other mosquitocidal strains.

Using antibody of *B. thuringiensis* serovar *entomocidus* INA288 crystal protein, I demonstrated that there is cross-reactivity among the parasporal inclusion proteins of *B. thuringiensis* serovar *aizawai* Bun 1-14 and serovar *israelensis* ONR60A. Antisera for whole *B. thuringiensis* serovar *entomocidus* INA288 parasporal inclusion has only cross-reactivity to 69 kDa proteins bands of *B. thuringiensis* serovar *aizawai* Bun 1-14 and 130 kDa proteins bands of serovar *israelensis* ONR60A. These results suggest that there is partial similarity to cry genes between *B. thuringiensis* serovar *entomocidus* INA288, serovar *aizawai* 1-14 and serovar *israelensis* ONR60A. However, Kawalek et al. (1995) demonstrated that using antibodies of B.thuringiensis serovar israelensis ONR60A showed weak cross-reactivity to serovar

*jegathesan.*Indeed, *B. thuringiensis* serovar *jegathesan* included cryIVD and showed immunological similarity with antibodies of serovar *israelensis*.

PCR screening analysis suggests that the *B. thuringiensis* serovar *aizawai* Bun 1-14 and *aizawai* Bun 2-1 with specific primers failed to show a cry gene. However, the *aizawai* Bun 1-14 had novel cry gene with mosquitocidal activity. Anyhow, those isolates needed another specific primers set and under different condition for the next PCR treatment.

In southern hybridization analysis showed that almost 50 kb plasmid of *B. thuringiensis* serovar *aizawai* Bun 1-14 has a littlehomology with serovar *entomocidus* INA288 cry gene, while the fragment (20 kb) of aizawai Bun 1-14 digested with *Bam*Hi has a little homology with that gene.

In conclusion, a new mosquitocidal *B. thuringiensis* serovar *aizawai* Bun 1-14 was found and characteristics of toxic protin from this strain were demonstrated. Since cry gene serovar *aizawai* Bun 1-14 has only partial homology with the cry gene from *entomocidu*s INA288, it seems a novel cry gene. In the near future, it becomes very important and very useful to clear the mechanism of mosquitocidal activities of these two isolates. These strains should be a alternative or supplement insecticidal agenst to the application for the biological control of mosquitoes.

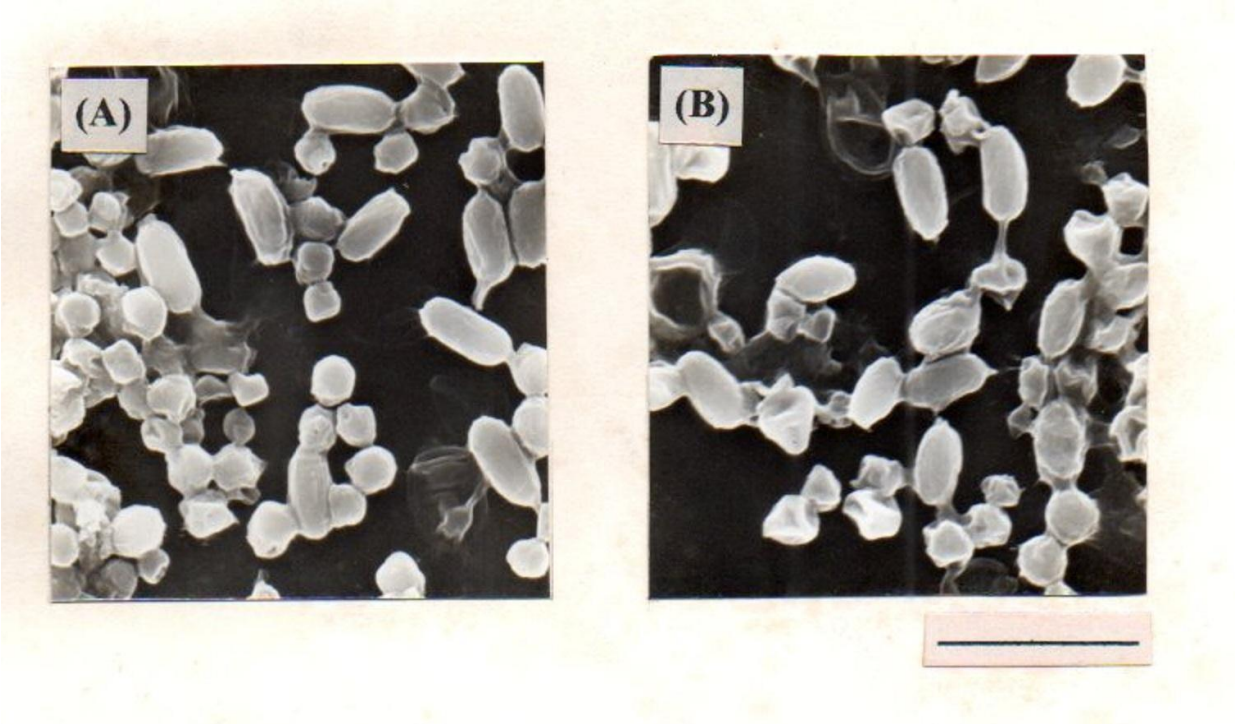

Fig. 6. Scanning electron microscopy showing spores and parasporal crystal of *B. thuringiensis* serovar *aizawai* Bun 2-1 and serovar *aizawai* Bun 1-14 Bar 3 um.

Table 4. Toxic of serovar aizawai strains against five insect species

Strain	Lepidopteracidal			Dipteracidal	
	B, m,.	***P.x.***	***S.l.***	***A.a.***	***C.q.***
aizawai IPL	+	+	+	-	-
aizawai Bun 1-14	-	-	-	+	+
aizawai Bun 2-1	-	-	-	-	-

B.m: Bombyx mori, P.x: Plutella xylostella, S.l: Spodoptera litura
A.a: Aedes aegypty, C.q: culexquinque fasciatus

Table 5. Toxicity of crystal proteins from *Bacillus thuringiensis* strains to 2nd-instar larvae of *Anopheles stephensi.*

Serovar	LC50 (ug/ml{fiducial limit}
israelensis	0.027 {0.014-0.060}
aizawai IPL	> 5 (insufficient)
Aizawai Bun 1-14	0.057 {0.035-0.088}
aizawai Bun 2-1	> 5 (insufficient)

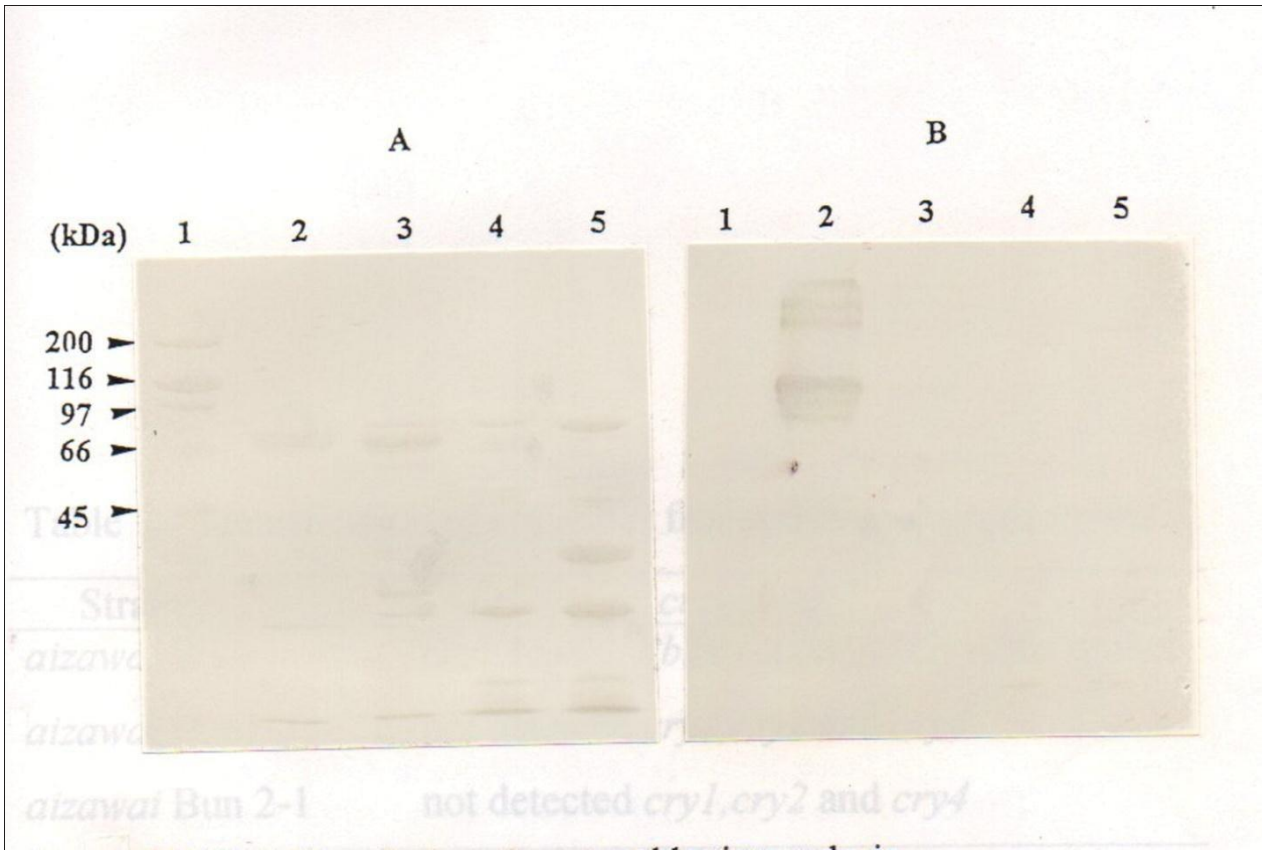

Fig. 7. SDS-PAGE analysis and western blotting analysis

(A) SDS-PAGE gel (10%) stained with Coomassie brilliant blue

(B) Western blotting and EIA analysis

Lane 1: molecular weight marker

Lane 2: *serovar entomocidus* INA288

Lane 3: *serovar aizawai* Bun 1-14

Lane 4: *serovar aizawai* Bun 2-1

Lane 5: *serovar israelensis*

Table 5. Identification of cry genes from serovar *aizawai* strains

Straincry genes	
***aizawai* IPL**	***cry IAa, cry I Ab, cry I C, cry I D, cry 2 Aa, cry 2 ab***
***aizawai*Bun 1-14**	**not detected *cry I, cry 2*, and *cry 4***
***aizawai* Bun 2-1**	**not detected *cry I, cry 2*, and *cry 4***

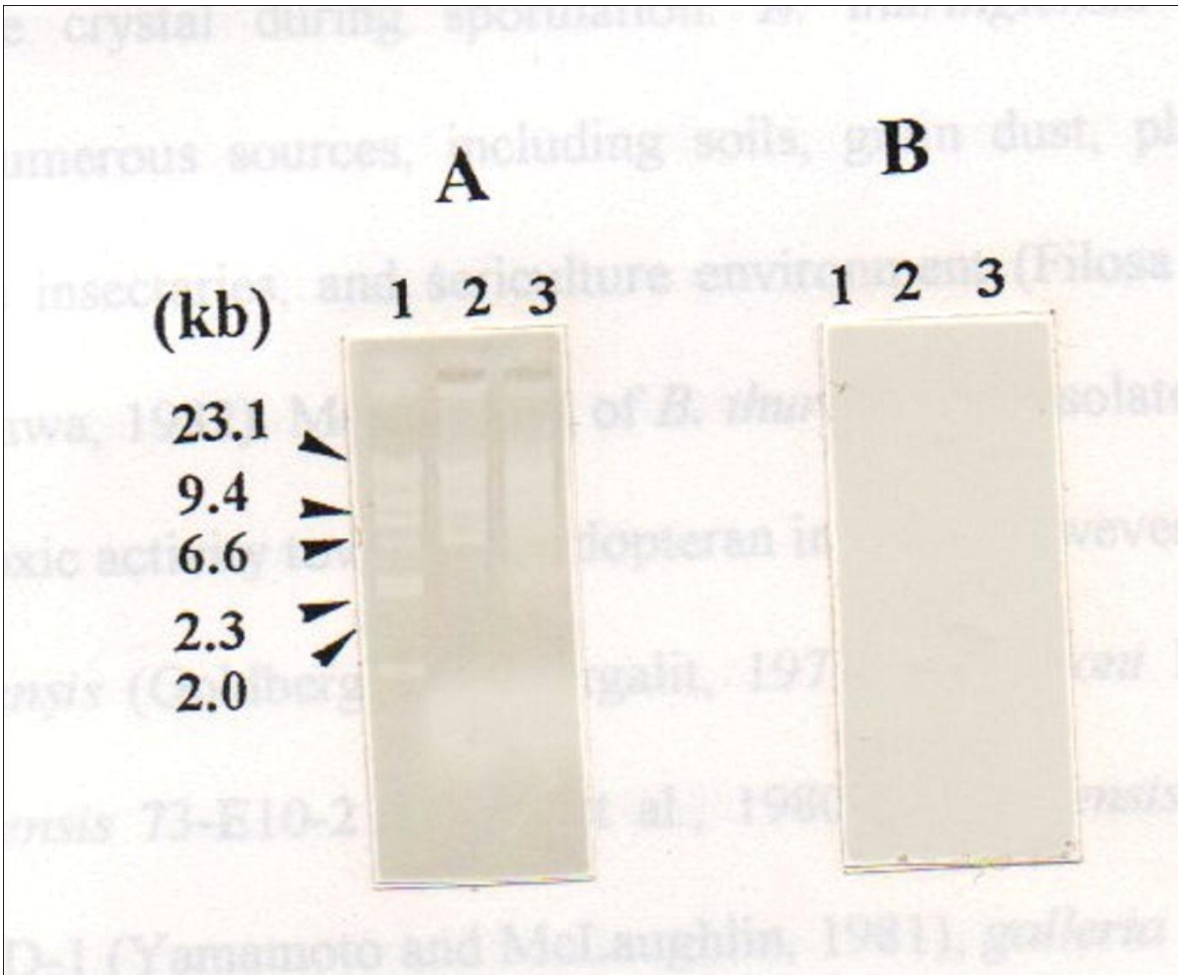

Fig. 8.A. DNA of *B. thuringiensis* serovar *aizawai* Bun 1-14 was analyzed on a 0.8 agarose gel

Lane 1: molecular weight marker

Lane 2: DNA of *aizawai* Bun 1-14

Lane 3: DNA of *aizawai* Bun 1-14 digested with *Bam*Hi

B. Southern blot hybridization

Lane 1: molecular weight marker

Lane 2: DNA of *aizawai* Bun 1-14 undigested

Lane 3: DNA of *aizawai* Bun 1-14 digested with BamHi

Chapter IV. GENERAL DISCUSSION

Bacillus thuringiensis, a gram-positive soil bacterium, is characterized by its ability to produce crystal during sporulation. *B. thuringiensis* isolates have been recovered from numerous sources, including soil, grain dust, plant leaves, diseased insect larvae from insectaries, and sericulture environment (Filosa and Dengler, 1972; Saga and Yanagisawa 1982). Most strains of *B. thuringiensis* isolated early time prior to 1972 showed toxic activity towards lepidopteran insects. However, a few serovar, such as serovar *israelensis* (Goldberg and Margalit, 1977), *morrisoni* PG-14 (Padua et al., 1984), *darmstadiensis* 73-E-10-2 (Padua et al., 1980), *kyushuensis* (Ohba and Aizawa, 1979), *kurstaki* HD-1 (Yamamoto and Mc Laughlin, 1981), *galleria* (Ahmad et al., 1989),*fukoukaensis* (Yu et al., 1991), *medellin* (Orduz et al., 1992), *jegathesan* (Kawalek et al., 1995), *higo* (Ohba et al., 1995), and *canadensis* (Ohba, 1997) which produce crystal proteins were found as mosquitocidal strains to dipteran insects.

In recent years, formulation of mosquitocidal *B. thuringiensis* serovar *israelensis* and serovar *sphaericus* have been used on a large scale in the field and in the various countries. This initial success has been tempered by the result obtained for resistance to *B. thuringiensis* serovar sphaericus (Georghiou et al., 1993). Numerous investigators have explored a new strain of *B. thuringiensis* as alternatives to the application of *B. thuringiensis* serovar *israelensis*. During joint research of Iizuka and Lay (Institut Pertanian Bogor), they isolated hundreds of *B. thuringiensis* strains from soil sample and mulberry leaves. In the present study I found the unique strains, *B. thuringiensis* serovar *entomocidu*s INA288 and serovar *aizawai* strains, both of which were isolated in Indonesia. Morphology of the crystals from serovar *entomocidus* original strains has been reported by Iizuka et al. (1982) and Faust et al. (1982) that serovar *entomocidus* original strain produced bipyramidal-form crystal. However, observation conducted using high resolution Scanning Electron Microscope (SEM) revealed that the original strain produced irregular-form crystal. On the other hand, serovar *entomocidus* INA288 produced large cuboidal-form crystal. Interestingly, serovar *aizawai* Bun 1-14 and Bun 2-1 produced only irregular shaped crystal proteins, which were similar in shape to the crystal produced by serovar i*sraelensis* ONR60A (Iizuka et al., 1982)., while *aizawai* IPL had bipyramidal and irregular shaped crystal proteins (Iizuka et al., 1982).

B. thuringiensis serovar *israelensis* ONR60A had mosquitocidal activity, which was composed of major protein of 130 kDa peptides. On the other hand, *B. thuringiensis* serovar

entomocidus INA288 and aizawai Bun 1-14 also had mosquitocidal activity, while *aizawai* Bun 2-1 both did not have. In addition, neither serovar *entomocidus* original strain nor serovar a*izawai* IPL had lepidopteracidal activity. SDS-PAGE profiles of *entomocidus* INA288 crystals indicated that the polypeptide of 70 kDa was dominant, while *entomocidus* original strain was constituted of 130-65 kDa. However, *B. thurngiensis* serovar aizawai Bun 1-14 was composed of polypeptides of 69 kDa, while *entomocidus* Bun 2-1 was constituted of 130-65 kDa. Interestingly, using antibody of *B. thuringiensis* serovar *entomocidu*s INA288 crystal protein demonstrated that there is cross-reactivity among the parasporal inclusion proteins of *B. thuringiensis* serovar *aizawai* Bun 1-14, *israelensis* ONR60A, and *fukoukaensis.* However, Kawalek et al. (1995) demonstrated that using antibodies of *B. thuringiensis* serovar *israelensis* ONR60A showed weakly cross-reactivity to serovar *jegathesan*. Indeed, *B. thuringensis* serovar jegathesan included *cryIVD* and showed immunological similarity with antibodies of serovar israelensis. While, *entomocidus* INA288 contained a novel crystal protein gene *cryINA288*, on plasmid. In addition, the similarity of amino acid sequence between *cryINA28*8 and *cry4Aa* was 38%. On the other hand, the characteristics of toxic protein of *B. thuringiensis* serovar *aizawa*i demonstrated that cry gene from serovar *aizawai* Bun 1-14 has only partial homology with the cry gene from serovar *entomocidus* INA288, and that it seems a novel *cry* gene.

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