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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Bacillus *thuringiensis* from Indonesia as a Unique Insecticidal

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Chapter I. C.ENERAL 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 bnth human and the environment. In addition, target insect pests rapidly develop biological resistance especiall y 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 toxicit y to an y organism and their low specific toxicit y to any organism and their slight decomposition in nature (Shore y and Hall, 1962). Therefore, many biological control s of insects have been investigated. Currently, researches on the use of pathogenic micrcinrganisms to control insect pests are increasing. Micmbial 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. lt was a study of flacherrie of the sil kworm, *Bomh v. mori* (Burges and Husse y, 197 I and Burges, 198 I). lshiwata (190 I) 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 *Btu.illus thurinpien.st.s* for a species of bacillus which was isolated from the diseased larvae of the Mediterranean flour moth Anagasta (Ephestia) *kuhniell‹i* Zell. Later, Berliner (1915) noted infection of the larvae after the ingestion of the bacillus or its spores, described and named it *B‹i‹ illus thurinpiensis* (1927) isolated the same bacillus from the same insect host, which Berliner had found earlier. This strain is now maintained as *B. thurinpieusis* serovar *thurinpiensis* (serot ype H- I). 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 bndies any role in the disease process caused by ingestion of spnrulating *B. thurinpiensis.*

B. thurinpieusis 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. *thurinpieusis* 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 (196 I) has defined various toxic substance produced b y *B. thurinpiensis a s* follow: (a) thermolabile endotoxin; (b) thermostable exotoxin; (c) bacillogenic antibiotic; (d) lecithinase; (e) proteinase.

B.thru ingirn.st. has been studied world wide over the past decades, mainly because this gram positive bacteriu m producer significant amounts of crystal proteins with toxic activit y against economically important insect larvae (DeSouza et al, 1993).

The most attractive characteristics of the *B. thurinpieusis* proteins for insect control are their specificit y 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. thurinpieusis* from soil samples, plant surfaces, dead insects, and stored grains from all over the world. The isolated strains show a wide range of specificit y against different insect orders (Lepidotera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phithiraptera or Mallophaga, and Acari) and other invertebrates (Nemathelminthes, Plat yhelminthes, and Sarcomastigophora) (Feitelson, 1993). Currently 45 different serot ypes have been classified (Table I) by Lecadet et al, (1994). The first classification of insecticidal cr ystal protein genes (cr y gene) (Tabel 2) was based on insecticidal activity (Hofte and Whitele y, 1989)., with different cry proteins denoting their gnes tixic to various insect and vertebrate groups as follow: *‹u vI* toxic to lepidopterans, *‹u vII* toxic to lepidopterans and dipteran, *‹ rvIII* toxic to coleopterans, *‹u vlV* toxic to dipterans, and ‹ yV and *‹u vVI* toxic to nematodes (Feitelson et al., 1992).

Most strains of *B. thurinpiemsis* produce delta-endotoxin crystals toxic to lepidopteran insects such as moth (Dulmage, 1981). But some strains of *B. thurin pien.st.s* 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 comprised of 130 kDa proteins (Calabrese et al., 1980; Bulla et al., 1977) while dipterans-specific delta-endotoxin are comprised of several proteins (Pfannenstiel et al., 1984; Lee et al., 1985). Yamamoto and McLaughlin (198 I) has reported P I (135 kDa) and P2 (65 kDa) proteins from serovar kurstaki HD- I 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 propert y as commercial insecticides is due to 28 kDa protein of dipteran-specific delta-endotoxin (Ward and Ellar, 1987; Pfannenstiel., 1986). The insect specificit y of the *B. thuripien.ti.t* insecticidal proteins has, however, limited their usefulness in man y important agricultural situations. None of the proteins encoded by the previously characterized *B. thurinpien.ti.t* 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 *Btu. illu.s* strains possessing a high larvicidal activity, specificit y for mosquitoes, from the soil of mosquito-breeding site in Israel. On the basis of this strain, de Barjac (1978) established *Btu.illus thuringiensis* serovar *i.sr‹ielensis* (H antigen 14).

Thereafier, many workers have reported the occurrence of highl y mosquitocidal *B. thru inpirnsis* .ttarins, belonging to serovar israelensis (Balararnan et al., 198 I ; 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. thurinpiensis,* with moderate to low toxicit y, is also disseminated in natural environments. Recenl y, however, several workers have shown that *B. thru inpirnsis* is also widely distributed in natural soils of various area. Delucca et al., (1981) reported that *B. thurinpieusis* made up less than 0.5°/c 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 frequenc y at which *B. thurinpiensis* could be recovered from soils. However, Martin and Travers (1989) have recovered *B. thurinpieusis* from numerous soils obtained from around the world. While Ohba and Aizawa (1989) have reported recovery of over 300 *B. thurinpiensis* isolates from Japanese soils and sericulture areas. lt 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 abnut its distribution in other regions of Southeast Asia.

When author started this study, no mosquitocidal activity of *B. thurinpien.st.s* has been isolated yet in Indonesia. Howe ver, Sasaki et al. (1996) isolated *B. thurinpien.st.s* serovar kui:troki INA-02 from soil in Indonesia, which was toxic to lepidoteran insects, so 1 aimed at screening of mosquitocidal *B. thru inpirnsis* strains from soil and mulberry leaves in Indonesia, and to increase our knowledge of the distribution of *B. thurinpiensis* in the tropic Asian condition.

Table 1. Classifiction of *Bacillusthuringiensis*

SEROTYPE (H-ANTIGEN)	ESTERASE TYPE REFERENCE	SUBSPECIES	PRODN. OF B-E XOTOXIN lN
1	thuringien.si.s	thuringein.si.s	+
Subsp.			
2	*finitimu.s*	finitimu.s	
5a 3b	*ale.sti*	*alesti*	
4a 4b	*kur.staki*	*kurstaki*	
4a 4c	.sorro	.sorrn	
5a 5b	*kenyae*	*kenyae*	
5a *5c*	*galleriae*	*galleriae*	
6	canadian.si.s	*canadian.si.s*	
7	enromocidu.s	enrnmocidu.s	
8a 8b	morri.soni	*morri.soni*	+
8a 8c	o.srriniae	*o.striniae*	
8b 8d	*nigerien.si.s*	nigerian.si.s	
9	*tolworihi*	*tolworihi*	+
10	darm.sradien.si.s	darm.sradien.si.s	
11a 1lb	*toumanoffi*	*toumanoffi*	+
11a 12c	kyu.sheen.si.s	*k y shuen.si.s*	
12	*thorny.soni*	*thorny.soni*	

Table l.continued.....

SEROTYPE (H-ANTIGEN)		ESTERASE TYPE REFERENCE	SUBSPECIES	PRODN. OF B-EXOTOXIN IN
	13	ski.srani	*Paki.stani-*	
	14	*i.srae*fen.si.s	*i.srae* fen.si.s-	
	15	*dakota*	*Dakota*	
	16	*indiana*	*Indiana-*	
17		toI/okuerzsi.s	toI/okuerzsi.s	
18		kumamoroen.si.s	kumamoroen.si.s	+
19		tochigien.si.s	tochigien.si.s	
20a	20b	yunnanen.si.s	yunnanen.si.s	
20a	20c	pondicheriensis	pondicheriensis	
	21	*colmeri*	*colmeri*	
	22	.shadongien.si.s	.shadongien.si.s	
	23	jayonen.si.s	jayonen.si.s	
	24	neoleonen.si.s	neoleonen.si.s	
	25	coreanen.si.s	coreanen.si.s	
	26	.si/o	.si/o	
	27	mexicanen.si.s	mexcanen.si.s	

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

Table 2. Mosquitocidal *B‹i‹illus thru inpirnsis* delta-endotoxin genes

Name	Original	Accession	Reference	Year	Coding region
Cry2AaI	*cryIIA*	M31738	Donovan et al	1989	I 56-2054
Cry4AaI	*cryIVA*	Yoo423	Ward & Ellar	1987	I -3540
Cry4BaI	*cryIVB*	XO7423	Chungjatporn Chai et al	1988	I 57-3564
Cry10AaI	*cryIVC*	M 1 2662	Thorne et al	1986	941-2965
Cry11AaI	*cryIVD*	M31737	Donovan et al	1988	41- 1969
	Cry288		lizuka et al	1996	
	Cry19A		Rosso et al	1997	

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

INTRODUCTION

Btu. illus thurinpien.ti.t is gram-positive, spore-forming bacterium that produces parasporal crystal during the sporultaion stage. The cr ystal is made of one or more proteins toxic to some insect species. Golberg and Margalit (1977) isolated a *h‹i‹ illus* 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. thurinpiensis* serovar israelensis (H antigen 14) as highly mosquitocidal activity. Another investigators have reported the occurrence of highl y mosqutocidal *B. thurinpien.st.s* strains belonging t serovar israelensis (Balarman et al.,198 I ; Zhang et al., 1984; Shim et al., 1990; Abdel-Hameed et al., 1990). To date, most strains of *B. thurinpien.st.s* produce inclusions toxic to dipteran insects, though three are subspecies such as serovar *mot t i.sent* PG- 14 (Padua et al., 1984), /ukr›ukoruti.t (Yu et al., 1991), Sum.trodirutis (Kim et al., 1984), *k vushuensis* (Held et al., 1990), yrgor/ir.tan (Kawalek et al., 1995), /iigo (Ohba et al., 1995), and ‹onodirn.ti.t (lshii and Ohba, 1997), which produce toxins that are predominantly toxic to dipteran insects.

In this chapter, 1 describe the identification, characterization and profiles of the crystal protein peptides between mosquitocidal serovar *entomo‹ idus* lN A288 and other mosquitocidal strains. 1 found that *entomo‹ idus* lN A288 had mosquitocidal activity and similar genes between *B. thurinpiensis* serovar *i.truelensis* and serovar *fuLouk‹iensis.*

MATERIALS AND METHODS

Bacterial stains.

The strain of *B. thurinpien.st.s* used in the present study wereB. *thurinpiensisse ov'a i. r‹ielensis* ONR60A and *entomo‹ idmt* (orginal strain) and *B. thurinpiensis* serovar *entr›mr›‹* iduslNA288. The *B. thurinpien.st.s* serovarf /ukur›Rrn.ti.t, and *k vhusuen.st.s* were provided and cultured in our laborator y.

Isolation and identification.

B. thurinpiensis serovar lN A288 which had been isolated from Indonesia soil, was prepared according to the methcd. 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 spnrulated 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. thurinpiensis* lN A288 was identified by H-serot ype. In order to make antibody, H-antisera to the reference strain of *B. thuringiensisserov’arentomo‹ idus* original starin) were prepared according to the method. For H-serot yping of the strains, activel y motile bacteria were selected by passing trhoughcraigie’s tubes at 37"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°/c) 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. turinpiemsis* serovar *entomo‹idu.s* lN A288 were cultured on N-broth agar at 30"C until almost all cells lysed (overnight).The cr ystal and spores (about 100 mg wet weight) were washed in 10 ml of 50 mMTris-HCL (p H 8.0). The final precipitate was resuspended in I 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, *Plutello .cvlostell‹i* and .Syr›dr›yrrio *litur‹i.* The insect cultures were maintained in this laboratory. Toxicity

test with the Lepidopteran insect, *B. mori*, f. *lo. trllu* and .S. limbo, were done by introducing ten 3"-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 mortalit y. The *B. thurinpieusis* isolates were examined for oral insecticidal activity against the insects were prepared by the following procedures. Overnight culture of serovalNA288, and i.tier/ruti.tONR60A 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 I 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 mosquitcies, *Aedes ‹ie pvpti, Aedes* Joyr›nir u.t and *C’ule. quinine [‹red i‹itus.* Ten 2" -instar larvae were placed in a test tube containing 10 ml of the spore-parasporal inclusion suspension, respectively, under levels I ul/ml. The tubes were kept at 22”C for 24 hr without feeding.

SDS-PAC’.E and Western blotting

Parasporal inclusions were separated from spores and cell debris using Percoll (Pharmasia) as the following: Percoll solution were added I M NaCL solution and the *B. thurinpieusis* sample was added for separating of cr ystal proteins from spores b y centrifugation at I5.000 rpm at 4"C for 20 min. In the first step, Percoll solution (Percoll: 0.15 M NaCL = 8 :2) was prepared, and then I ml of cr ystals and spores mixture (10 m_V l 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 purit y of parasporal inclusions was monitored with a phase-contrast microscope. SDS-PAGE of parasporal inclusion proteins was done by the method" using a 10°/c running gel with a 3°/c stacking gel. After electrophoresis, gel was stained with 0.04°/c Coornasie brilliant blue (Sigma Chem.Co.). The following prestained reference proteins (Bio-Rad Laboratories) were used as molecular markers: m yosin (200 kDa), B-galactosidase (1 1 6 kDa), phosphorylate 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. thurinpiensis* serovar entomcicidus lN A288. Guinea pig was immunized b y 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 lN A288, respectively. The antiserum was centrifuged at 3000 rpm for 10 ruin, supernatant was collected and added 0.1°/c NaNs till storing of it at-20"C. Three days later, antiserum was cheked with SDS-PAGE and Western blowing.

For immunoblot anal ysis (Towbin et al., 1979), the separated proteins were transferred electrohoretical from SDS-Polyacrylamide gel to a ECL nitrcicellulose membrane (Amersham) using an electrophoreticaly tran-blott (Bio-Rad) according to the manufacturer's instructions. The membrane was blr›cked with 3°/c skim milk in TBST buffer (100 mM Tris-HCL (pH 7.5), 150 mM NaC1, and 0.1°/c (v/w) Tween 20) for 3 hr at 4"C, than the membrane was incubated in TBST buffer with I °/c of plyclonal antibodies of *B. thurinpiensis* serovar *entomo‹ idu.s* lN A288 for I hr. the nitrocellulose membrane was washed three times with TBST, and then incubated with perooxidase lgG guinea pig in I .5°/c TBST for I hr. The membrane was washed three times with TBS buffer and visualized with hydrogen peroxide (HCO) and 4-chloro- I naphthol as describe in the instructions from BlO-Rad.

DNA preparation

Overnight cultures of the *B. thurinpien.st.s* 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 t yptose phosphate broth (Difco) using 200 ml cultu ie flask and reincubated for 4-5 hr until the synch ronized culture had reached 10-I5°/c optical transmission. The cells are finally harvested b y centrifugation at 500 g for 10 min. Cells were suspended in approximately I ml of lysoz yme (10 mg/ml; Wako pure chemical Industries, Lid.), preincubated for 60 min at 37"C. Lysis was brou ght to incubate for 15 min at 60"C by the addition of 8°/c 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 I SD), 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 92^0C for 1 min, annealing is at 48^0C for 1 min and polymerization is at 72^0C for 2 min. The PCR technique was used to identify the contents of *B. thuringiensis*serovar*entomocidus* INA288, using oligonucleotide primers specific to *CryI*(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 *entomocidus* 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. thurinzirnsis* serovar *entomo‹idu.t* lN A288 was screened from that sample.

Identfication by H-seroty ping

In order to identifyserovar lN A288 strains by H -serot ype cell with broth, they were dropped to glass slide and the motilit y of cels was observed under phase -contrast microscope. Since isolate of .trrr›ror lN A288 gave positive reaction in the H 6 serum agglutination test, it was identified as *B. thurinpiensis* serovar *entomo‹ idu.s.*

Morphology of INA288 by Scanning Electron Microscope (SEM)

Morphology of the crystals from serovar *entomo‹ idus* (original starin) has been reported and the crystals are bipyramidal-formed (Fig.1). On the other hand, interestingly, *entomo‹ idu.s* lN A288 produced large cuboidal-form crystals (Fig.2).

Quantitative toxicity test of' isolates

The serovar *entomo‹ idu.t* .tit min and *i.tt oelensi.t GtNR60A* w rrr 6ir›‹zt.toyrd against 4"'-instar larvae of *B. mnri, P. vlostello, S. litur‹i, A.* yoyr›ni‹ o.t, *A. we pvpti ‹ind C'. guigue[‹is‹ i‹itu s.* Respectively, serovar *entomo‹ tin.s (original str min) showed* rr›.rim o‹ rir ir y against latter 3 former lepidoteran species, while *rntomo‹ idus* 1NA288 had also the, toxicit y against later 3 dipteran species *A. ‹iep vpti, A.* yoyr›ni‹o.t and C'. qeinqcr /o.t‹ torn.t) (Table 3). The 50°/c lethal concentration of crystal inclusions for each dipteran species was higher than of serovar *isr‹ielensis* ONR60A but lower than /ukur›koruti

SDS-PAC'.E analysis

The purit y of *B. thurinpiensis* serovar *entomo‹ idus* lN A288 parasporal inclusion by percoll showed more than 90°/c depending on monitoring with a phase-contrast microscope. SDS-PAGE

analysis of parasporal inclusion purified from *B. thru inpiru is* serovar *rntomo‹ idu.* INA288 and three other serovar with mosquitocidal activity, *i.trurlensis* ONR60A, k *vushueusis*, and /okur›korn.ti.t, demonstrated that serovar *entomo‹ idus* lN A288 has significant differences in protein composition, compared with serovar *i.sr‹ielensis* ONR60A (mainly 130 kDa, 70 kDa, and 27 kDa) *Lvushueusis* (mainly 140-25 kDa) (Held et al., 1990) and /ukur›korn.ti.t (mainly 90-27 kDa) (Fig.3 A). The cr ystals of serovar *entomo‹ idu.s* lN A288 indicated that the polypeptide 70 kDa was dominant, while *entomo‹idus* (original strain) consisted mainly of I 30-65 kDa.

Immunoblot analysis

B. thurinpieu is serovar *i.ti oelensis* ONR60A and /ukr›ukorn.ti.t peptides little croos-reacted with monciclonal antibodies formed against *B. thrurinpeiusis* serovar *entomo‹ idus I* N A288 (Fig.3B). An anti-70 kDa-peptide monoclonal antibody revealed little cross-reactivit y with a monoclonal antibody directed against the 130 kDa peptide of *B. thurinpien. i. se ov'* ar*i. r‹ielensis* ONR60A. The other two serovar, *B. thurinpieusis* serovar *entomo‹ idu.s* (original strain) and *k vushuensis* did not demonst rate cross-reactivit y with monciclonal antibody of *B.* thuringirn.ti.tserovarrntr›mr›‹ tin.tlNA288. The immunoblot analysis showed that 70 kDa peptides of *B. thurinpiensis* serovar *entomo‹ idus* lN A288 had similar cry gene with *B. thuringien.ti.tserov'ar i.trurleusis* ON R60A and /ukr›Aorn.ti.t.

Identification of Cry genes in *B. thurin#iemsissernvarentomocidus* INA288

The PCR was used to survey a number of *B. thurinpien.st.s* 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. thurinpiensis* serovar *entomo‹ idus* lN A288, *‹ r vI, ‹v vII,* and *‹ rvIV* primers were used. PCR with these primers and DNA template generated a uniquely size fragment for each cry t ype gene. Therefore, the size of PCR product indicated the presence of particular cr ystal protein genes. Previous reports concerning the occurrence of crystal protein genes were confirmed by this technique. Serovar *entomo‹ idus* lN A288 did not amplify *‹ rvIC', ‹ rvIIA, ‹v vIVA, ‹ rvIVB, ‹ rvIVC',* and *‹ rvIVD* genes. However, their cry genes had mosquitocidal activities. This fact shows that serovar *enomo‹ idus* INA288 contains novel cry genes for mosquitocidal activities.

Cloning and sequence of *e*rit*om ocidu.s* INA288

When the total DNA of *rnrr›mr›ridzt* 1N A288 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 cry4 B gene from serovar *i.srurlensis*. The predicted dipteracidal pol ypeptide of *entomo‹idus* IN A288 (cr ylN A288) consisted of 703 amino acids with conserved blocks and amino acid sequence was aligned to that of cry4 Aa (Fig. 4). Furthermore, an alignment of conserved blcick sequence among the crylN A288 and other mosquitcicidal proteins was performed (Fig. 5).

DICUSSION

In recent year.The discoveryof bacteria like *B. spheri‹ u.s* and *B. thurinpien.ti.tserov'ari.trurlensis* 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. thurnpiensisse ov'aisph‹ieri‹ us.* In the search for potential alternatives to the application of *B. thurin pien.ti.t .terov'ari.trurlensis,* isolation of novel mosquitocidal strains is very important. In present research, it is found that *B. thuringien.ti.tserov'arentomo‹ idu.s* lN A288 (belonging to serot ype H 6) isolated from soil in Indonesia, prossess novel mosquitocidal activity toxin. lt was observed that *B. thuringien.ti.tserov'arentomo‹idu.s W* A288 has cuboidal shaped crystal protein, while se*iov'* ar *rntomo‹ idu.s* (original strain) has bip yramidal and irregular shaped one. However, *B. thuringien.ti.tserov'ari.truelensis* ONR60A has irregular cr ystal protein. In this study, the parasporal inclusion from *B.* thurinpiensisserovarrntr›mr›‹ ido.tlNA288 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. thuringien.ti.tserov'aentomo‹ idu.t* INA288, this protein has no activity against the three species of lepidoteran insects and only toxic against the three species of dipteran insects. lnterestingl y, *B. thurinpien.st.sseiov'aientomo‹ idu.s* (original strain) is only toxic to the *B. mori, P.*.ry/r›stella and .'i. *litur‹i.* Paraspnralinclusion of *serov'arentomo‹ idus* lN A288 are slightly more toxic than

serovari.trnr/rnsisONR60A to all mosquito species, such as *A.* /oyr›ni‹o.t, *A. ‹up vyti,* and C'. quinqur/o.t‹ roms.

The molecular bands of these proteins suggest that there is no major overlap among an y these proteins with those of *B. thurinpieuti.t* serov'ar *i.ti oelensi.t, [ukoukoeuti.t* and *entomo‹ idu.* INA288.The antibodies of *B. thurinpiensisserov ar entomo‹ idus* IN A288 showed immunoblot reactivity to protein inclusions of *B. thurinpiensisserov'ar* i.tioelrn.ti.tONR60A, and serovar /okr›Aorn.ti.t.The results suggests that there are similarit y gene between serovar *entomo‹ idu.t* INA288, serovar *i.sr‹ielnsis,* and serovar /ukr›uRrn.ti.t. lshii and Ohba (1993) demonstrated that antibodies of *Lvushuensis* showed immunoblot ractivity to inclusion proteins of serovar *d‹itmst‹idiensis* 73-E- 10 2 and weak reactivit y to those of serovar ‹ wodruti.t.

The PCR screening results suggest that *‹v v4A‹i* had similarit y with cr ylN A288. Therefore, cry INA288 seems a novel mosquitocidal cry gene. However, serovarrntr›mr›‹ thus(original strain) encodes only *‹ rv IAW, ‹ rv lAh, ‹ r vlB* and *‹ rvIC',* which have not been thought to be di pteracidal activity.

Squencing anal ysis of the insert DNA revealed the amino acid sequence of the pol ypeptide encoded. The predicted dipteracidal polypeptide of lN A288 (crylN A288) consist of 703 amino acids with conserve blocks and the amino acid sequence is aligned to that of *‹u v4A‹i.* This strain contains a novel crystal protein gene, cr ylN A288 was 38°/c. Therefore, cr ylN A288 seems a novel mosquitocidal cry gene. However, after analyzing all amino acid sequences of this gene, amino acid comparison should be done for *‹v vINA28S, ‹v v4Au,* and ‹u v#Ab.

Fig. lficanning electron microscopy showing spores and paraspporal crystal of Baciffii.s *thurin#ien.st.s* serovar *entomocidu.s* (original strain). Bar indicates 3 um.

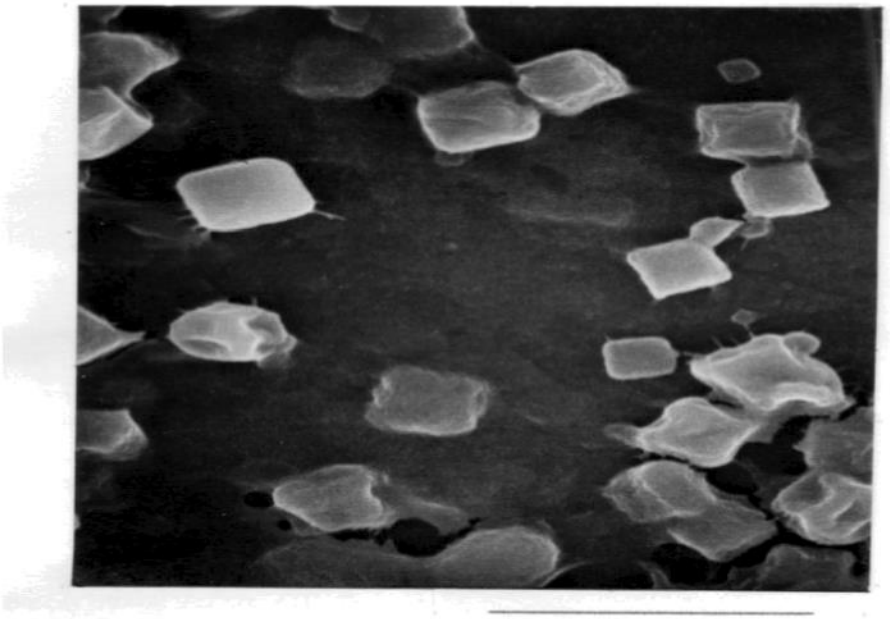

Fig. 2Scanning electron microscopy showing spores and parasporal crystal of *Baciffus thuringiensis* serovar *entomocidus* INA288. Bar indicates *3* um.

Fig. 3. SDS-PAC.E analysis and western blotting analysis

(A) SDS-PAC.E gel (10 to) stained with Coomassie brilliant blue

(B) Western blotting and EIA analysis

Lane 1: mnlecular weight marker

Lane 2: *.sero var entomocidus* INA288

Lane *3: sero var entom ocidus* original strain

Lane 4: *.sero var* isroefensis ONRfiO

Lane 5: *.sero var kyushuensis*

Lane fi: *.sero var fukouLaensis*

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

Strain	Lepidopteracidal			Dipteracidal		
	B, m,.	*Pa.*	*S.l.*	*A.j.*	*As.*	*C p.*
Entomocidusoriginal	+	+	+			
Entomocidus TN A2*88*						
israelensis ONR60A						

B.m: Bombyxmori, P.x: Plutellaxylostella,S.1: Spodopteralitura
ñ.j:Aedes japonicas,A.a: Aedesaegypty,C.q: culexquinquefasciatus

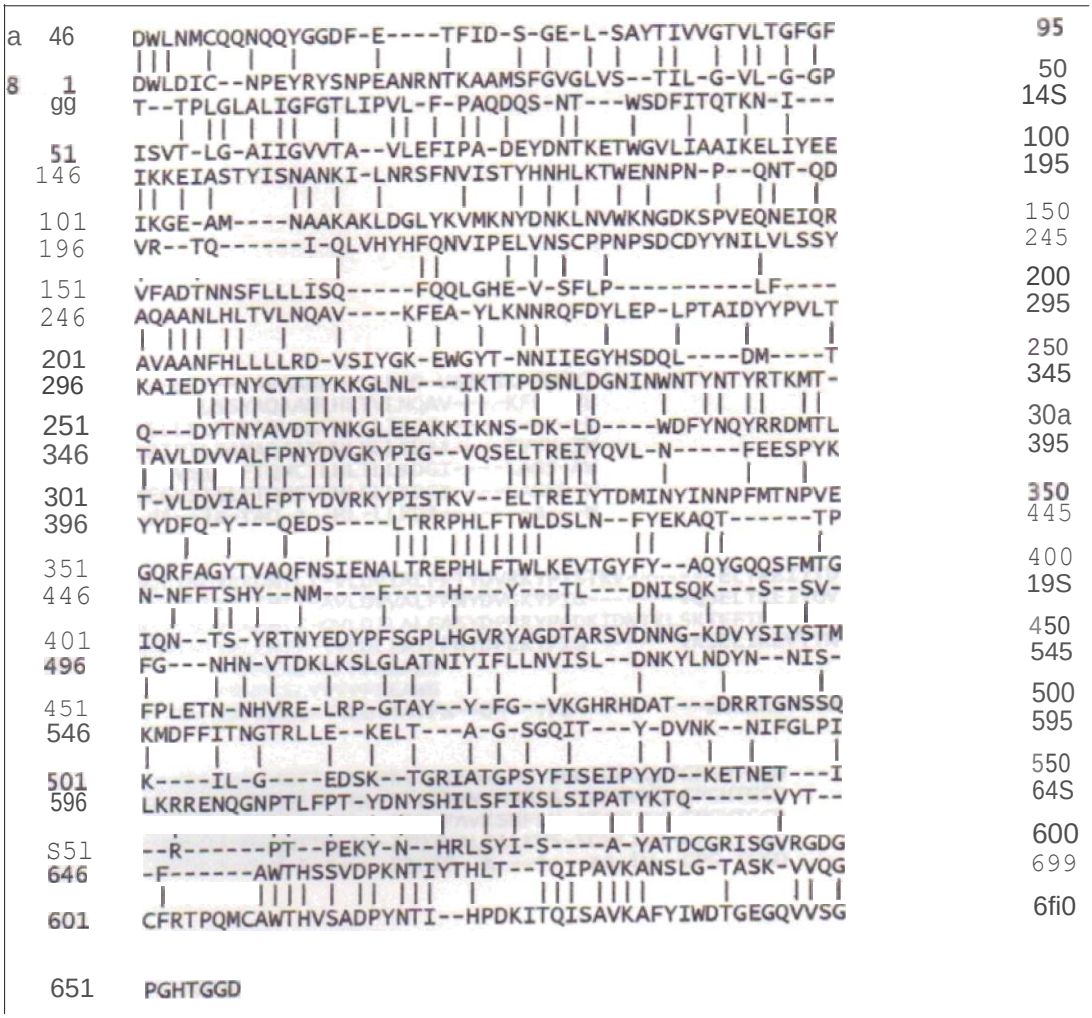

Fig. 4. Amino acid comparison between cry4Aa and cryINA288

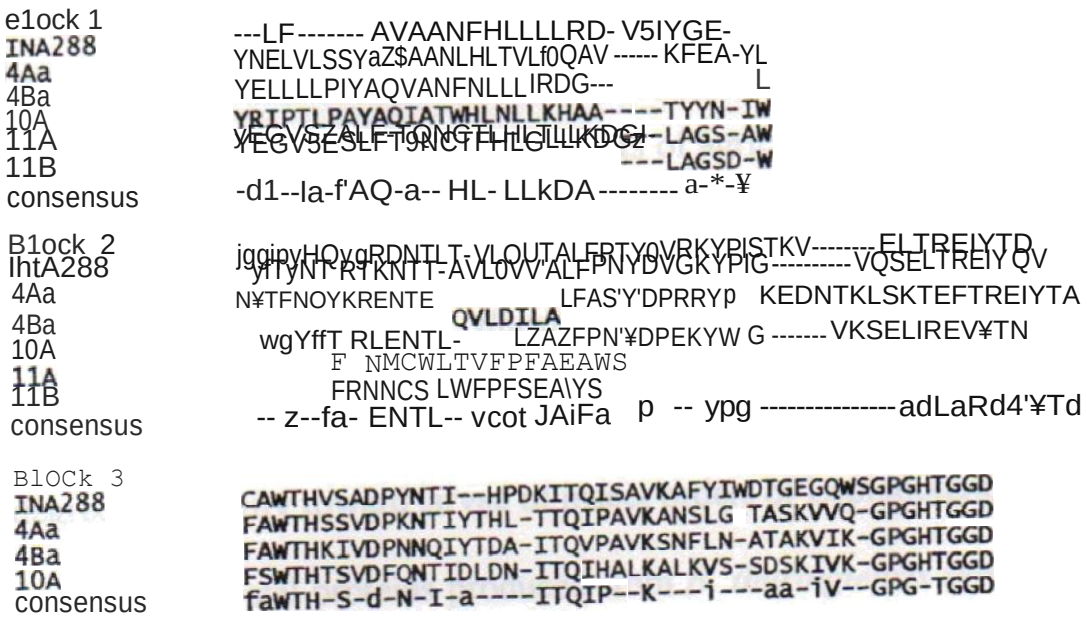

Fig. 5. Conserved blocks 1, 2, and 3 among mosquitocidal cry proteins.

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

Chapter III. Novel Baciffii.s *tIiuringieusi.s* **serovar aizawai strains isolated from mulberry** leaves **in Indonesia**

INTRODUCTION

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

The crystal proteins produced from *B. thurin piensis* 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. thurinpiensis isolates have been recovered from numerous sources, including soils, grain dust, plant leaves, diseased insect larx'ae from insectaries, and sericultural environments (Filosa and Dengler, 1972; Saga and Yanagisawa, 1982). The principal source of novel *B. thurinpiensis* isolates has been soil. Hastowo et al.(1992) reported that 135 strains of *B. thui in pirn.st.s* were isolated from soils of sericultural insect aries and environments of various regions in Indonesia. Distribution of *B. thurinpiensis* in the soil of Indonesia is wider than in the soil of Japan (lizuka et al., 1995), because the tropical regions, as Indonesia, offered a higher chance of isolating *B. thurinpeusis*. Smith and Couche (199 I) isolatedB. *thurinpieusis* strains from the phyllopl ane. In recent years, Ohba (1996) has reported the *B. thurinpien.st.s* strains were isolated from the mulberry leaves in Japan.

During joint research of lizuka and La y (lnstitut Pertanian Bogor), they isolated hundreds of *B. thurinpien.st.s* strains from soil sample and mulberry leaves in Indonesia, and these strains have been maintained in Laborator y of Apllied Molecular Entomology, Facult y of Agriculture, Hokkaido University. In this stud y, 1 report that a novel isolate (serovar aizawai Bun I - 14) with highl y 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. thurinpien.st.s* serovar aizawai lPL and serovar israelensis ONR60A (lizuka et al., 1982) and *entomo‹ idus* lN A288 (lizuka et al., 1996) have been maintained and cultured in this laborator y&. *thurinpiensis* serovar or ount Bun I - 14 was originally isolated from mulberr y leaves in West Java, Indonesia by lizuka and Lay (not published).

Identification by H-serotypeing

The isolate of *B.* r/iuringiruti.tBun I - 14 was identified by H-serot ype. In order to make antibody, H-antisera to the reference strain of *B. thurinpiensisse ov'a wit uw‹ii* lPL (lizuka et al., 1982) were prepared according to the method of Ohba and Aizawa (1978). For H-serot yping of the strains, actively motile bacteria were selected by passing t hroughcraigie's tubes at 37"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°/c) 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 5-800 Scanning Electron Microscope (SEM) at a magnification of 10,000x, according to the method *B. turinpiemsis* serovar *entomo‹idu.s* lN A288 were cultured on N-broth agar at 30"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 (p H 8.0). The final precipitate was resuspended in I 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 carbnn and gold, it was observed and photographed with SEM.

Biological activity for *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 laborator y. Toxicit y test with the mosquito, *A. stephensi,* was done by introducing ten 2" -instar larvae into a test t ube containing 10 ml bacterial suspension.The *B. thurinpiensis* isolates were examined for oral insecticidal activity against the A. stephensi, which were prepared by the following procedures. Overnight culture of serovar aizawai Bun I - 14, and *isr‹ielensis* 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. Spnrulated 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 I 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°/c lethal concentration values (LC,t,i) were determined b y probit analysis (Finne y, 197 I).

SDS-PAC’.E 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 I M NaCL solution and the *B. thurinpiensis* sample was added for separating of crystal proteins from spores by centrifugation at I 5.000 rpm at 4"C for 20 min. In the first step, Percoll solution (Percoll: 0.15 M NaCL = 8:2) was prepared, and then I ml of crystals and spores mixture (10 mz l dry weight /ml) pored carefully the top of 30 ml of percoll solution, and centrifuged at I 5.000 rpm (30,000 g) for 30 min. After recovering the crystal protein, cr ystal protein was immediately washed three times by distilled water. Finally, the pellet was suspended with 500 ul sterile distilled water. The purit y of parasporal inclusions was monitored with a phase-contrast microscope. SDS-PAGE of parasporal inclusion proteins was done by the methcd " using a 10°/c running gel with a 3°/c stacking gel. After electrophoresis, gel was steined with 0.04°/c 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), phosphorylate b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (45 kDa).

For immunoblot anal ysis (Towbin et al., 1979), the separated proteins were transferred electrophoretically from SDS-Polyacr ylamide 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°/c skim milk in TBST buffer (100 nM Tris-HC L {pH 7.5}, 150 mM NaC L, and 0.1°/c (v/w} Tween 20) for 3 hr at 4"C, then the membrane was incubated in TBST buffer with I °/c of polyclonal antibodies of B. thuringiensis serovar entomocidus INA288 for I hr. The nitrocellulose membrane was washed three times with TBST, and ten incubated with peroxidase lgG guinea pig in I .5°/c TBST for I hr. The membrane was washed three times with TBS buffer and it was v'isualized with hydrogen peroxide (H O) and 4-Chloro- I -napht hol 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. thurinpiensis* serovar *entomo‹ idus* lN A288. 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 *entomo‹idus* INA288, respectively. The antiserum was centrifuged at 3000 rpm for 10 ruin, supernatant was collected and added 0.1°/c NaNs till storing of it at-20"C. Three days later, antiserum was cheked with SDS-PAGE and Western blotting.

For immunoblot anal ysis (Towbin et al., 1979), the separated proteins were transferred electrohoretical from SDS-Polyacrylamide gel to a ECL nitrcicellulose membrane (Amersham) using an electrophoreticaly tran-blott (Bio-Rad) according to the manufacturer's instructions. The membrane was blr›cked with 3°/c skim milk in TBST buffer (100 mM Tris-HCL (pH 7.5), 150 mM NaC1, and 0.1°/c (v/w) Tween 20) for 3 hr at 4"C, than the membrane was incubated in TBST buffer with I °/c of plyclonal antibodies of *B. thurinpiensis* serovar *entomo‹ idu.s* lN A288 for I hr. the nitrocellulose membrane was washed three times with TBST, and then incubated with perooxidase lgG guinea pig in I .5°/c TBST for I 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 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°C for 1 min, annealing is at 48°C for 1 min and polymerization is at 72°C for 2 min. The PCR technique was used to identify the contents of *B. thuringiensis* serovar *entomocidus* 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 or ount Bun I -14 was digested with *B‹imHl,* then incubated at 30”C for I hr. The digest solution was electrophoresed using Agarose 0.8°/c, 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 I hr, l00V. 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 lN A288B probe labeled with (”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°/r SDS at 50”C for I 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 I -14 and Bun 2- I strains b y H-serot ype cells with broth, they were dropped to glass slide and the motilit y of cells was observed under phase-contrast microscope. Since isolates of Bun I -14 and Bun 2- I gave a positive reaction in the H 7 serum agglutination test, both of tern were identified as *Btu. illu. thurinpien. i.* serovar oigoivoi.

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

Morphology of the cr ystal from serovar or ount lPL has been reported by lizuka et al. (1982). They reported that serovar or ount lPL produced bipyramidal and irregular shaped crystal proteins. However, observation conducted using high resolution SEM revealed that *B. thurinpiensis* serovar or ount Bun I - 14 and serovar or ount 2- I produced onl y irregular-shaped proteins (Fig.6). someB. *thuringien.ti.t* strains produced irregular shaped crystal proteins.

Quantitative toxicity test of isolates

Toxicit y of inclusion of *B. thru inpirnsis* serovar lPL, Bun I -14 and Bun 2- I was shown in Table 4, against the *B. mori, P. vlostello, A. Sep vpti, C’. guingue[‹is‹ i‹itus,* and .S. *litur‹i.* By the result, *B. thuringiensis se ov’ai ‹ii-uw ‹ii* lPL showed toxic activity to *B. mori, P. vlostell‹i,* and .'i. *litur‹i,* even though was not toxic to *A. ‹ie pvyti,* and C’yuinqur/o.t‹torn.t. on the other hand, aizawai Bun I - 14 was toxic to the dipteran insects, *B. mori, P. vlostell‹i,* and .S. *litur‹i,* while or ount Bun 2- I was not toxic to any insects of bnth groups.

As the strain of or ount Bun I - 14 had a mosquitocidal activity, differences in the toxic activity to mosquito larvae between Bun I -14 and another mosquitocidal strains, serovar or ount strains and serov’ar *i.sr‹ielensis* ON R60, were demonstrated using 2" -intar lars’ae of *Anopheles stepheusi.* Sporulated cultures were washed three times for removing the B-exotoxin activity by I M NaCL and dried at room temperature for one week. Serovar or ount Bun I - 14 and israelensis ONR60 showed highly toxic activities against *A. steyheusi,* while both serovar

or owoi lPL and or owoi Bun 2- I 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 toxicit y against mosquito larvae. The lethal concentration (LC,t,) value of *B. thurinpiensis* serovar aizawai Bun I - 14 was 0.0057 ug/ml, and an almost similar activity was seen in the case of *B. thurinpieusis* serovar *i. r‹ielensis* ONR60A (0.027 ug/ml) (Table 5). This fact showed that serovar or ount Bun I - 14 was a novel isolate from mulberry leaves.

SDS-PAC'.E analysis

The purit y of *B. thurinpien.st.s* serovar or onoiBun I - 14 and or ount Bun 2-1 parasporal inclusion by percoll showed more than 90°/c, depending on monitoring with a phase -contrast microscope.

SDS-PAGE analysis of parasporal inclusions purified from serovar or ount Bun 1- 14 demonstrated that the protein composition of its srain is significantly different from that of *B. thurinpien.st.s* serovar *i.sr‹ielensis* (mainl y 130 kDa, 70 kDa, and 27 kDa) and serovar *entomo‹ idu.s* INA288 (70 kDa). The crystal of serovar or ount Bun I -14 consisted mainly of 69 kDa peptides, while serovar or ount Bun 2- I consisted mainly of I 30-65 kDa peptides (Fig. 7A).

Immonublot analysis

To investigate the antigenic relationship among the *B. thurinpiensis* serovar or ount Bun I - 14 and serovar or ount Bun 2-1 crystal toxins, and two other serovar *entomo‹idu.s* lN A288 and *isr‹ielensis* ONR60A with known mosquit cicidal activity, western blotting and enz yme immunoassay (immunoblotted) were demonstrated (Fig. 7B). the antibody of entomocidus INA288 had cross-reactivities against *B. thurinpieusis* serovar or ount Bun I - 14 and serovar *isr‹ielensis* ONR60A. The immunoblot analysis showed that the 69 kDa peptides of *B. thurinpiensis* serovar or ount Bun I -14 had encoded in similar cry genes with *B. thurinpieu is* serovar *i. r‹ielensis* ONR60A (130 kDa peptides). However, immunoblot profiles of the serovar or ount bun 2- I was quite different from those strains.

Identification of Cry genes in *B. thuringieusi.s* serovar ***ai:yr*** *wai*

The PCR method was used to survey a number of *B. thurinpien.st.s* 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. thurinpiensis* serovar or on'oi *IPL, ‹ii-uw ‹ii* Bun 1- 14 and or ount Bun 2- I , *‹u vI, ‹u vII,* and *‹v vI V* primers were used. PCR with these primers and DNA template generated a uniquely size fragment for each cry t ype gene. Therefore, the DNA size of PCR product indicated the presence of particular crystal protein genes. Both of serovar oizoivoi Bun I - 14 and or ount Bun 2-1 did not amplify‹ yiC', *‹u vIIA, ‹u vIVA, ‹u vIVB, ‹u vIVC',* and *‹ rvIVD* 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 or ount Bun I - 14 had a highl y toxicit y against *A. stephensi.*

Southern blot hybridization

Southern hybridizati on signal was detected from plasmid DNA of or ount Bun I - 14 and its DNA was digested with *B‹imHl* (Fig. 8), which were probed of *entomo‹ idus* lNA288B. The result showed that the toxin genes from or ount Bun I - 14 had a little homology to the toxin genes from *B. thurinpiensis* serovar *entomo‹ idu.s* lN A288B. The 1NA288B probe was hybridization with a large plasmid and *BomHl* 20 kb fragment of or ount Bun I -14.

DISCUSSION

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

In the morphological study by using SEM, *B. thru ingirusis* serovar oi¿onoi lPL as the t ype or ount strain has bipyramidal and irregular shaped cr ystal proteins (lizuka et al., 1982). lnterestingl y, or ount Bun I -14 and aizawai Bun 2-1 produced only irregular shaped crystal proteins, which were similar to the forms produced by serovar *i.sr‹ielensis* ONR60A (lizuka et al., 1982), serovar *mot t i.sent* PG- 14 (Padua et al., 1984) and serovar *L vushurnsis* (Held et al., 1990). In generally, *B. thrurine piensis,* which produces irregular shaped crystal proteins, has a mosquitocidal activity.

The insecticidal activity of *B.thurnpieusis* serovar aizawai lPL showed onl y on toxicit y to the leidopteran insects *{Bomhi. mori, Plutell‹i vloetell‹i,* and âyr›dr›yrrio *litur‹i).* However, or ount Bun I - 14 was t oxic to the dipteran insects *Aedes Aep vpti*, *Anopheles stephensi* and *C'ule. quingue[‹is‹ i‹itu.s),* while or ount Bun 2- I was not toxic to lepidopteran insect and dipteran insect species. The LC 50 of or ount Bun I - 14 against *A. stephensi* was calculated to compare the

toxicities of *B. thru inpirnsis* serovar *i. r‹irlrnsis* ONR60A and serovar *rntomo‹ idus* INA288. The mosquitocidal activity of this isolate was almost similar to those of *B. thurinpeusis* serovar *isr‹ielensis* ONR60A and serovar *entomo‹ idu.s* lN A288. The results indicated that this strain has highl y toxic actix'it y against *A. stephensi*. lizuka et al. (1996) reported that serovar *entomo‹idu.s* INA288 as toxic rr› *A.* yoyr›ni‹o.t, *A. ‹ie pvpti* and C'. quinqur/o.t‹ torn.t. Ohba et al. (1995) reported that *B. thurinpiensis* serovar *hipo* (fl gellar serot ype 44) as also toxic to *A. stephensi* and C". *fifteen molestus*. A new mosquitocidal *B. thurinpiensis* serovar *je p‹ithes‹in,* which as composed of polypeptides of 77 kDa had toxic to C'. quinqop/‹ztr torn.t, *A. we pvpti, A.togoi. A. olhopi‹ tu.s, A. m‹i‹ ul‹ites,* and *M‹insoni‹i uni[ormis* (Kawalek et al., 1995). On the other hand, *B. thurinpiensis* serovar *medellin* (flagellar serot ype 30) isolated in Columbia includes peptides of 100 to 30 kDa and showed a toxicit y to C'. *quingue[‹is‹ iotus, C'. fifteen, A. ‹ie pvpti*, and *A. stephensi. B. thurinpiensis* serovar /Ar›Aornsi.t, which consisted of polypeptides of 90 to 27 kDa, was toxic to A. aegypti, and C. quinquefasciatus (Yu et al., 199 I). In addition, lshii and Ohba (1997) reported that *B. thurinpieusis* serovar ‹wodrn.ti.t comprised of major proteins of 65 kDa had toxic to *A. ‹iep vpti.* Moreover, *B. thurinpiensis* serovar *i. r‹ielensis* ONR60A had constituted of mosquitocidal 130 kDa, 70 kDa, and 27 kDa proteins, and serovar *entomo‹idus* lN A288 constituted of mosquitcicidal 70 kDa proteins, respectively. *B. thurinpieu is* serovarWrmriodirn.ti.t 73-E- 10-2, which were comprised of polypeptides of 1 25 to 28 kDa, had mosquitocidal activity to *A. ‹iep vpti* (Kim et al., 1984; Drobniewski et al., 1989). In the present study, it was cleared that the cr ystal protein of serovar oi¿onoi Bun I - 14 was constituted of 69 kDa peptides and this was not the same peptide wit other mosquit cicidal strains.

Using antibody of *B. thurinpien.st.s* serovar *entomo‹ idu.s* lN A288 cr ystal protein, 1 demonstrated that there is cross-reactivity among the parasporal inclusion proteins of *B. thui inpirnsis* serovar ni ntvni Bun I -14 and serovar *i.sr‹irlrnsis* ONR60A. Antisera for whole *B. thurinpien.ti.t* serovar *entomo‹idu.t* lN A288 parasporal inclusion has only cross-reactivit y to 69 kDa proteins bands of *B. thurinpiensis* serovar or ount Bun I - 14 and 130 kDa proteins bands of serovar *i. r‹ieleusis* ONR60A. These results suggest that there is partial similarit y to cry genes between *B. thurinpieusis* serov'ar *entomo‹idu.s* lN A288, serovar or ount 1- 14 and serovar *isr‹ielensis* ONR60A. However, Ka walek et al. (1995) demonstrated that using antibodies of B.thuringiensis serovar israelensis ONR60A showed weak cross-reactivity to serovar

*jr*piithrsan.Indeed, *B. thru inp rmsis* serovar *jr p‹ithrson* included crylVD and showed immunological similarit y with antibodies of serovar *i.trurlensis.*

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

In southern h ybridization analysis showed that almost 50 kb plasmid of *B. thurinpieu is* serovar or ount Bun I - 14 has a littlehomology with serovar *entomo‹idu.s* lN A288 cry gene, while the fragment (20 kb) of aizawai Bun I - 14 digested with *B‹imHi* has a little homology with that gene.

In conclusion, a new mosquitocidal *B. thurin piensis* serovar or ount Bun I - 14 was found and characteristics of toxic protin from this strain were demonstrated. Since cry gene serovar or ount Bun I - 14 has only partial homology with the cry gene from *entomo‹ idus* lNA288, it seems a novel cry gene. In the near future, it becomes very important and very usefu 1 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 rnosquitoes.

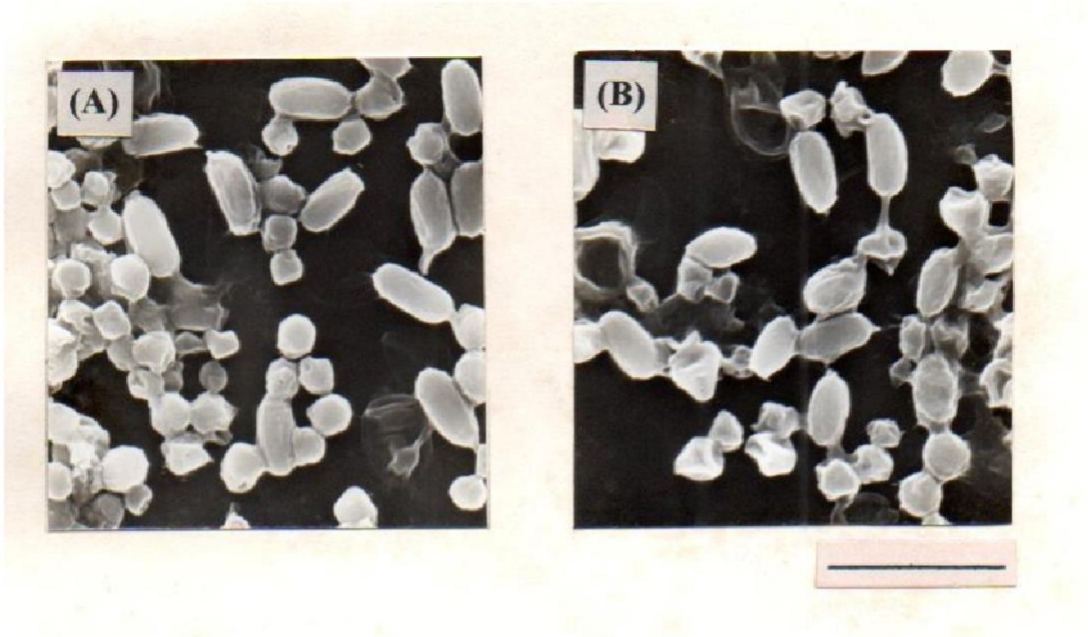

Fig. 6. Scanning electron microscopy showing spores and parasporal crystal of *B. thuringiensis* serovar *aizawai* Bun 2-1 and serovar *aizawai* Bun 1-14 Bar 3 um.

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 S. Toxicity of crystal proteins from Baciffii.s *thuringien.si.s* strains to 2^n -instar larvae of *Anophefes stephensi.*

Serovar	LC50 (ug/ml{fiducial limit}
israelensis	0.027 {0.014-0.0fi0}
aizawai IPL	> 5 (insufficient)
Aizawai Bun 1-14	0.057 {0.035-0.088}
aizawai Bun 2-1	> 5 (insufficient)

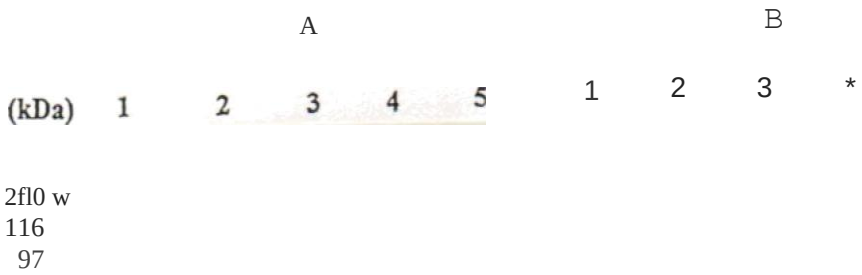

Fig.7. SDS-PAC'E analysis and western blotting analysis

(A) SDS-PAC'E gel (10 to) stained with Coomassie brilliant blue

(B) Western blotting and EIA analysis

Lane 1: molecular weight marker

Lane 2: *.zero var entomocidus* INA288

Lane *3: sero var ai:yr wai* Bun 1-14

Lane 4: *.sero var ai:yr wai* Bun 2-1

Lane 5: *.sero var* isroefensis

Table 5.Identification of cry genes from serovar or owoi strains

Straincry genes	
aiza wai IPL	*cry IAa, cry I lb, cry I C, cry I D, cry 2 Aa, cry 2 at*
ai:yr waiBuo 1-14	not detected *cry I, cry 2,* and *cry 4*
ai:yrwai Bun 2-1	not detected *cry I, cry 2,* and *cry 4*

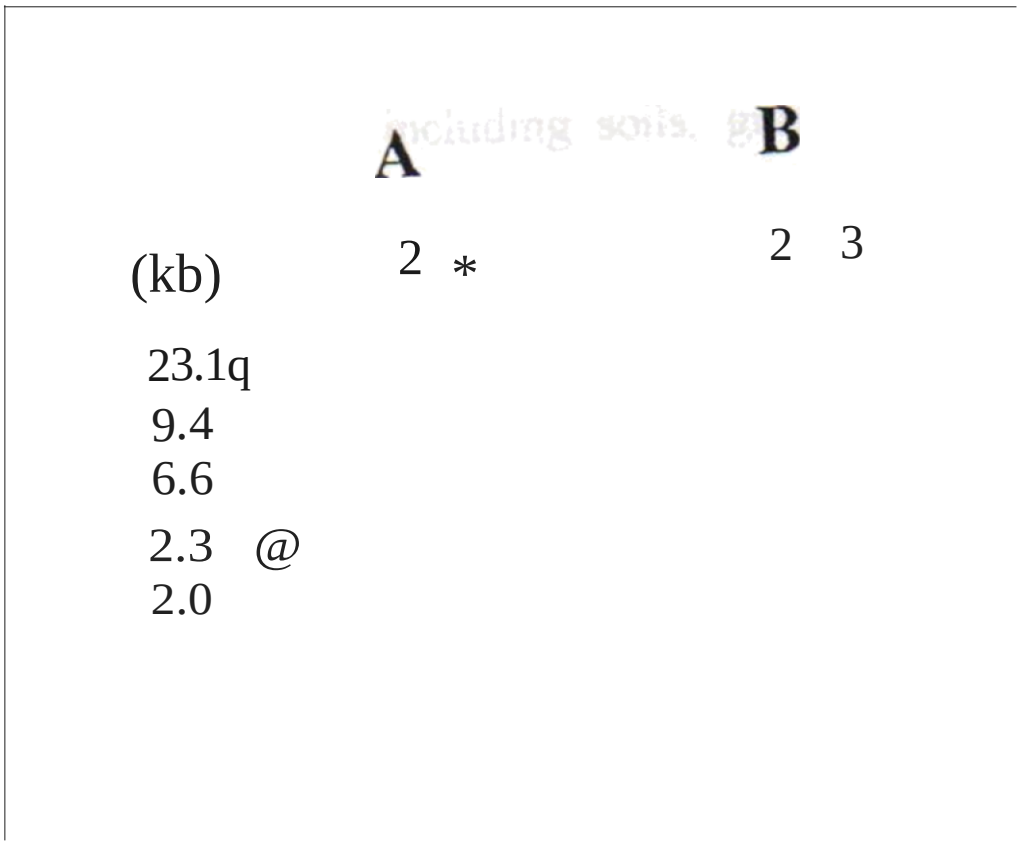

Fig. 8.A. DNA of *B. tfiiiringiensis* serovar *ai*m *wai* Bun 1-14 was analyzed on a OF agarose gel

Lane 1: mnlecular weight marker

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

Lane 3: DNA of ***ai:yr wai*** Bun 1-14 digested with BamHi

B. Southern blot hybridization

Lane 1: mnlecular weight marker

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

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

Chapter IV. C.ENERAL DISCUSSION

Btu. illus thurinpien.st.s, a gram-positive soil bacterium, is characterized by its abilip to produce crystal during sporulation. *B. thurinpiensis* isolates have been recovered from numerous sources, including soil, grain dust, plant leaves, diseased insect larvae from insectaries, and sericulture environment (Fil osa and Dengler, 1972; Saga and Y anagisawa 1982). Most strains of *B. thurinpien.st.s* isolated early time prior to 1972 showed toxic activity towards lepidopteran insects. However, a few serovar, such as serovar *i.sr‹ielensis* (Goldberg and Margalit, 1977), *mot t i.sent* PG- 14 (Padua et al., 1984), Sum.tiodirn.ti.t 73-E- 10-2 (Padua et al., 1980), *k vu. huensis* (Ohba and Aizawa, 1979), kur.tram HD- I (Y amamoto and Mc Laughlin, 198 I), *p‹illeri‹i* (Ahmad et al., l989),/ukr›ukorn.ti.t (Yu et al., 199 I), *medellin* (Orduz et al., 1992), *jr p‹itheson* (Kawalek et al., 1995), *hipo* (Ohba et al., 1995), and ‹ onMrn.ti.t (Ohba, 1997) which produce crystal proteins were found as mosquitocidal strains to dipteran insects.

In recent years, formulation of mosquitocidal *B. thurinpien. i.* serovar *i.sr‹ielrnsis* and serovar *sph‹ieri‹ u.s* 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. thurinpien.st.s* serovar sphaericus (Georghi ou et al., 1993). Numerous investigators have explored a new strain of *B. thurinpiensis as* alternatives to the application of *B. thurinpiensis* serovar *i.sr‹ielensis.* During joint research of lizuka and Lay (lnstitut Pertanian Bogor), t hey isolated hundreds of *B. thurinpien.st.s* strains from soil sample and mulberry leaves. In the present study 1 found the unique strains, *B. thru ingirn.st.s* serovar *rntomo‹ idus* INA288 and serovar oi¿onoi strains, both of which were isolated in Indonesia. Morphology of the cr ystals from serovar *entomo‹ idu.s* original strains has been reported by lizu ka et al. (1982) and Faust et al. (1982) that serovar *entomo‹ idus* original strain produced bipyramidal-form crystal. However, observation conducted using high resolution Scanning Electron Microscope (SEM) revealed that the original strain prrxluced irregular-form cr ystal. On the other hand, serovar *entomo‹ idu.s* INA288 produced large cuboidal-form cr yst al. Interestingly, serovar sign met Bun I - 14 and Bun 2- I produced onl y irregular shaped crystal proteins, which were similar in shape to the cr yst al produced by serovar i.si *or/mets* ONR60A (lizuka et al., 1982)., while or ount IPL had bipyramidal and irregular shaped cr yst al proteins (lizuka et al., 1982).

B. thurinpieu is serovar *i.ti oelensis* ONR60A had mosquitocidal actis'it y, which was comprised of major protein of 130 kDa peptides. On the other hand, *B. thurinpiensis* serovar

entomo‹ id» INA288 and aizawai Bun I - 14 also had mosquitocidal activity, while or owoi Bun 2- I both did not have. In addition, neither serovar *entomo‹idu.s* original strain nor serovar at ount IPL had lepidopteracidal activity. SDS-PAGE profiles of *entomo‹ idu.t* lN A288 crystals indicated that the polypeptide of 70 kDa was dominant, while *entomo‹ idu.s* original strain was constituted of I 30-65 kDa. However, *B. thurnpien.st.s* serovar aizawai Bun I - 14 was composed of polypeptides of 69 kDa, while *entomo‹ idus* Bun 2- I was constituted of I 30-65 kDa. lnterestingl y, using antibcd y of *B. thurinpien.ti.t* serovar *entomo‹ idus* lN A288 cr ystal protein demonstrated that there is cross-reactivity among the parasporal inclusion proteins of *B. thurinpiensis* serovar or ount Bun I - 14, *i.sr‹ielensis* ONR60A, and *[ukouL‹iensis.* However, Kawalek et al. (1995) demonstrated that using antibodies of *B. thurinpien.st.s* serovar *i.sr‹ielensis* ONR60A showed weakly cross-reactivit y to serovar *jegothes‹in.* Indeed, *B. thuringen.ti.t* serovar jegathesan included *‹u vIVD* and showed immunological similarit y with antibodies of serovar israelensis. While, *entomo‹idu.s* lN A288 contained a novel crystal protein gene *‹u vINA288,* on plasmid. In addition, the similarit y of amino acid sequence between *‹u vINA28S* and *‹v v4A‹i* was 38°/c. On the other hand, the characteristics of toxic protein of *B. thurinpiensis* serovar or onoi demonstrated that cry gene from serovar or ount Bun I - 14 has onl y partial homology with the cry gene from serovar *rnrr›mr›‹* thus lN A288, and that it seems a novel ‹u ygene.

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