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Bacillus Thurungiensis isolates from indonesia toxic to mosquito insects

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Abstract

B. thuringiensis has been studied worldwide over the past decades, mainly because this gram-positive bacterium produce significant amount of crystal proteins with toxic activity against economically important insect larvae. Most strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to lepidopteran insects such moth. But some strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to dipteran insects such as mosquitoes and blackflies. One gram of soil samples was suspended in 9ml of sterile distilled water and shaken for 5min. the upper layer of the soil suspension was transferred to a test tube and heated at 80°C for 5min 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., it was observed and photographed with (SEM). The results that serovar *entomocidus* (original starin) produced bipyramidal-formed and *entomocidus* INA288 produced large cuboidal-form crystals. The PCR screening showed that cry4 Aa had a novel mosquitocidal *cry* gene and had 70 kDa peptide, but serovar *entomocidus* (original strain) encodes only *cry1Aa, cry1Ab, cry1B* and *cryIC*, which have not been thought to be dipteracidal activity.they had differences form crystal protein, and unique as insecticidal to control the same orders (mosquitocidal).

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Introduction

The insecticidal properties of *Bacillus thuringiensis* were recognized many years before the bacterium was identified, with some account suggesting that *B. thuringiensis* spores may have already been in used an ancient Egypt. In the modern era, the bacterium was isolated in 1901 by the Japanese biologist Shigetane Ishiwatari during an investigation into wilt disease in silk worm, and he named it *Bacillus sotto* (Burges, 2001; Aroson, 2002). Ten years later, the same bacterium was isolated by Ernst Berliner from disease Mediterranean flour moth (*Ephestia kuehniella*) in the German province of Thuringia, and it was named *Bacillus thurimgiensis* (Boonsem *et al.*, 2006). The defining feature of *B. thuringeinsis* is its ability to produce proteinaceous crystal during sporulation. *B. thuringiensis* is a member of the Bacillus cereus group of gram positive, spore-forming soil bacteria, and occasionally the bacteria lose their ability to form crystals and then become indistinguishable from *B. cereus* itself. Similarly, *B. cereus* can be transformed into *B. thuringeinsis*, and investigations into the transformation mechanism led to the discovery that crystal formation is conffered by genes carried on a plasmid. The genes, which encode Cry/Cyt proteins, become active during sporulation because they are controlled by a dedicated RNA polymerase that is also synthesized specifically while spores are forming up to 20% of the spore protein is represented by these Cry/Cyt toxins (Aroson, 2002; Rizali, 1998).

B. thuringiensis is a gram-positive soil bacterium, and produce a crystalline inclusion body during sporalation (Bulla, 1977; Dulmage, 1992). This parasporal body is composed of proteins termed "delta-endotoxin", and specifically toxic to insects. In addition, *B. thuringiensis* produce another toxin namely: alpha-exotoxin, beta-exotoxin, and gamma-exotoxin. All of the toxic substances may not be present in the bacterium (Golberg *et al.*, 1977; Hastowo, 1992). In another hand, various toxic substance produced by *B. thuringiensis* as follow: (a) thermolabile endotoxin; (b) thermostable exotoxin; (c) bacillogenic antibiotic; (d) lecithinase; (e) proteinase (Ishii *et al.*, 2013; Iizuka *et al.*, 1995), but some strains of *B. thuringiensis* produce delta-endotoxin crystals toxic to dipteran insects such as mosquitoes (Golberg *et al.*, 1977) and Lepidopteran-specific delta-endotoxin are composed 130 kDa proteins (Bouquet, 2004; Held *et al.*, 1990), while dipteran-specific delta endotoxin are composed of several protein (Pazos, *et al.*, 2009; Kwalek *et al.*, 1995). Since isolate of *Bacillus* strains possessing a high larvacidal activity, specific for mosquitoes, from the soil of mosquitoes-breeding site in Israel (Golberg *et al.*, 1977). The objective of the studies to identify and bioassay the *B. thuringiensis* against mosquito insects.

Materials and methods

Bacterial stains

The strain of *B. thuringiensis* used in the present study were *B. thuringiensis* serovari *israelenisis* ONR60A and *entomocidus* (orginal strain) (Iizuka *et al.*, 1982; Iizuka *et al*, 1984: Iizuka *et al.*, 1995) and *B. thuringiensis* serovar *entomocidus* INA288 (Iizuka *et al.*, 1982). The *B. thuringiensis* serovar *fukuokaensis*, and *kyhusuensis* were provided and cultured in our laboratory.

Isolation and identification

B. thuringiensi sserovar INA288 which had been isolated from Indonesia soil, was prepared according to the method (Hastowo *et al.*, 1992). One gram of soil samples was suspended in 9ml of sterile distilled water and shaken for 5min. the upper layer of the soil suspension was transferred to a test tube and heated at 80°C for 5min 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-serotypeing

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* serova *entomocidus* (original starin) were prepared according to the method (Ohba and Aizawa, 1978). For H-serotyping of the strains, actively motile bacteria were selected by passing trhough craigie's tubes at 37°C for 24hr.

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 5min 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 (Iizuka *et al.*, 1982). *B. turingiensis* serovar *entomocidus* INA288 were cultured on N-broth agar at 30°C until almost all cells lysed (overnight).The crystal and spores (about 100mg wet weight) were washed in 10ml of 50 mMTris-HCL (pH 8.0). The final precipitate was resuspended in 1 ml of distilled water, and 20ul of the suspension was air-dried on a glass disk (O 10mm). 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 Leppidopteran insect, *B. mori*, *P. xylostella* and *S. litura*, were done by introducing ten 3rd-intar larvae were fed on an articial diet dropped with 0.3ml of the bacterial suspension and rear at 25°C for 48hr 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 serovar INA288, and *israelensis* ONR60A were grown on 2ml of nutrient broth at 30°C using tube glass. Then, 200ul of the overnight culture was plating on nutrient agar, reincubated for 4 days at 30°C. Sporulated cultures were harvested by centrifugation at 10,000g 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*, *Aede sjaponicus* and *Culex quinquefasciatus*. Ten 2nd-instar larvae were placed in a test tube containing 10 ml of the spore-parasporal inclusion suspension, respectively, under levels 1ul/ml. The tubes were kept at 22°C for 24hr without feeding.

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°C for 20min. In the first step, Percoll solution (Percoll: 0.15 M NaCL = 8:2) was prepared, and then 1 ml of crystals and spores mixture (10mg [dry weight]/ml) pored carefully the top of 30ml of percoll solution, and centrifuged at 15.000 rpm (30,000g) for 30min. After recovering the crystal protein, crystal protein was immediately washed three times by distilled water. Finally, the pellet was suspended with 500ul 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 Laemmli (1970) 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).

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 2min. The PCR technique was used to

identify the contents of *B. thuringiensi* serovar *entomocidus* INA288, using oligonucleotide primers specific to *Cry1* (Kalman *et al*., 1993), *CryI* (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 base on unique-size DNA fragments amplified by PCR for each cry gene (Asano, 1996).

Result

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 identify serovar INA288 strains by H-serotype cell with broth, they were dropped to glass slide and the motility of cells 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 by Iizuka *et al.*, (1982) and Faust *et al.*, (1982) and the crystals are bipyramidal-formed (Fig.1A). On the other hand, interestingly, *entomocidus*INA288 produced large cuboidal-form crystals (Fig.1B).

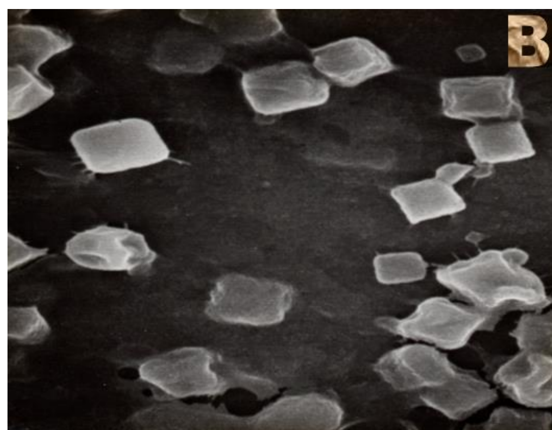

Fig. 1. Scanning electron microscopy (SEM) showing spores and parasporal crystal of *Bacillus thurin giensis*serovar*entomocidus* INA288. Bar indicates 3 um.

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. quiquefasciatus*. Respectively, serovar *entomocidus (original strain) showed toxic activity* against latter 3 former lepidoteran species, while *entomocidus* INA288 had also the, toxicity against latter 3 dipteran species (*A. aegypti, A. japonicas* and *C. quinquefasciatus*) (Table 1). The 50% lethal concentration of crystal inclusions for each dipteran species was higher than of serovarisraelensis ONR60A but lower than *fukuokaensis.*

Table 1. 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: *culex quinquefasciatu*

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) (Iizuka *et al*, 1982) *kyushuensis* (mainly 140-25 kDa) (Held *et al*., 1990) and *fukuokaensis* (mainly 90-27 kDa) (Yu *et al*., 1991) (Fig. 2A). The crystals of serovar *entomocidus* INA288 indicated that the polypeptide 70 kDa was dominant, while *entomocidus* (original strain) consisted mainly of 130-65 kDa.

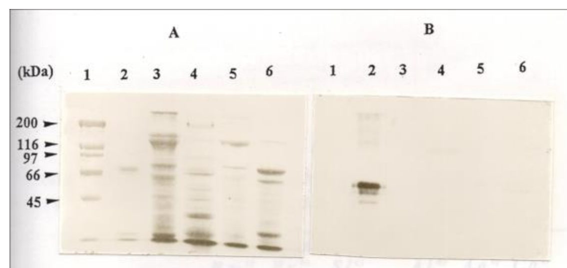

Fig. 2. SDS-PAGE analysis and western blotting analysis.

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

B. Western blotting and EiA analysis

Lane 1: moleculer weight marker

Lane 2: serovarentomocidus INA288

Lane 3: serovarentomocidus original strain

Lane 4: serovarisraelensis ONR60A

Lane 5: serovarkyushuensis

Lane 6: serovarfukoukaensis

Immunoblot analysis

B. thuringiensis serovar *israelensis* ONR60A and *fukoukaensis* peptides little croos-reacted with monoclonal antibodies formed against *B. thruringeinsis* serovar *entomocidus* INA288 (Fig. 2B). 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 (Kalman *et al*., 1993; Asano, 1996). 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 *entomocidus* INA288 contains novel cry genes for mosquitocidal activities.

Discussion

In recent years. The discovery of bacteria like *B. sphericus* and *B. thuringiensis* serova r*israelensis* which are highly toxic to dipteran larvae, and these formulation 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 *sphericus* (Georhiou *et al*., 1993). In the search for potential alternatives to the application of *B. thuringiensis* serovar *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* serova r*israelensis* ONR60A (Iizuka *et al*., 1992) 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 (Poopathi and Abidha, 2010). the isolates with bipyramidal and cuboidal-shape predominated on the soil and dead insect (Lee *et al*, 1985). In spite of 70 kDa peptide in *B. thuringiensis* serovar *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*. Parasporal inclusion 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 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* (Donovan *et al.*, 1988 and yamamoto *et al.*, 1991).

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 (Asano, 1990).

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