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Cloning and analysis sequence of *Bacillus thuringiensis ENTOMOCIDUS* INA 288

Akhmad Rizali*

Department of Agroechotechnology, Agriculture Faculty, Lambung Mangkurat University, Indonesia

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Abstract

B. thuringiensis has been studied worldwide over the past decades, mainly because this gram-positive bacterium produces significant amounts of crystal protein 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. Numerous chemical insecticides have been used to control some insects. While chemical insecticides have knock down effect, they are too expensive in the developing countries and harmful to both humans and the environment. In addition, target insect pests develop biological resistance rapidly especially at higher rates of application. Thus, the increase in pesticidal application to control this pest has urged to researcher to search for biological control alternatives that would be a good component of Integrated Pest Management. To clone and analyze *B. thuringiensis* gene fragments isolated by restriction digest PCR. Specific primers were designated to amplify the genes of *B. thuringiensis* serovar *entomocidus* INA 288, and the PCR products were classified and re-amplyfied by PCR to obtained the fragments for subsequent purification and cloning into the pGEM-T vectors, followed by rapid identification. The recombinant plasmid were extracted from positive clones and the target gene fragments were sequenced. The sequence analysis showed that *cry4Aa* had similarity with *cryINA 288*. It seems a novel mosquitocidal cry gene. It may be concluded that dipteracidal polypeptide of *entomocidus* INA 288 (*cryINA 288*) consisted of 703 amino acids with conserved blocks and amino acid sequence was aligned to that of *cry4Aa*. Furthemore, an aligment of the conserved block sequences among the cry INA 288 and other mosquitocidal proteins was performed.

***Corresponding Author:** Akhmad Rizali ✉ arizali25@yahoo.com

Introduction

In recent years, formulation of mosquitocidal *B. thuringiensis* serovar *israelensis* and *B. sphaericus* have been used on large scale in the field and in the various countries. This initial success has been tempered by result obtained for resistance to *B. thuringiensis* serovar sphaericus. (Georghiou *et al.*, 1993), In the search for potential alternative to the application of *B. thuringiensis* serovar *israelensis*, isolation of novel mosquitocidal strains is very important.

Bacillus thuringeinsis has been used an effective bio-insecticide. The specificity of *B. thuringiensis* is showed highly beneficial in agriculture biotechnology. Unlike most insecticides, *B. turingiensis* insecticides are highly toxic against target insect towards beneficial insect, non-target organisms such as human and wildlife (Bravo *et al.*, 2011 and, Brar *et al.*, 2006). It is also not harmful to the environment. *B. thuringiensis* has been used as an alternative to chemical pesticides for decades by organic farmers to control insect. At present, *B. thuringiensis* is the only "microbial insecticide" in widespread use (Cherif *et al.*, 2003, and Dong *et al.*, 2002). For more than 50 years, a variety of *B. thuringiensis* (Bt) products have been utilized worldwide as microbial insecticides (Li *et al.*, 2003, Asano *et al.*, 1996, and Iizuka *et al.*, 1984).

The bacterium *B.thuringiensis* widely used as bio-pesticide. The specific toxic activity to insect and another microbe(s) (Qi *et al.*, 2016 and Asano *et al.*, 1996). Numerous chemical insecticides have been used to control some insects. While chemical insecticides have knock down effect, they are too expensive harmfull to both humans and the environment. In addition, target insect pests develop biological resistance rapidly especially at higher rates of application. Thus, the increase in pesticidal application to control this pest has urged to researcher to search for biological control alternatives that would be a good component of Integrated Pest Management (Asano *et al.*, 2006).

Bacillus thuringiensis is a gram-positive, spore-forming bacterium that produce parasporal crystal during the sporulation stage. The crystal is made of one or more proteins toxic to some insect species. Most strains of *B. thuringiensis* produce delta-endotoxin crystal toxic to lepidopteran insect such as moth (Baravo *et al.*, 2016, Bulla *et al.*, 1997 and Kim *et al.*, 1994).

The name proposed of *Bacillus thuringiensis* for a species of *bacillus* which was isolated from diseased larvae of Mediterranean flour moth *Angasta* (*Ephestia*) *kuhniella* Zell (Sanahuja *et al.*, 2011, Kim *et al.*, 1984, Ohba *et al.*, 1986, and Zhang *et al.*, 1984). Later, noted infection of the larvae after the ingestion of the *bacillus* or its spores, described and named it *Bacillus thuringiensis* (Berliner 1915 and Mattes 1927). It isolated the same *bacillus* from the same insect host, which had found earlier. This strain is now maintained as *B. thuringiensis* serovar *thuringiensis* (serotype H-1). They noticed that the vegetative remains of sporulating cells assumed a rhomboid shape (Dong *et al.*, 2002 and Bulla *et al.*, 1985). Hedescribed this crystalline inclusion in the sporangium of the organisme 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 the disease process caused by the ingestion of sporalating *B. thuringiensis* (Sanahuja *et al.*, 2011).

Material and methods

Bacterial stains

The strain of *B. thuringiensis* used in the present study were *B. thuringiensis* serovar *entomocidus* INA 288.

DNA Preparation

Overnight cultures of *B. thuringiensis* strains were grown in 2ml of nutrient broth at 30°C using glass tube. Oneml of the overnight culture was inoculated into 50ml of tryptose phosphate broth (Difco) using 200ml culture flask and re-incubated for 4-5 hr until the synchronized culture had reached 10-15% optical transmission. The cell are finally harvested by centrifugation at 500g for 10 min. cells were suspended in approximately 1ml of lysozyme (10mg/ml; Wako pure chemical Industries, Ltd.), pre-incubated for 60 min at 37°C. Lysis was brought to incubate for 15 min at 60°C by addition of 8% SDS (Sodium Dodecyl Sulfate) buffer in TES and 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 (1ug/ml), and pre-incubated 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 500ul electrophoresis buffer.

Polymerase Chain Reaction (PCR) Procedures

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 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 te contents of *B. thuringiensis* serovar *entomocidus* INA 288, using oligonucleotide primer specific to *cryI* (Iizuka *et al*., 1995), *CryII* (Asano *et al*., 19934), and *CryIV* (Cherif *et al*., 2003 and Iizuka *et al*., 1982). Identification of cry genes in genomic DNA sample extracted from *B. thuringiensis* serovar *entomicidus* INA 288 strains was based on unique-size DNA fragments amplified by PCR for each cry gene (Asano, 2006).

Cloning and Squencing of the cry gene amplipied from serovar entomicidus INA 288.

The amplified gene of *B. thuringiensis* serovar *entomocidus* INA 288 was ligated into pGEM-T (Promega Co.). DNA sequences were obtained by dideoxy chain termination method (Cherif *et al*., 2003, Li *et al*., 2003, and Cherif *et al*., 2008) with {@-^{32}P} dATP (Amersham) and a sequenase version II kit from U.S. Biochemicals.

Results

Identification of cry genes in B.thuringiensis serovar entomocidus INA 288

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* INA 288, *cryI, cryII*, and *cryIV* primers and a total DNA template generated a uniquely sized fragment for each cry type gene. Therefore, the sizes of PCR products indicated the presence of crystal protein genes were confirmed by this technique. Serovar *entomocidus* INA 288 did not amplify *cryIC, cryIIA, cryIVA, cryIVC* and *cryIVD* genes. However, their cry genes had mosquitocidal activities. This fact shows that serovar *entomocidus* INA 288 contains novel cry genes for mosquitocidal activities.

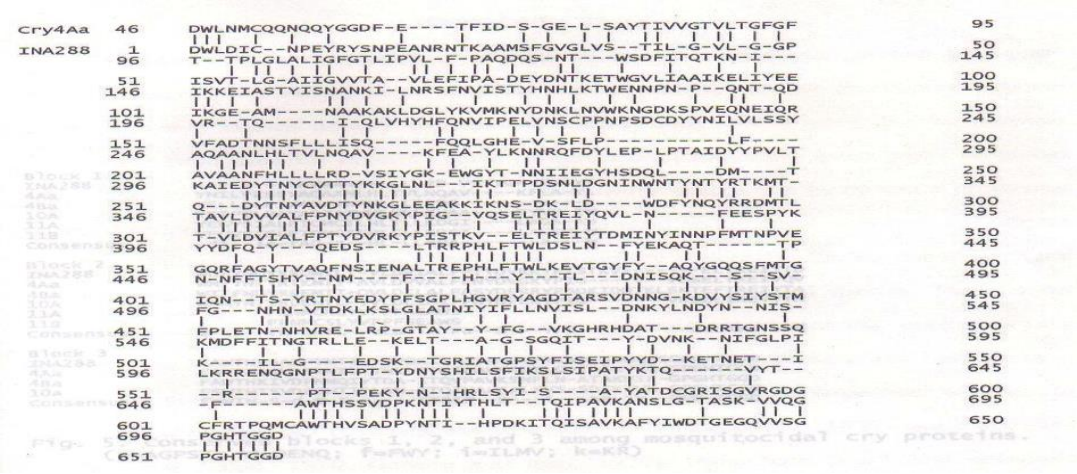

Fig. 1. Amino acid comparison between *cry4Aa* and *cryINA 288*.

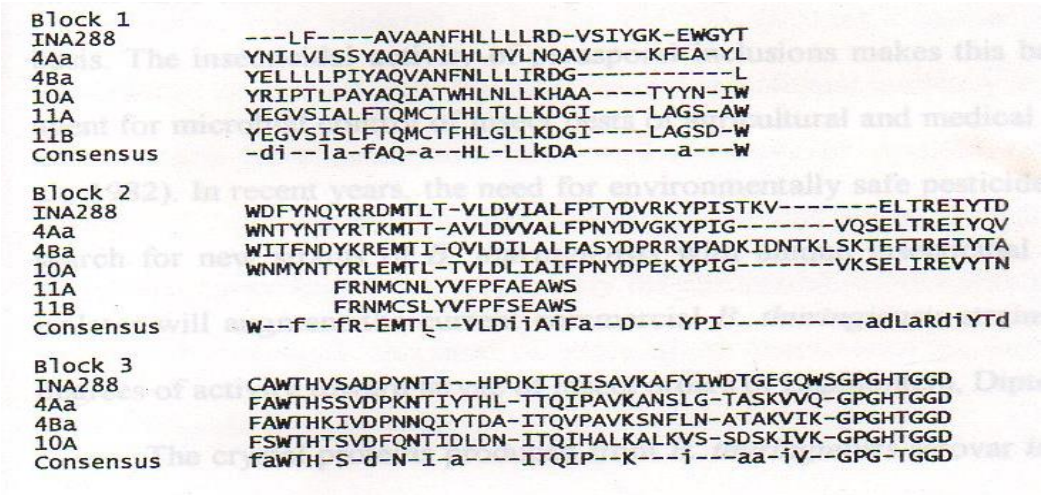

Fig. 2. Conserved block 1, 2, qnd 3 among mosquitocidal cry proteins.
(a=AGPST ; d=DENQ ; f=FWY ; i=ILMV ; k=KR)

Cloning and Squence of entomocidus INA 288

When the total DNA of *entomocidus* INA 288 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* INA 288 (*cryINA 288*) consisted of 703 amino acids with conserved blocks and amino acid sequence was aligned to that of *cry4Aa* (Fig.1). Furthemore, an alignment of the conserved block sequences among the *cryINA* 288 and other mosquitocidal proteins was performed (Fig. 2).

Discussion

The screening results suggest that *cry4Aa* had similarity with *cryINA 288*. Therefore, *cryINA 288* seems a novel mosquitocidal cry gene. However, It has already reported that serovar *entomocidus* (original strain) encodes only *cryIAa, cryIAb, cryIB*, and *cryIC*, which have not been thorough to be dipteracidal, implying that *entomocidus* INA 288 codes another protein with dipteracidal activity (Iizuka *et al*., 1982, Asano *et al*., 1993 and Iizuka *et al*., 1984).

Sequencing analysis of insert DNA revealed the amino acid sequence of the polypeptide of INA 288 (*cryINA 288*) consist of 703 amino acids with conserve blocks and amino acids sequence is aligned to that of *cry4Aa*. This strains a novel crystal protein gene, *cryINA 288*, on plasmid. *cryINA 288* consists of 703 amino acids, which include 3 conserve blocks. The similarity of amino acid sequence between *cryINA 288* and cry4Aa was 38%. Therefore, *cryINA 288* seems a novel mosquitocidal cry gene. However, after analyzing all amino acid sequences of this gene, amino acid comparison should be done for *cryINA 288*, *cry4Aa,* and *cry4Ab*.

Conclusion

It may be concluded that dipteracidal polypeptide of *entomocidus* INA 288 (*cryINA 288*) consisted of 703 amino acids with conserved blocks and amino acid sequence was aligned to that of *cry4Aa*. *cryINA 288* seems a novel mosquitocidal cry gene.

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