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Characterization of Bacillus thuringiensis Berl. indigenous from soil and its potency as biological agents of Spodoptera litura (Lepidoptera: Noctuidae)

by Dr.ir.yulia Pujiastuti, Ms.

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Characterization of *Bacillus thuringiensis* Berl. indigenous from soil and its potency as biological agents of *Spodoptera litura* (Lepidoptera: Noctuidae)

Y Pujiastuti[1*], D T Astuti[2], S R Afriyani[2], S Suparman[1], C Irsan[1], E R Sembiring[3], S Nugraha[3], Mulawarman[1] and N Damiri[1]

[1]Department of Plant Protection, Faculty of Agriculture, Sriwijaya University, Jalan Padang Selasa, Palembang, Indonesia

[2]Graduate School of Agriculture, Faculty of Agriculture, Sriwijaya University, Jalan Padang Selasa, Palembang, Indonesia

[3]Genomic and Crop Improvement Laboratory, Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI), Jalan Raya Bogor Km. 46 Cibinong, Bogor, Indonesia

[*]Email: ypujiastuti@unsri.ac.id

Abstract. The objective of the study was to characterize the molecular weight of protein in order to be utilized as biological agent of *S.litura* and their cell or spores production. To investigate the molecular weight of protein was done by SDS-Page electrophoresis. Growth media used for producing *B. thuringiensis* were T3, LB broth and agricultural waste. The results showed that the molecular weight of protein ranged from 37 to 140 kDa. In DLM and DLKK23 isolates were found ranging from 37 to 40 kDa and from 110 to 130 kDa, respectively. KJ3R5 and KJ3P1 isolates were obtained having three protein bands ranging from 43 to 45, 73 to 80 and 110 to 130 kDa and 45-50, 75-80 and 130-140 kDa, respectively. It was predicted that isolates *B. thuringiensis* were belonging to Cry IA, Cry IIA, Cry IVC and Cry15c. These crystal proteins were toxic to *S. litura*. There was no protein bands found in the two last isolates (KJ3R3 and KJ3J4). Production of spore after sporulation in agricultural waste media ranged from 0.5 to 10^6 - 2.67 x 10^7 spores/ml showing medium level of toxicity to *S. litura*.

Key words : *control, electrophoresis, entomopathogene, pest, protein.*

1. Introduction

Bacillus thuringiensis is an entomopathogenic bacteria, which can produce protein and spores at the sporulation. The protein often refers to Cry toxin, because its shape resembles crystals. Cry toxins plays a role in midgut epithelial cells by inserting into the target membrane and forming pores [1]. These have a potency to control a number of insect pests belonging to Lepidoptera, Diptera and Coleoptera and are safe to the ecosystem as they do not kill any useful insect, natural enemies and human being [2 - 4]). Crystal protein was the main substance which toxic to insect pest, therefore this crystal should be isolated. The amount and molecular weight of *B. thuringiensis* crystalline proteins (Cry) was estimated by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [5].

Recent study showed new indigenous *B. thuringiensis* strains were isolated and examined so that these alternatives were used to support the current commercial strains, such as *B. thurigiensis* subsp. Aizawai / cry proteins in use [6]. Research on *B. thuringiensis* bacterial isolate from South Sumatera has been started since 15 years ago [7]. Isolates of *B. thuringiensis* were identified, such as SMR-02 and SMR-04 [8] indicating a high level of toxicity to armyworm *Spodoptera litura*. Unfortunately, isolation of protein has never been conducted due to lack of laboratory facilities. The objective of the study was to characterize the molecular weight of proteins in order to be utilized as biological agents of *S. litura* and their cells or spores production. To investigate the molecular weight of protein was done by SDS-Page electrophoresis.

2. Materials And Method

Soil sampling was taken from Musi Banyuasin regency of South Sumatera. Isolation of *B. thuringiensis* was conducted in Phytopathology Laboratory of Plant Protection Faculty of Agriculture Sriwijaya University and molecular weight of protein with SDS-PAGE electrophoresis was done in Genomic and Crop Improvement Laboratory, Research Center for Biotechnology- Indonesian Institute of Sciences LIPI Cibinong West Java. The study was conducted from March to July 2017.

Methods.

2.1. Isolation of Bacillus thuringiensis .

Isolation of B. thuringingiesn was conducted according to the method of Ozturk *et al.* [9] with some modifications. Five gram of soil samples was diluted well in 15 ml H_20 in test tube, by shaking well until perfectly diluted. One ml of upper part of dilution was taken in eppendorf tube, added by 1 µl Triton X-100, and heated in water bath 85º C for 15 minutes. With a sterile spatula, the solution was streaked on the medium NaCl Glycine Kim and Goepfert (NGKG) on petridish. Petridish was incubated at 30ºC, for 24 hours. Colonies of *B. thuringiesnsis* growed in white colour. After 24-72 hours incubation, proteinaceous parasporal inclusion bodies will be present. Identification of *B. thuringiensis* refers to Thiery and Frachon [10], by microscopic observation of bacterial cells test and gram staining.

2.2. Isolation of Bacillus thuringiensis protein

One colony of *B.thuringiensis* in the slant agar stock was transferred using a wire needle heated until red then cooled, into 1 ml T3 medium and shaked at 25 ºC overnight. This culture was then removed to three kinds of growth media, namely : 1). 10 ml of T3 medium [11], 2). 10 ml LB medium (5 g yeast extract, 10 g Tryptone and 10 g NaCl in 1000 ml volume, pH 7.2) and 3). Agricultural waste (coconut water and rice water = 1 : 1 (V/V) were shaken at 25ºC for 2 days. The cells were harvested by centrifugation at 13 K rpm for 10 min, washed in distilled water twice and collected white pellets. The pellet was suspended in distilled water. Precipitation was collected by centrifugation at 13 krpm for 10 min. It was re-suspended in water and added the same volume of 0.5 M NaCL. For collecting precipitation it was centrifuged down at 10 krpm for 15 min. This step (NaCl wash) was done at least two times and continued with distilled water wash two times. The cells were re-suspended in 5 ml of 10 mM Tris HCl pH 8 containing 10 mM EDTA and disrupted by a sonicator. This solution was kept on ice for 30 min after addition of 100 µl of mercapto-ethanol and adjustment of pH 10.5. To collect supernatant contained protein, it was centrifuged down at 10 krpm for 10 min. The last step was adjusting to pH 4.4 and kept the crystal protein on 4 ºC for long storage.

2.3. Weighing molecular mass of crystal protein by SDS-PAGE analyses

Crystal proteins isolated from *B. thuringiensis* strains, namely DLM5, KJ3R32, KJ3R53, KJ3J44, KJ3P15 and DLKK236, were analyzed by sodium dodecyl polyacrilamide gel electrophoresis (SDS-PAGE) [5]. SDS-PAGE was carried out by the method of Laemmli [12] using 10% running and 4% stacking gels. The crystal proteins was prepared as follows: 10 µl of crystal protein kept at pH 4.4 was centrifugation down at 10 k rpm for 1 min to collect precipitation. It was suspended in the equal

volume of TE. After the addition of SDS sample buffer (1:4, v/v), protein sample was boiled for 3 min. The centrifugation at 7 krpm for 1 min was done to purify the protein solution. Samples were ready for electrophoresis. The 10% gel SDS-PAGE consisted of two layers, separating gel and stacking gel. The sample proteins and marker (PM1500 SMOBIO range 10-180 kDa) were loaded in the gel and run at current 60 V 30 mA 180 minutes in SDS electrophoresis buffer. After bromo phenol blue tracking dye had reached the bottom of separating gel, it needed to disconnect with power supply. The gel was removed from the glass, clean with dH20 and stained with co-massive blue overnight at room temperature. The co-massive blue was poured out and the de-staining was poured in with shaker incubation at 55 °C for 30 min. The fresh de-staining solution was added and continued de-staining until blue bands and clear background was obtained. After SDS-PAGE electrophoresis, it was analyzed the content of protein by comparing with PM1500, product of SMOBIO marker.

2.4. Cell production / spores B. thuringiensis.

Seed culture was required for the propagation of *B. thuringiensis*. The preparation was as follows: a needle isolate *B. thuringiensis* inserted into 100 ml of Nutrient Broth medium (NB) then shaken by using a shaker for 12 hours at 200 rpm. After it was taken 10 ml to put in 100 ml NB, then shaken again by using a shaker for 12 hours with a speed of 200 rpm. Furthermore, seed culture is ready for bio insecticide. Media multiplication of 50 ml coconut water + 50 ml rice water and 50 mg $CaCl_2$, 50 mg $MgSO_4$, 50 mg K_2HPO_4 and 50 mg KH_2PO_4 was prepared. The medium was inserted into a 250 ml Erlenmeyer tube. Tube was covered with aluminum foil and plastic and tied with rubbers. Further sterilized was done by insertion into the autoclave for 20 minutes with a temperature of 121°C and at a pressure of 1 atm. After cooling process, 5 ml seed culture of each Erlenmeyer was aseptically added. Furthermore, it was shaken by using a shaker for 72 hours with a speed of 200 rpm. The number of cells was produced after sporulation was calculated using a haemocytometer.

2.5. Bioassay of cell and spores of B. thuringiensis towards Spodoptera litura.

Bioassay was conducted with third instar larvae of *S. litura*. No-choice test was applied by dipping soybean leaves on 10^7 spores/ml *B. thuringiensis* medium for 5 minutes. The leaves were air-dried and placed it in a petri-dish. Third instar of Spodoptera larvae was individually inserted into the petri-dish. Each treatment was repeated 30 times or required as many as 30 larvae. Observations were made every day for 7 days, calculated the number of larvae that died or did not develop into 4^{th} larval instar. Mortality percentage was calculated by number of insects died divided by number of insect treated x 100%.

3. Results And Discussion

3.1. Number of B. thuringiensis isolates

Among 50 isolates of *B. thuringiensis* obtained, through the process of color testing, morphology, gram positive test and spore staining was obtained 6 isolates suspected *B. thuringiensis*. Six isolates were tested for toxicity to armyworm *S. litura*. The isolates were coded as follows: DLM 5, KJ3R3, KJ3R5, KJ3J4, KJ3P1 and DLKK2 3.

3.2. Character of Molecular weight of B. thuringiensis protein

The observations on the SDS Page gel electrophoresis showed a clear band. The band indicated as a sign of protein presence in accordance with the guidelines on the PM 1500 SMOBIO marker. In Figure 1, the medium grew was T3 broth which was a suitable medium for the growth of B. thuringiensis. The results obtained that DLM5, KJ3R5 and KJ3P1 isolates possessed some clear bands. Some parts of the band looked thick showing protein content was abundance.

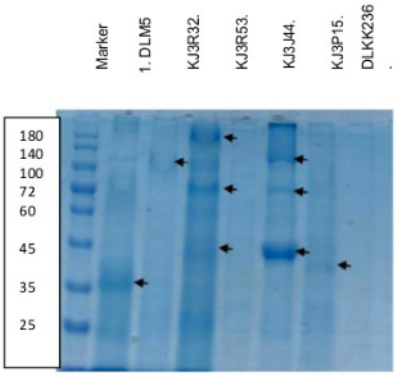

Figure 1. Profile of molecular weight of Protein of *Bacillus thuringiensis* harvested from T3 growth medium.

In Figure 2, Bt protein was produced from T3 growing medium mixed with waste materials namely coconut water and rice water. It was shown that the band was not very clearly visible. There was a relationship between isolate and growth media. Bt sporulation did not complete, leading in less production of cell/spores/crystal protein. There may be suspicion of prolong the time in the shaker. Growing with medium T3 was done for 2 days in producing perfect sporulation, whereas in waste medium waste may took longer time. It is also encountered by Palma *et al* [13] bio cidal of *B. thuringiensis* was influenced by the time in sporulation process. Furthermore it was possible that use of coconut waste and rice water was not sterilized, therefore there was a possibility for other bacteria to grow. Thus, the production of cells / spores and protein can be found in small amounts.

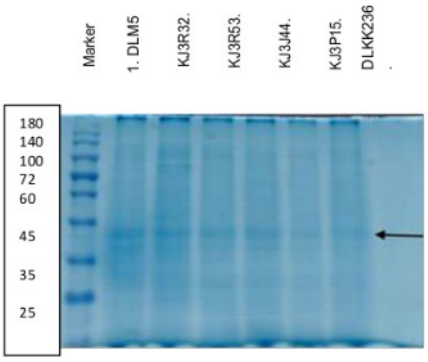

Figure 2. Profile of molecular weight of Protein of *Bacillus thuringiensis* harvested from T3 medium mixed with agricultural waste growth medium.

Figure 3 below, *B. thuringiensis* was grown in LB broth media. In the fifth column (KJ3P15) a thin band appeared at about 45-50 kDa. Bands was very light to see. The use of LB may be less suitable for growth and development of *B. thuringiensis* cells compared with T3 medium.

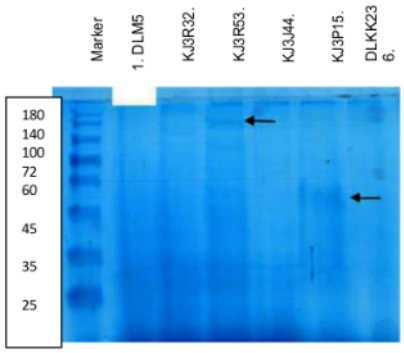

Figure 3. Profile of molecular weight of Protein of *Bacillus thuringiensis* harvested from LB broth growth medium.

3.3.Estimated weight of protein molecules

The weight of the protein molecule is one of the important indicators in determining the grouping of a strain of *B. thuringiensis*. The analysis of *B.thuringiensis* crystal composition is useful in determination of inclusion toxicity [5]. From the expected molecular weight, the targeted target insects can be determined, as stated by Hofte and Whiteley [14] and Crickmore et al. [15]) in Table 1 below.

Table 1. Molecular weight of Protein, gene type and insect target.

Gene type	Protein Type	Predicted molecular weight protein (kDa)	Host Range
cryIA(a)	CryIA(a)	133.2	Lepidoptera/Coleoptera/Diptera
cryIA(b)	CryIA(b)	131.0	Lepidoptera/Coleoptera/Diptera
cryIA(c)	CryIA(c)	133.3	Lepidoptera/Coleoptera/Diptera
cryIB	CryIB	138.0	Lepidoptera/Coleoptera/Diptera
cryIC	CryIC	134.8	Lepidoptera/Coleoptera/Diptera
cryID	CryID	132.5	Lepidoptera/Coleoptera/Diptera
cryIIA	CryIIA	70.9	Lepidoptera/Diptera
cryIIB	CryIIB	70.8	Lepidoptera
cryIIIA	CryIIIA	73.1	Coleoptera
cryIVA	CryIVA	134.4	Diptera
cryIVB	CryIVB	127.8	Diptera
cryIVC	CryIVC	77.8	Diptera
cry IVD	Cry IVD	72.4	Diptera
cyt A	Cyt A	27.4	Diptera/cyt.

Moreover, the crystalline toxins are classified into groups with amino acid similarity ([15]. Protein Cry1, Cry8, Cry9, Cry15, Cry22, Cry32, and Cry51 may cause negative effect to Lepidotera [14]. Based on band position in gel of SDS-PAGE, it was shown that the protein contained 1-8 protein with the molecular weight of protein was ranged of 37 – 1[illegible] kDa. This number of protein was similar to the result reported by Konecka et al. [5]. They found crystals contained 1–8 proteins with molecular

eight of 36–155 kDa isolated during epizootics in *Cydia pomonella* L. Reyaz et al. [6] found by SDS-PAGE investigation of the spore–crystal mixture demonstrated Bt strains contained proteins of various molecular weights ranging from 150 to 28 kDa. Two protein bands at 130 kDa and 65 kDa were detected with SDS-PAGE analysis from B. thuringiensis isolated from environments of boron [15]. In the isolate of DLM and DLKK23 isolates were found one i.e. 37-40 kDa and 110-130 kDa, respectively. KJ3R5 and KJ3P1 isolates were obtained that have three protein bands namely 43-45, 73-80 and 110-130 kDa and 45-50, 75-80 and 130-140 kDa, respectively. It was predicted that isolates B. thuringiensis were belonging to Cry IA, Cry IIA, Cry IVC and Cry15c (Table 2).

Table 2. Molecular weight of Proteins of *Bacillus thuringiensis* and predicted host.

Isolate Code	Similarity of position: Sample loading in SDS-PAGE	Position	Prediction of molecular weight	Predicted Cry protein Group	Predicted host
DLM 5	Volume 20 µl running 1	Band 1 : 37-38	37-40	Cry 15Aa *	Lepidopetra
	Volume ±30 µlµl running 3	Band 1 : 38-40			
KJ3R3	No similarity				
KJ3R5	Volume 20 µl running 1	band 2 : 75	73-80	Cry IIA	Lepidoptera/ Diptera
	Volume 8 µl running 2	band 2 : 73-75			
	Volume ±30 µl running 3	band 2 : 75-80			
	Volume 8 µl running 2	band 1 : 110-130	110-130	CryIA	Lepidoptera
	Volume ±30 µlrunning 3	band 1 : 110-130			
	Volume 8 µl running 2	band 3 : 43-45	43-45	Cry 15Aa *	Lepidoptera
	Volume ±30 µl running 3	band 3 : 45			
KJ3J4	No similarity				
KJ3P1	Volume 20 µl running 1	band 1 : 130	130-140	CryIA	Lepidoptera
	Volume 8 µl running 2	band 1 : 140			
	Volume ±30 µlµl running 3	band 1 : 140			
		Band 3 : 75			
	Volume 20 µl running 1	(light)	75-80	CryIVc	Diptera
	Volume ±30 µlµl running 3	Band 3 : 75-80			
	Volume 20 µl running 1	Band 4 : 45	45-50	Cry 15Aa *	Lepidoptera
	Volume ±30 µlµl running 3	Band 4 : 47-50			
DLKK2 3	Volume 8 µl running 2	Band 2 : 110	110-130	CryIA	Lepidoptera
	Volume ±30 µlµl running 3	band 2 : 130			

3.4. Toxicity to target insect and spore production in growth media

In general, liquid waste media in the form of coconut water and rice water was used to grow *B. thuringiensis* cells. This technique was resulted from the multiplication of cells / spores /crystal proteins that may be done with easy-to-obtain media, useless waste and low cost, so it can be made in non-aseptic circumstances. Spore density obtained ranged from 0.50 x 10^6 to 2.52 x 10^7 spores / ml. Isolates with code KJ3R5 showed the highest result (2.63 x 10^7 spores / ml). When focused I production of crystal protein, the isolate significantly indicated the presence of three types of protein bands, ie. 43-45, 73-80 and 110-130 kDa. Similarly, KJ3P1 isolates showing spore production of 2.52 x 107 spores / ml had three bands at 45-50, 75-80 and 130-140 kDa levels. The results are presented in Table 3.

Table 3. Cell/spore production of *Bacillus thuringiensis* harvested in agricultural waste and their toxicity towards *Spodoptera litura*.

Isolate Code	Cell Production (cells/ml)	Toxicity (%)
DLM 5	2.11×10^7	53.33
KJ3R3	1.50×10^6	46.67
KJ3R5	2.63×10^7	60.00
KJ3J4	0.50×10^6	46.67
KJ3P1	2.52×10^7	53.33
DLKK2 3	2.50×10^7	46.67
Control (dH20)	-	15.00

In bioassay test of *S. litura* obtained the highest mortality result was isolate of KJ3R5. While on KJ3R3, KJ3J4 and DLKK2 3 showed similar mortality rate. Bioassay was a preliminary test which was followed by more detailed assays taking into number of the spores content possessed by the isolates. This data showed that every single isolates was toxic against lepidopteran insects. This results were supported with other finding of other researchers. Ninety percents of mortality in third instar larvae of diamond backmoth *Plutella xylostella* larvae treated for 72 hours was caused by a protein content of 11.1 g / l [16]. Research on insect bioassays with first larvae of *S. litura* showed that the purified Cry2Al1 was toxic to *S. litura* with LC_{50} 2.448 mg / ml [6]. Shah et al. found that some isolates with cry protein range 30-150 kDa were toxic against *Amsacta albistriga* (Lepidoptera:Arctiidae) [17].Therefore, it was no doubt that *B. thuringiensis* isolates indigenous South Sumatera were toxic to diamond backmoth *P. xylostella* and armyworm *S. litura*.

4. Conclusion

The molecular weight of protein ranged from 37 to 140 kDa. Isolates of DLM and DLKK23 contained two protein bands, their were 37-40 kDa and 110-130 kDa, respectively. KJ3R5 and KJ3P1 isolates had three protein bands, i.e. 43-45, 73-80 and 110-130 kDa and 45-50, 75-80 and 130-140 kDa, respectively. It was predicted that isolates *B. thuringiensis* were belonging to Cry IA, Cry IIA, Cry IVC and Cry15c. These crystal proteins were toxic to *S.litura*. Production of spore after sporulation in agricultural waste media was ranged 0.5×10^6 - 2.67×10^7 spores/ml showing medium level of toxicity to *S. litura*.

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