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Sodium dodecyl sulphate polyacrylamide gel electrophoresis of toxin proteins of *Bacillus thuringiensis* isolated from soil at the Liwa Botanical Garden

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Abstract

Bacillus thuringiensis is a spore-forming bacterium known to have the ability to synthesize proteins that are toxic against insect pests. Currently, the bacterial protein toxins are widely used as pesticides because they are environmentally friendly and safe for health. As a cosmopolitan microbe *B. thuringiensis* is widely distributed in nature with soil as its main habitat. Liwa Botanical Gardens is one among the conservation areas in Lampung Province of Indonesia where the soil type and conditions expected to good for the bacterial life of *B. thuringiensis.* This study aims to determine toxin proteins extracted from *B. thuringiensis* isolated from the soil of the Liwa Botanical Garden by their molecular weights. The proteins were quantified using Lowry method and the protein profiling performed using SDS PAGE electrophoresis. The results showed that there were two isolates, Bt 3 and Bt 5 which showed close protein concentrations, namely 0.679 mg/ml and Bt 5 with 1.313 mg/ml respectively. Both isolates also exhibit different molecular weights. Bt 3 isolate has a molecular weight of 110.59 kDa, while Bt 5 has a molecular weight of 76.97 kDa.

Keywords: Protein crystal; Bacillus thuringiensis; Liwa Botanical Garden; SDS PAGE

1. Introduction

Bacillus thuringiensis is a type of gram-positive bacteria that has been widely used in the field of agricultural biotechnology because it has toxicity on several plant insect pests such as the order Diptera and Lepidoptera [1]. There are at least 70 different subspecies or varieties of *B. thuringiensis* known to produced more than 300 Cry proteins from 1000 strains and only a few strains have been utilized [2]. The ability of *B. thuringiensis* in synthesizing protein toxins makes this bacterium categorized as entomopathogenic. The toxicity of this bacterium is supported by protein crystals that are formed when the cells sporulate [3]

During sporulation, *B. thuringiensis* forms parasporal crystals composed of insecticidal crystal proteins (ICPs) that typically represent up to 25% of the cell dry mass of the sporulating cells. ICPs have been used as bioinsecticides to combat insect pests of agricultural crops. In addition, *B. thuringiensis* has various forms of protein that are correlated with its toxicity. The shape of the protein crystals is influenced by the molecular weight of the protoxin produced by *B. thuringiensis* [4].

In addition to its characteristic of the protein crystals, the habitat of *B. thuringiensis* is also a distinguishing factor of the toxin protein produced by this bacterium. *B. thuringiensis* can be found in many types of environment including soil, insect carcasses, stored product dust, plant leaves and aquatic environments. However, the normal habitat of the organism is soil. Soil conditions with high humidity will be a good habitat for *B. thuringiensis* [5]. The state of the soil ecosystem of the Liwa Botanical Garden with an annual average rainfall of 2500-3000 mm with a relative humidity of 50-80% [6]. is very likely to have a typical type of bacterial toxin proteins produced by *B. thuringiensis* [7].

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The current study is aimed at determining toxin proteins extracted from *B. thuringiensis* strains isolated from the soil of the Liwa Botanical Garden carried out by Janah et al., 2022 using sodium dodecyl sulfate polyacrilamid gel electrophoresis (SDS-PAGE) [7] This study is a part of the efforts to explore Indonesia's biological resources, especially soil containing bacteria.

2. Material and methods

2.1. Isolates preparation

The isolate samples of *B. thuringiensis* used in this study were taken from collections of Microbiology Laboratory at the Department of Biology, University of Lampung. Those bacterial isolates were isolated by Janah et al., 2022 from soil at the Liwa Botanical Garden, West Lampung Regency the Lampung Province of Indonesia [7]

Two isolates coded Bt3 and Bt5 were selected and rejuvenated with Luria Bertani media [8], containing 10 g tripton, 5 g yeast extract, 10 g NaCl in 15 g agar [9]. Isolates were taken aseptically using ose needle, inoculated into Luria bertani agar slant medium by streak method and incubated at room temperature for 24 hours. The growing colonies were observed until uniform colonies were obtained. The rejuvenated isolates were then inoculated in T-3 agar plates (per-liter containing 16 g nutrient broth, 2 g KCl, 0.5 g $MgSO_4.7H_2O$, 2 mL 50% glucose, 1 mL $Ca(NO_3)2$ 1 M, 1 mL $MnCl_2$ 0.1 M, 1 mL $FeSO_4$ 1 mM in 17 g agar), then incubated for 48 - 72 hours until the colonies grown.

2.2. Isolation of toxin protein

Once the isolates colonies grown, the spores are transferred using an ose needle into microtube containing 1 ml of cold 0.5 M NaCl, immersed in ice and shaken until it becomes a homogeneous suspension. The suspension was centrifuged at 13000 g for 10 minutes, the supernatant was discarded and only the pellet was taken. The pellet was rinsed with 1 mL of 1% NaCl and re-centrifuged. The pellet obtained was then suspended with 140 µl of 1% SDS0.01% β-mercaptoethanol mixture and heated at 100°C for 10 minutes. The suspension was then centrifuged again at 10000 g for 10 min to obtain the supernatant containing the crude protein extract.

2.3. Quantification of Protein Concentration

Sample of 400 µl were added with 400 µl of 2x Lowry Concentrate containing Reagent A (20 g Na2CO3 in 260 mL dH_2O, 0.4 $CuSO_4.5H_2O$ in 20 mL H_2O) and Reagent B (0.2 g Na.K.tartrate in 20 mL H_2O, 4 g NaOH in 100 mL H_2O). The mixture then incubated at room temperature for 10 minutes. Next, 200 µl of 0.2 N Folin Ciocalteu was added and then homogenized with a vortex after each addition. The mixture was incubated for 30 minutes at room temperature and read with a spectrophotometer at a wavelength of 595 nm using BSA (bovine serum albumin) solution as a comparison.

2.4. Standard Curve Preparation

Bovine Serum Albumin (BSA) of 100 mg was dissolved ino 100 ml of ddH_2O to obtain a BSA stock solution of 1 mg/ml. Furthermore, a series of standard solution dilutions were made with concentrations of 0 mg/ml; 0.02 mg/ml; 0.04 mg/ml; 0.06 mg/ml; 0.08 mg/ml; and 0.1 mg/ml. From each concentration, 0.1 ml of standard solution was taken and 5 ml of Bradford solution was added and after being left for 30 minutes the absorbance was measured at λ 595 nm. Then, the data was plotted using Microsoft excel to obtain a standard curve using a linear regression equation y= mx + c (information: y: absorbance; m: slope; x: protein concentration and c: intercept)

2.5. Protein molecular weight analysis trough SDS-PAGE

SDS PAGE (sodium dodecyl sulfate polyacrilamid gel electrophoresis) was performed using the standard method with the Mini-Protean II Slab Cell Electrophoresis tool (Bio Rad). Protein samples (crude extract) were denatured by adding sample buffer (Tris-Cl 150mM pH 6.8, SDS 6.25%, mercaptoethanol, glycerol 25%, bromophenol blue 2.5 mM) with a ratio of protein and buffer 2:1, and boiled for 10 minutes and centrifuged for 5 minutes. Electrophoresis equipment was prepared, polyacrylamide gel was made from acrylamide & bisacrylamide stock solution (30%T, 2.67C), stacking buffer (Tris-HCl 0.5M pH 6.8), resolving buffer (Tris-HCl 1.5M pH 8.8), 10%SDS, APS and TEMED as catalyst. After the bottom gel (resolving gel) is formed, the stacking gel is inserted at the top and a mold is made to place the sample protein. Gel formulation for resolving gel is 12% while for stacking gel is 4%. Electrophoresis of samples was carried out at a voltage of 150 volts for 60 minutes following the marker protein as a comparison. For protein staining, Coomasie briliant blue 0.1% (w/v) was used. The staining results were washed in a solution of methanol: acetic acid (40%: 7.5%). The stained proteins were photographed with a digital camera, for further analysis.

3. Results and discussion

3.1. Isolates and the colonies growth

The bacteria isolate samples of *B. thuringiensis* used in the research were coded Bt3 and Br5. The origins of the two isolates according to the soil type of their habitat in the Liwa Botanical Gardens [10] are presented in Table 1.

Table 1 *B. thuringiensis* isolate samples by the soil type of their habitat

Soil type	Isolate Code
Litter soil	Bt 3
Araceae land soil	Bt 5

It is the differences in the type of soil from which the isolates are taken that are the focus of further exploration of toxin proteins synthesized by two bacterial isolates of *B thuringiensis*. As it was known that the difference soil type of the habitat will affect the biosynthesis process of toxin protein in *B. thuringiensis* [11]. (Shively, 2006). Each *B. thuringiensis* strain can carry one or more crystal toxin genes, and therefore, strains of the organism can synthesize one or more crystal proteins. Plasmid transfer among *B. thuringiensis* strains is the main mechanism for generating toxin gene diversity [12].

Isolates that have been re-cultured in Luria Bertani media were then transferred to *B. thuringiensis* sporulation selective media, namely T-3 media, until they sporulated for seven days, resulting in the colonies displayed in Figure 1. The isolate colonies formed shown in Figure 1 confirm the findings of previous researchers that *B. thuringiensis* incubated in the T-3 selective medium were grew uniformly with yellowish-white color, rough and slightly shiny [9]. On medium T-3, the colonies that grew are colonies that have produced spores. The sporulation process is an effort by crystallized bacteria to defend themselves from unfavorable environmental changes [3].

Colonies formed that were Gram stained with safranin exhibit a purple color. Gram-positive cells look purple because their cell walls bind violet crystals more strongly, while gram-negative cells contain more lipids so that the pores easily enlarge and violet crystals easily dissolve when washed with alcohol. Microscopic observations confirmed that *B. thuringiensis* is a gram-positive bacterium, rod-shaped, 3-5 μm long [13].

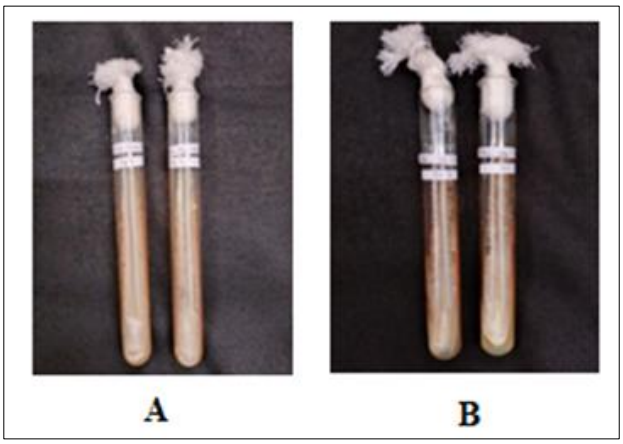

Figure 1 Colonies of *B. thruingiensis* in test tubes inoculated in duplicates grown on T-3 specific medium. (A) Bt3 isolate and (B) Bt5 isolate

3.2. The toxin proteins quantity

The results of protein quantification that have been separated from isolate spores using a spectrophotometer at a wavelength of 595 nm are presented in Table 2.

Table 2 Absorbance values and concentrations of protein in test bacterial samples

Sample	Absorbance (λ 595 nm)	Concentration of protein (mg/ml)
Bt3	0.568	0.679
Bt5	0.919	1.313

The separation of spores and crystalline proteins is done by adding a mixture of 1% SDS 0.01% beta mercaptoethanol [9]. This mechanism is also described by [10] that the crystalline proteins mixed with spores will be separated due to the presence of beta mercaptoethanol which is able to break disulfide bonds in proteins without affecting their primary structure through the reduction of S-S bonds by producing two cysteine residues [13]. Crystal proteins that have been separated will be dissolved because they are bound by SDS which is an ionic detergent that can dissolve hydrophobic molecules that give a negative charge to the overall protein structure.

The protein content obtained showed that the two isolates, Bt 3 and Bt 5, had relatively close protein concentrations Bt3 with 0.679 mg/ml and Bt5 1.313 mg/ml. Of the two samples, the average concentration of all samples from soil in the Liwa Botanical Garden was 1.0045 mg/ml. This difference in concentration refers to the theory that *B. thuringiensis* is an entomopathogenic bacterium whose development and defense are influenced by several factors including rainfall, erosion, humidity and temperature [15].

3.3. The proteins profiles

The application of the SDS PAGE method to determine profile and molecular weight of proteins synthesized by isolate resulted in the data shown in Figure 2 and Table 3.

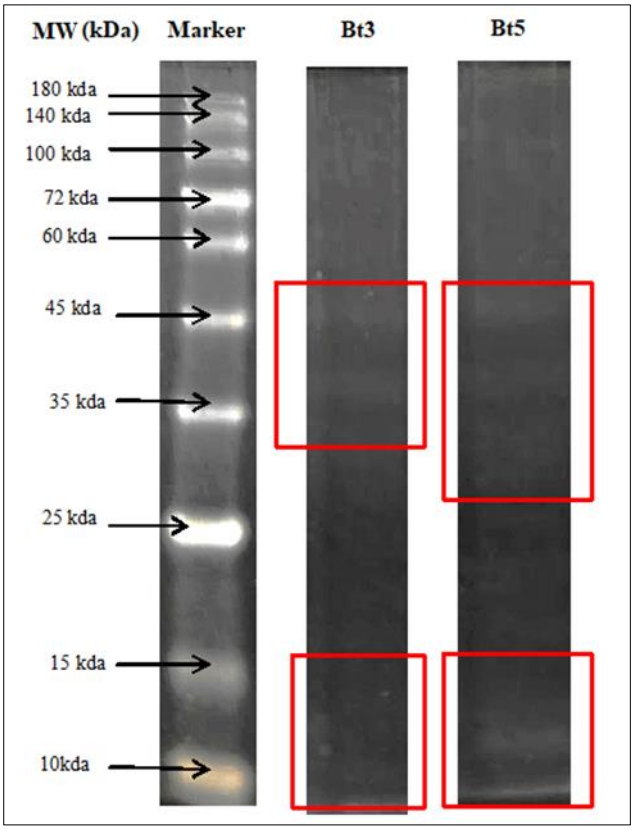

Figure 2 SDS-PAGE profiles of toxin proteins extracted from *B. thuringiensis* isolated from soil at Liwa Botanical garden. MW: molecular weight; Bt3 and Bt5: type of isolate samples; while the red rectangle indicates the assumed molecular weight of the protein in the samples analysed

Table 3 Molecular weight of marker and proteins extracted from isolate samples in kDa

No. Band Distance	Molecular weight marker	Bt 3	Bt 5
1	180	110.59	76.97
2	140.0	90.45	59.43
3	100	80.17	52.23
4	72	68.25	33.94
5	60	55.82	24.05
6	45	43.85	17.79
7	35	37.34	15.63
8	25	29.33	
9	195	24.97	
10	10	21.26	

*Values marked in grey are the assumed weight of the protein measured in sample by assumption that it is still in the form of protoxin that has not been broken. While the data marked in yellow is the assumed weight of the protoxin that has been broken into fragments of active toxin so that it is assumed to have smaller weight.

Refers to the SDS PAGE results depicted in Figure 3 the molecular weight of 10 markers protein (All Blue Regular Range Protein Marker PM1500) are 180, 140, 100, 72, 60, 45, 35, 25, 15, and 10 kDa. From each sample well, a band is formed which shows the assumption that the protein sample is comprised of several protein parts that have different molecular weights [2]. This assumption is in line with the statement that the protein is still in the form of protoxin.

Sample Bt3 has an assumed weight of 110.59 while Bt5 has an assumed weight of 76.97. Based on these weight assumptions, two types of toxin proteins were obtained from measurements using SDS PAGE. The sample with an assumed weight of 110.59 was identified as Cry1A for the type of protein and this type of protein is a toxin for the Lepidoptera order [16]. Meanwhile, for the assumption of weight 76.97 is the Cry 22 protein type, this is based on research by [17]. In addition, based on the type of protein that has been identified, it can be seen that the Cry 22 protein type is toxic to Coleoptera insect pests [17]

Figure 3 shows 2-4 rows of bands that are brighter than the other rows of bands shown in the blue box. According to Kitada et al., 2005 the *B. thuringiensis* toxin protein structure has three active regions (domains) of the polypeptide chain due to the differences in structure and functions that result in insect death and this domain will be split into active toxins [17]. The process of protoxin rupture occurs in the intestine and is dissolved by protease enzymes so that it becomes smaller parts in the form of active toxins. It's all due to the nature of crystalline proteins will dissolve in the midgut of insect larvae and are proteolytically converted into toxic core fragments in the form of active toxins [18].

The SDS PAGE results shown Table 3 confirms previous research findings done by [9]. The mechanism of action of the Cry protein is through direct ingestion by insects. The Cry protein becomes a toxin after undergoing proteolysis, then causes osmotic disturbances so that cells swell and burst and then cause death in insects [19]. (Masta, 2020). The life cycle of Bt is divided into phases, namely phase I: vegetative BM Marker Marker Bt 3 Bt 5 180 kda 140 kda 100 kda 72 kda 60 kda 45 kda 35 kda 15 kda 25 kda 10kda growth; phase II: transition to sporulation; phase III: Sporulation; phase IV: spore maturation and cell lysis [20]. The description of the phase and mechanism of *B. thuringiensis* Cry protein is provided in Figure 3.

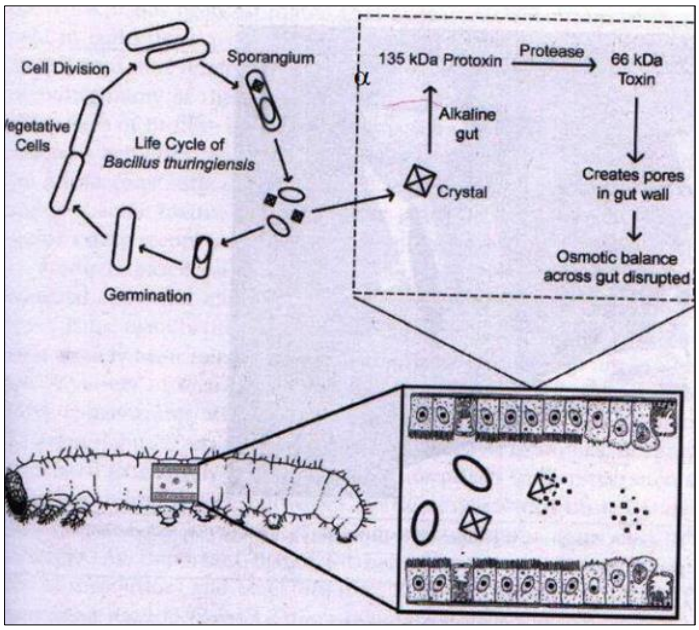

Figure 3 Phase and mechanism of action of bacillus thuringiensis (Berbert-molina et al., 2008)

The results of the assumption of molecular weight with SDS PAGE are in accordance with what was done by [9]. The mechanism of action of the Cry protein is through direct ingestion by insects. The Cry protein becomes a toxin after undergoing proteolysis, then causes osmotic disturbances so that cells swell and burst and then cause death in insects [19]. The life cycle of Bt is divided into phases, namely phase I: vegetative growth; phase II: transition to sporulation; phase III: Sporulation; phase IV: spore maturation and cell lysis [20].

4. Conclusion

The toxin protein synthesized by isolate sample Bt3 has a molecular weight assumption that tends to be identical of 110.59 kDa and is thought to be a Cry1A type protein, whereas isolate sample of Bt5 synthesized a different molecular weight assumption of 76.97 and suspected as a Cry22 type protein. The results of this research still need to be followed up by conducting in vitro study to determine the the toxin protein activities of *Bacillus thuringiensis* isolate collected against several orders of insects.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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