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Original Article

Characterization and toxicity of *Bacillus thuringiensis* serovar *chanpaisis* (H46): A serovar from Thailand

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Abstract

A new strain of *Bacillus thuringiensis* serovar *chanpaisis* was isolated from soil samples in Thailand. It was classified and characterized in terms of the crystal proteins, carbohydrates fermentation patterns, *cry* gene content, and its toxicological properties against the species of Lepidoptera, Coleoptera, and Deptera. This strain was identified as *Bacillus thuringiensis* according to morphological and biochemical characteristics. The strain produced bipyramidal crystal proteins consisting of major bands at about 130 kDa, 85kDa, 64kDa, 50kDa, and 20 kDa. A PCR analysis of the *cry* genes revealed that only *cry*32 genes could be detected and localized on chromosomal DNA and plasmid DNA of the organism. The bioassay showed that *B. thuringiensis* serovar *chanpaisis* had no larvicidal activity against any species of the Lepidoptera or Coleoptera but exhibited mortality level against *Aedes aegypti* and *Culex quinquefasciatus* with LC_{50} values of 1.48×10^4 spores mL^{-1} and 1.00×10^4 spores mL^{-1}, respectively.

Keywords: *Bacillus thuringiensis*, *cry* gene, crystal protein, mosquitoes, Thailand

1. Introduction

Bacillus thuringiensis is a Gram-positive, spore-forming soil bacterium of considerable economic importance and can be used as a biocontrol agent (Ben-Dov, 2014). *B. thuringiensis* strains can synthesize crystal (Cry) and cytolytic (Cyt) toxins, (also known as δ-endotoxins) at the onset of sporulation during the stationary growth phase of crystal proteins (Höfte and Whiteley, 1989; Schnepf *et al.*, 1998). In addition to δ-endotoxins, *B. thuringiensis* produces soluble factors including chitinases (Driss *et al.*, 2005), proteases, vegetative insecticidal proteins (VIP) (Mesrati *et al.*, 2005), alpha-exotoxin, and beta-exotoxin (Porcar and Juárez-Pérez, 2003) which contribute to its insecticidal activity. To date, more than 67 serotypes of *B. thuringiensis* have been deposited in the International Entomopathogenic Bacillus Centre (I.E.B.C) at Pasteur Institute (France) (Frutos *et al.*, 1999) and at least 600 *cry* genes have been reported and identified (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html).

Cry proteins have similar mechanisms of action. The crystals are solubilized in the midgut lumen and converted to active toxins that bind to specific receptors on the brush-border (apical) membrane where they form pores. As a consequence, the epithelial midgut cells swell and lyse causing the larvae to stop feeding and eventually die by septicemia or starvation (Fiuza *et al.*, 1996; Iracheta *et al.*, 2000). These toxins are highly toxic to many insect targets and friendly to non-target organisms and environment. Therefore, the toxins have been widely used as an alternative to chemical pesticides or genetically engineered into crops to provide constant protection (Bravo *et al.*, 2011).

From a previous report, Chanpaisaeng *et al.* (1993) discovered the serovar *chanpaisis* from soil samples in

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Narathiwat Province, Thailand. The preliminary characterization on toxicity property of the new serovar was performed (Chanpaisaeng *et al.*, 1996; Lecadet *et al.*, 1999). It was found that serovar *chanpaisis* did not yield mortality in *Heliothis armigera, Spodoptera exigua, Plutella xylostella* or *Bactrocera dorsalis*. Interestingly, this strain contains bipyramidal crystal proteins which are produced by *cry* genes. Furthermore, the study reported that only *cry*IA gene was not detected on both chromosomal and plasmid DNA. Therefore, there must be other *cry*IA genes on serovar *chanpaisis* which generate bipyramidal crystal proteins. Thus, the aims of this study were to examine the biochemical characteristics, *cry* gene content, crystal protein profile, and evaluate the larvicidal activity against a number of insect pests in order to gain further molecular and toxicity understanding of this isolate.

2. Materials and Methods

2.1 Bacterial strains

The standard strain of *B. thuringiensis* serovar *chanpaisis* (JC51) and serovar *israelensis* were obtained from our own collection, whereas *B. thuringiensis* serovar *aizawai* and *B. thuringiensis* serovar *tenebrionis* were isolated from commercial products manufactured by Valent Biosciences Corporation under the trade names of Xanthari and Novodor, respectively. All strains of *B. thuringiensis* were maintained at 20 °C in nutrient broth supplemented with 30% glycerol.

2.2 Examination of the cells, spores, and crystal proteins

The morphology of the vegetative cells, spores, and crystal proteins of the sporulated bacterial cells were examined under a phase-contrast microscope. The colony sample was firstly inoculated into nutrient agar (NA). The culture was then incubated at 30 °C for 48 h. This procedure allowed the bacterial cells to sporulate. Wet mount slides were prepared from the inoculated culture after 48 h of incubation to visualize the cells, endospores, and the crystal protein morphology under a phase contrast microscope. For electron microscopy, the isolate was cultured in NA at 30 °C for 72 h, then a spore-crystal mixture was air dried on a cover glass and coated with gold. Spores and crystal proteins were examined with JOEL JSM-35CF scanning electron microscope (SEM) operated at 10 kv.

2.3 Carbohydrate fermentation pattern

B. thuringiensis serovar *chanpaisis* was subjected to characterization depending on carbohydrate fermentation pattern by the API identification system 50 CHB (Bio Mérieux, France). The carbohydrate fermentation tests were based on the principle of pH change. Substrate utilization and other biochemical reactions exhibited color changes. The whole kit consisted of 49 different tests of carbohydrate. The results were determined at 24- and 48-h incubation times. The data were analyzed using the apiweb™ database available on https://apiweb.biomerieux.com

2.4 Larvicidal activity against target insect

Larvicidal activity of the novel serovar were tested against the economically important insect pests which belong to three orders: Lepidoptera, Coleoptera, and Diptera. The bioassay was carried out on the larvae of four lepidopterous pests (*Spodoptera litura, Spodoptera exigua, Plutella xylostella,* and *Galleria mellone*lla), two Coleoptera (*Tenebrio molitor* and *Tribolium castaneum*), and four Diptera, (*Aedes aegypti, Culex quinquefasciatus, Bactrocera dorsalis*, and *Musca domestica*). Bacterial strains used as positive controls for coleopterans, mosquito larvae, and lepidopteran were *B. thuringiensis* serovar *tenebrionis*, *B. thuringiensis* serovar *israelensis*, and *B. thuringiensis* serovar *aizawai*, respectively. Three replicates were made for each strain including the control.

To prepare *B. thuringiensis* stock suspensions, sporulating cultures of *B. thuringiensis* releasing spores and crystals were produced in the nutrient broth (NB) medium at 30 °C for 72 h. Spore crystal mixtures were washed at 10,000*g* for 10 min at 4 °C twice in sterile water. The resulting pellets were resuspended in sterile water and consequently supplemented with 0.1% of the wetting agent (Tween® 80). As a control, the insects were treated with water plus the same concentration of Tween® 80.

The lepidopterous species, *S. litura, S. exigua*, and *G. mellonella*, were obtained from the laboratory colonies of the Department of Agriculture, Thailand while *P. xylostella* larvae were collected from field populations in Supanburi Province, Thailand. Bioassays of *S. litura, S. exigua*, and *P. xylostella* larvae were performed using the leaf dipping method (Chanpaisaeng *et al.*, 1996). Briefly, Chinese kale leaf discs (32-mm diameter) were dipped for 10 s into a *B. thuringiensis* stock suspension and air dried. After which the leaf discs were inoculated with five larvae per leaf. A bioassay of *G. mellone*lla larvae was performed using the diet-incorporated method (Chanpaisaeng *et al.*, 1996). Five second-instar larvae of *G. mellonella* were placed in plastic cups (30 mm diameter) and fed honey bee wax supplemented with 300 µL of the *B. thuringiensis* stock suspensions.

The larvae of *T. molitor* and *T. castaneum* were maintained on chicken feed at the insectary conditions (26±2 °C, 60±5% relative humidity [RH], and 12 h day length). The bioassay was performed as described by Beron and Salerno (2006). Batches of 10 second-instar larvae which were placed into petri dishes were fed 1 g of chicken feed supplemented with 300 µL of the *B. thuringiensis* stock suspensions.

The larvae of *A. aegypti and C. quinquefasciatus* were obtained from laboratory colonies of the Ministry of Public Health, Thailand while *B. dorsalis* larvae were obtained from the laboratory colonies of Department of Agriculture, Thailand. Batches of 20 mosquito larvae in glass assay containers holding 50 mL of water were added with 300 µL of the *B. thuringiensis* stock suspensions (Chowana disai *et al.*, 1995). Toxicity tests were performed on the second-instar larvae of *B. dorsalis* following Alberola *et al.* (1999). Ten larvae were transferred to petri dishes containing 300 µL of the *B. thuringiensis* stock suspensions and mixed with 1 g of artificial diet (wheat bran 26%, sugar 12%, dried yeast 3.6%, sodium benzoate 0.1%, methyl paraben 0.1%, acetic acid 0.2%, and water 58%).

A laboratory population of *M. domestica* was originally started with females collected in the field and reared in accordance with the method described by Shoukry and Radi (1988). The bioassay was performed following Zhong *et al.* (2000). Ten neonates were placed into petri dishes containing 1 g of wet cat food supplemented with 300 µL of the *B. thuringiensis* stock suspensions. The bioassay was conducted at 25 °C and 60-70% RH with a 12-h photoperiod. Mortality of the larvae was recorded daily until pupation.

2.5 PCR primers, sample preparation, and amplification

Molecular characterization through PCR was performed to identify the toxin-coding genes using a variety of oligonucleotide pairs specific for the following genes/gene families: *cry*1, *cry*2, *cry*3A, *cry*4A, *cry*5, *cry*9, *cry11A*, *cry*12, *cry*13A, *cry*14A, and *cry*32 (Table 1). Genomic DNA and plasmid DNA were extracted following the method described

Table 1. Sequences of primers used in the detection of *cry* genes.

Primer pair	Sequence[a] (5'-3')	Product size (bp)	Source
*cry*1Aa	1Aa (TTCCCTTTATTTGGGAATGC)(d) I(-) (MDATYTCTAKRTCTTGACTA)(r)	1286	Juárez-Pérez *et al.*, 1997
*cry*1Ab	1Ab(CGGATGCTCATAGAGGAGAA)(d) I(-) (MDATYTCTAKRTCTTGACTA)(r)	1371	Juárez-Pérez *et al.*, 1997
*cry*1Ac	1Ac (GGAAACTTTCTTTTTAATGG)(d) I(-) (MDATYTCTAKRTCTTGACTA)(r)	844	Juárez-Pérez *et al.*, 1997
*cry*1Ad	1Ad (ACCCGTACTGATCTCAACTA)(d) I(-) (MDATYTCTAKRTCTTGACTA)(r)	1212	Juárez-Pérez *et al.*, 1997
*cry*1Ae	1Ae (CTCTACTTTTTATAGAAACC)(d) I(-) (MDATYTCTAKRTCTTGACTA)(r)	1169	Masson *et al.*, 1998
*cry*1B	1B (GGCTACCAATACTTCTATTA)(d) I(-) (MDATYTCTAKRTCTTGACT)(r)	1323	Juárez-Pérez *et al.*, 1997
*cry*1C	1C (ATTTAATTTACGTGGTGTTG)(d) I(-) (MDATYTCTAKRTCTTGACT)(r)	1176	Juárez-Pérez *et al.*, 1997
*cry*1D	1D (CAGGCCTTGACAATTCAAAT)(d) I(-) (MDATYTCTAKRTCTTGACTA)(r)	1138	Juárez-Pérez *et al.*, 1997
cry1E	1E (TAGGGATAAATGTAGTACAG)(d) I(-) (MDATYTCTAKRTCTTGACT)(r)	1137	Juárez-Pérez *et al.*, 1997
cry1F	1F (GATTTCAGGAAGTGATTCAT)(d) I(-) (MDATYTCTAKRTCTTGACT)(r)	967	Juárez-Pérez *et al.*, 1997
cry1G	1G (GCTTCTCTCCAAACAACG)(d) I(-) (MDATYTCTAKRTCTTGACT)(r)	1128	Juárez-Pérez *et al.*, 1997
cry1H	1H (ACTCTTTTCACACCAATAAC)(d) I(-) (MDATYTCTAKRTCTTGACT)(r)	567	Porcar *et al.*, 2014
cry1I	V(+)(ATGAAACTAAAGAATCCAGA)(d) V(-) (AGGATCCTTGTGTTGAGA)(r)	1137	Masson *et al.*, 1998
cry1J	1J (GCGCTTAATAATATTTCACC)(d) I(-) (MDATYTCTAKRTCTTGACT(r)	1089	Porcar *et al.*, 2014
cry1K	1K (TGATATGATATTTCGTAACC)(d) I(-) (MDATYTCTAKRTCTTGACTA(r)	1132	Porcar *et al.*, 2014
cry2A	II(+)(TAAAGAAAGTGGGGAGTCTT)(d) II(-) (AACTCCATCGTTATTTGT(r)	1556	Masson *et al.*, 1998
cry3A	CGTTATCGCAGAGAGATGACATTAAC(d) TGGTGCCCCGTCTAAACTGAGTGT(r)	951	Ben-Dov *et al.*, 1997
cry4A	TCAAAGATCATTTCAAAATTACATG(d) CGGCTTGATCTATGTCATAATCTGT(r)	459	Ibarra *et al.*, 2003
cry5	(TAAGCAAAGCGCGTAACCTC)(d) (GCTCCCCTCGATGTCAATG)(r)	322	Ejiofor and Johnson 2002
cry9	GTTGATACCCGAGGCACA(d) CCGCTTCCAATAACATCTTTT(r)	571	Bravo *et al.*, 1998
cry11	TTAGAAGATACGCCAGATCAAGC(d) CATTTGTACTTGAAGTTGTAATCCC(r)	305	Bravo *et al.*, 1998
cry12	(CTCCCCCAACATTCCATCC)(d) (AATTACTTACACGTGCCATACCT)(r)	363	Ejiofor and Johnson 2002
cry13A	(CTTTGATTATTTAGGTTTAGTTCAA)(d) (TTGTAGTACAGGCTTGTGATT)(r)	313	Bravo *et al.*, 1998
cry14	(ATAATGCGCGACCTACTGTTGT)(d) (TGCCGTTATCGCCGTTAT)(r)	456	Ejiofor and Johnson 2002
cry32	TGGTCGGGAGAGAATGGATGGA(d) ATGTTTGCGACACCATTTTC(r)	676	Ibarra *et al.*, 2003

[a] d and r, direct and reverse primers, respectively.

by Harwood and Cutting (1990) and Birnboim and Doly (1979), respectively. PCR amplification was carried out in a 25 mL reaction volume: 1 mL of DNA, 1x buffer deoxynucleoside triphosphate (0.2 mM each), oligonucleotide primer (0.2 mM each), and Taq polymerase (5U). The PCR cycling conditions were 5 min at 94 °C, 30 cycles at 94 °C for 1 min, annealing for 45 sec, and 72 °C for 2 min. An aliquot of the reaction mixture (5 µL) was analyzed by agarose gel (1%) electrophoresis.

2.6 Purification of crystal protein and electrophoresis

B. thuringiensis isolates were incubated in Luria–Bertani (LB) medium (1% tryptone, 0.5 % yeast extract and 1% NaCl; pH 7.0) at 30 °C with shaking at 250 rev/min for 72 h. Purified crystal protein was prepared by centrifugation in a discontinuous sucrose gradient according to the method previously described by Debro *et al.* (1986). Purified crystal proteins were analyzed for protein composition by electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide (SDS–PAGE) gels with an acrylamide/N, N-methylene bis-acrylamide ratio of 10:1 (Laemmli, 1970). The gels were stained in a solution containing 50% (v/v), ethanol, 10% (v/v) acetic acid, and 0.1% (w/v) Coomassie Brilliant Blue R250 for 40 min and de-stained with a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol. The molecular mass of the proteins was determined by comparison with a prestained SDS-PAGE standard broad-range protein marker (Bio-Rad).

3. Results and Discussion

3.1 Morphology of cell, spore and crystal protein

Based on phase-contrast microscopy observation, the released crystal proteins could be distinguished from the spores since the crystal proteins were a bipyramidal shape, whereas the spores were an elliptical shape. The electron micrograph of crystal protein showed freely released crystal proteins which were bipyramidal in shape and in different sizes. A large crystal protein was approximately 1.25 µm in length and 0.62 µm in diameter while a small crystal protein was approximately 1 µm in length and 0.5 µm in diameter (Figure 1). Cell morphology, spore position, and shape of the strain were similar to those previously described in *B. thuringiensis* strains (Sneath, 1986). The bipyramidal-shaped crystals exhibited great variation in size within and among the *B. thuringiensis* isolates, but the cuboidal- or spherical-shaped crystals exhibited small variation in size (Attathom *et al.*, 1995). Most of the *B. thuringiensis* isolates from Thailand produced bipyramidal crystal protein (Attathom *et al.*, 1995; Chanpaisaeng *et al.*, 1996; Thaphan *et al.*, 2008). Likewise in Syria, the most common crystal shapes of the *B. thuringiensis* strains in all environments were bipyramidal and cuboidal (42.9%) (Meihiar *et al.*, 2012). The bipyramidal-and cuboidal-shaped crystals were predominant in the *B. thuringiensis* isolates which were obtained from soil and dead insects in Columbia (López-Pazos *et al.*, 2009).

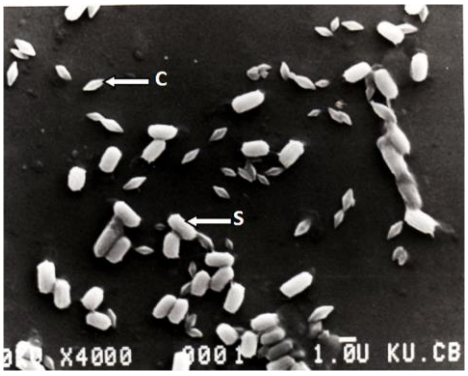

Figure 1. SEM of *Bacillus thuringiensis* serovar *chanpaisis.* Arrows indicate spores (s) and crystal protein (c).

3.2 Carbohydrate fermentation pattern

After testing *B. thuringiensis* serovar *chanpaisis* for carbohydrate fermentation using API50 CHB, this serovar was able to hydrolyze glycerol, arbutin, esculin, salicin, glycogen, starch, N-acetyl-glucosamine, and amygdalin and was able to ferment D-manose, inositol, and others like most *B. thuringiensis* strains (Table 2). With the API kit database and biochemical characteristics, the closest match for this strain was proposed to be *B. thuringiensis* with 99.7% identity. The API pattern of *B. thuringiensis* serovar *chanpaisis* was different from *B. thuringiensis* serovar *kurstaki* (Logan and Berkeley, 1984). However, the API pattern was different among *B. thuringiensis* isolates (Hernandez *et al.*, 1998; Swiecicka *et al.*, 2002). Based on carbohydrate fermentation, *B. thuringiensis* is very similar to *B. cereus* but can be differentiated by the presence of parasporal crystals that may be toxic for insects and other invertebrates. (Gillespie & Hawkey, 2005)

3.3 Larvicidal activity against target insects

The standard techniques (i.e., leaf dipping and diet incorporated techniques) are commonly used for insect bioassay. These methods are the most efficient techniques for toxicity studies since they permit the products to be evenly distributed on the diet. Furthermore, they are less expensive and require less time to perform (Bacci *et al.*, 2009).

In qualitative insect toxicity assays, highly concentrated spore-crystal mixtures of the strain produced 100% mortality in only *Ae. aegypti* and *Cx. quinquefasciatus* but did not show any evidence on pathogenicity against other insects: four Lepidoptera (*S. litura, S. exigua, P. xylostella, and G. mellonella*); two Coleoptera (*T. molitor and T. castaneum*) and two Dipera (*B. dorsalis* and *M. domestica* (Table 3). For the second test, *B. thuringiensis* serovar *chanpaisis* was assayed against second-instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* to determine the median lethal concentration (LC_{50}) values by probit analysis (Finney, 1971). The LC_{50} values for the tested isolate were 1.48×10^4 spores mL^{-1} and 1.00×10^4 spores mL^{-1} for *Ae. aegypti* and *Cx. quinquefasciatus*, respectively (Table 4). *Bacillus thuringiensis* serovar *israelensis* was more toxic than *B. thuringiensis* serovar *chanpaisis* to both mosquito larvae species.

Table 2. Carbohydrate fermentation patterns of *Bacillus thuringiensis* serovar *chanpaisis* compared with the reference strain, *Bacillus thuringiensis* serovar *aizawai*.

Test	serovar *chanpaisis*	serovar aizawai	*B. cereus**	Test	serovar *chanpaisis*	serovar aizawai	*B. cereus*/*
Glycerol	+	+	+/-	Salicin	+	+	+
Erythriol	-	-	-	D-Celiobiose	+	+	+/-
D-Arabinose	-	-	-	D-Maltose	+	+	+
L-Arabinose	-	-	-	D-Lactose (bovine origin)	-	-	+/-
D-Ribose	+	+	+	D-Melibiose	+	-	-
D-Xylose	-	-	-	D-Saccharose (Sucrose)	+	+	+/-
L-Xylose	-	-	-	D-Treharose	+	+	+
D-Adonitol	-	-	-	Inulin	-	-	-
Methyl-βD-Xylopyranoside	-	-	-	D-Melezitose	-	-	+/-
D-Galactose	-	-	-	D-Raffinose	-	-	-
D-Glucose	+	+	+	Amidon (starch)	+	+	+/-
D-Fructose	+	+	+	Glycogen	+	+	+/-
D-Mananose	+	+	+/-	Xylitol	-	-	-
L-Sorbose	-	-	-	Gentiobiose	-	-	+/-
L-Rhamnose	-	-	-	D-Turanose	-	-	+/-
Dulcitol	-	-	-	D-Lyxose	-	-	-
Inositol	-	-	+/-	D-Tagatose	-	-	-
D-Manitol	-	-	-	D-Fucose	-	-	-
D-Sorbitol	-	-	-	L-Fucose	-	-	-
Methyl-αD-Mannopyranoside	-	-	-	D-Arabitol	-	-	-
Methyl-αD-Glucopyranoside	-	-	+/-	L-Arabitol	-	-	-
N-Acetylglucosamine	+	+	+	Potassium gluconate	+	+	+/-
Amygdaline	+	-	+/-	Potassium 2-ketogluconate	-	-	-
Arbutin	+	+	+/-	Potassium 5-ketogluconate	-	-	-
Esculin ferric citrate	+	+	+				

(+); positive reaction, (-); Negative reaction.
* Logan and Berkeley (1984)

Table 3. Larvacidal activity of *Bacillus thuringiensis* serovar *chanpaisis* to an economically important insect pest.

Insect species	Mortality (%)			
	serovar *chanpaisis*	serovar *kurstaki*	serovar *israelensis*	serovar *tenebrionis*
Spodoptera litura	0	100	0	0
Spodoptera exigua	0	100	0	0
Plutella xylostella	0	100	0	0
Galleria mellonella	0	100	0	0
Bactrocera dorsalis	0	0	0	0
Aedes aegypti	100	0	100	0
Culex quinquefasciatus	100	0	100	0
Musca domestica	0	0	0	0
Tenebrio molitor	0	0	0	40
Tribolium castaneum	0	0	0	60

Table 4. Larvacidal activity of *Bacillus thuringiensis* serovar *chanpaisis* against *Aedes aegypti* and *Culex quinquefasciatus*.

B. thuringiensis strains	LC_{50} (spores mL^{-1})*	
	Aedes aegypti	*Culex quinquefasciatus*
B.thuringiensis subsp.*chanpaisis*	1.48×10^4 (7.49×10^3-2.70×10^4)	1.00×10^4 (5.21×10^4-1.78×10^4)
B.thuringiensis subsp.*israelensis*	3.64×10^3 (19-2.33×10^4)	9.56×10^3 (4.13×10^3-1.89×10^4)

*The fiducial limit at the 95% level is given in parentheses.

The LC_{50} values of *B. thuringiensis* serovar *chanpaisis* were similar to previous reports using the same methodology in this study. De Barjac and Coz (1979) reported LC_{50} values of *B. thuringiensis* serovar *israelensis* that fell within the range of 4×10^3 to 4×10^4 viable spores/mL for controlling *Ae. aegypti, Ae. caspius, Ae. albopictus, Ae. polynesiensis, Anopheles stephensi,* and *An. gambiae*. Chen *et al.* (1984) found that the LC_{50} values for *Cx. pipiens pallens, An. Sinensis,* and *Ae. albopictus* species were 0.55×10^4, 2.05×10^4, and 6.37×10^4 spores/mL, respectively. The LC_{50} value of *B. thuringiensis* serovar *chanpaisis* obtained from this study was similar to the previous report described above. This indicated that the potential of this isolate can be used as an alternate to *B. thuringiensis* subsp. *israelensis* for mosquito control.

3.4 *Cry* gene analysis

PCR analysis of *B. thuringiensis* serovar *chanpaisis* using the general and specific primers designed for *cry* and *vip* genes showed that both chromosome DNA and plasmid DNA reacted positively only to *cry*32 by producing a fragment of 676 bp. The PCR product of *cry*32 gene were cloned, sequenced, and subjected to BLAST search at NCBI. Sequence comparison with other *cry*32 genes in the database at NCBI with Blast-N program showed these sequences had high homology with *cry*32 genes (M97880.1) comprising 97% of chromosomal DNA and 95% of plasmid DNA. Parts of the *cry*32 gene sequence were deposited in Genbank under accession no. KX685157. Based on PCR analysis, only the *cry* 32 gene was detected in serovar *chanpaisis* but not *cry*1Ab, *cry*1Ac, *cry*1C, *cry*1D, *cry*1E, *cry*1I, and *cry*2A which are frequently reported in *B. thuringiensis* isolates in Thailand (Poojitkanont *et al.*, 2008, Thaphan *et al.*, 2011). These results presented evidence of the larvicidal activity since serovar *chanpaisis* was found to be toxic only to *Ae. aegypti* and *Cx. quinquefasciatus.* Similarly, *cry* 32B *cry*32C, and *cry*32D gene-encoded proteins were also stated to be toxic to insects in the order Diptera, especially mosquito larvae (van Frankenhuyzen, 2009). Nevertheless, the results from protein composition of crystal proteins showed an unknown polypeptide. Hence, further study needed to be pursued.

The novel *cry*32Aa gene was initially found and characterized in *B. thuringiensis* subsp. *yunnanensis* (Bala subramanian *et al.*, 2002) and later detected from *B. thuringiensis* isolated from different orchards of Hebei Province, China in 2010 by Lei *et al.*, (2010). In 2015, Yu *et al.*,(2015) found the *cry*32 gene from *B. thuringiensis* isolated from forestland soil in Chengdu, China. Interestingly, the *cry*32 gene was obviously found in Asia but not in Europe and America. Ibarra *et al.*, (2003) searched for the genes *cry*10, *cry*24, *cry*27, *cry*29, *cry*32, and *cry*40 in strains cultured from soil in Latin America, but they found only *cry*10, *cry*17, *cry*27 and *cry*30. Monnerat *et al.*, (2007) found only *cry*1 and *cry*2 from 1400 strains of *B. thuringiensis* but did not detect other *cry* genes such as *cry*4, *cry*32, *cry*39, and *cry*40. Konecka *et al.*, (2014) reported that none of the *B. thuringiensis* strains from Poland carried *cry*1J, *cry*1K, *cry*15, *cry*16, *cry*17, *cry*18, *cry*19, *cry*20, *cry*22, *cry*24, *cry*26, *cry*27, *cry*28, *cry*29, *cry*30, *cry*32, *cry*39 or *cry*40.

When compared with the *cry*1 gene, *cry*32 showed narrowed distribution revealing only the *cry*32 gene in this group (*cry*32Aa1-*cry*32Ya1) (http://www.btnomenclature.info/). It was suggested that the distribution of the *cry* gene was different depending on the geographical zone as reported by Ibanez-Bernal *et al.,* (1996) who discovered that the dipteran-active *cry*11 and *cyt* genes were more frequently found in the tropical rainy regions than in the semiarid regions. This distribution correlated with the distribution of dipteran insects. Similarly, the *cry1E* and *cry1F* genes were found only in the tropical rainy regions where there was an outbreak of *S. littoralis* and *S. exigua* larvae (Chamber *et al.*, 1991; Visser *et al.*, 1990).

3.5 Protein composition of crystals

SDS–PAGE analysis was employed to compare the polypeptide composition of crystal proteins from *B. thuringiensis* serovar *chanpaisis*. Post-sporulation samples of this serovar contained five polypeptides of around 130 kDa, 85 kDa, 64 kDa, 55 kDa, and 20 kDa while purified crystal protein showed the existence of 130 kDa, 85 kDa, 64 kDa, 50 kDa, and 20 kDa (Figure 2). The results revealed that *B. thuringiensis* serovar. *chanpaisis* generated proteins with molecular weights of 20-130 kDa, similar to the reference strains of *B. thuringiensis* serovar. *yunnanensis* with a *cry32Aa* gene producing 139.2 kDa polypeptides. In addition, *B. thuringiensis* serovar. *yunnanensis* was reported to be toxic only to the larvae of *P. xylostella*, but not to mosquito larvae (Balasubramanian *et al*., 2002). Lei *et al*. (2010) reported that *cry*32 gene on *B. thuringiensis* isolated from China produced proteins at around 130 and 60 kDa. According to the review by van Frankenhuyzen (2009), *cry*32 gene on serovar *chanpaisis* was probably *cry*32Ba, *cry*32Ca or *cry*32Da due to its toxicity to *Ae. aegypti*.

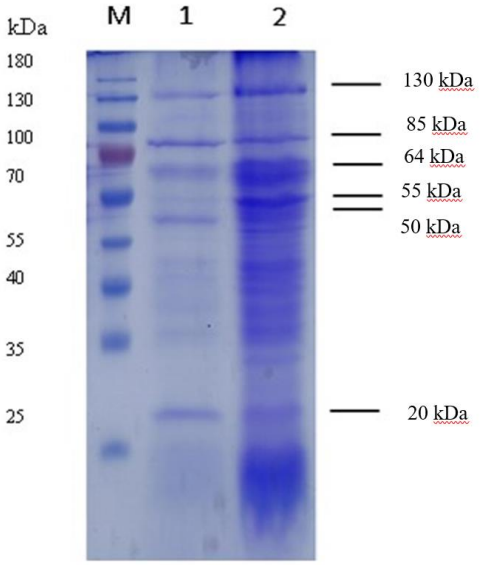

Figure 2. SDS-PAGE analysis of protein profiles of crystal proteins from Bacillus thuringiensis serovar chanpaisis. M: molecular weight markers; Lane 1: A crystal proteins (after purification); Lane 2: spore and crystal protein (before purification).

B. thuringiensis serovar *chanpaisis* showed toxicity to mosquitoes larvae but the protein profile was dissimilar to those of two Diptera-specific referenced strains (*B. thuringiensis* serovar *israelensis* and *B. thuringiensis* serovar *kyushuensis*) and also different from the mosquitocidal strains of *B. thuringiensis* serovar *jegathesan* (77, 72, 68, 55, 38, 35, 27, and 23 kDa) (Kawalek *et al*., 1990), *B. thuringiensis* serovar *medellin* (95, 67, and 30 kDa) (Ragni *et al*., 1996), and *B. thuringiensis* serovar *darmstadiens* 72E10-2 (125, 50, 47, and 28 kDa) (Drobniewski and Ellar,1989). This suggested that *B. thuringiensis* serovar *chanpaisis* was different from that previously characterized in the mosquitocidal strain.

Usually, mosquito-specific *B. thuringiensis* strains produce two families of δ-endotoxin, Cry and Cyt proteins. The SDS-PAGE profile of strain *B. thuringiensis* serovar *chanpaisis* contained a similar-sized protein of about 20 kDa which could be recognized as Cyt. The toxicity of *B. thuringiensis* serovar *chanpaisis* to *Ae. aegypti* and *Cx. quinquefasciatus* was probably derived from either the Cry 32 or Cyt protein since van Frankenhuyzen (2009) reported that Cry32Ba, Cry32Ca, and Cry32Da were toxic to *Ae. aegypti*. Several *B. thuringiensis* strains synthesize smaller (20-28 kDa) Cyt endotoxins (Koni and Ellar, 1994). Cyt endotoxins have been classified into three primary ranks (Cyt1, Cyt2, and Cyt3) and most were reported to be against some mosquitoes and black flies (Soberon *et al*., 2013). It was established that the Cyt protein dramatically enhanced the mosquitocidal activity of co-existing Cry proteins (Delécluse *et al*., 2001).

4. Conclusions

The colony of *B. thuringiensis* subsp. *chanpaisis* was flat, dry, and white with uneven borders. This strain utilized glycerol, arbutin, esculin, salicin, glycogen, starch, N-acetyl-glucosamine, amygdalin, D-manose, inositol, and others. Like most *B. thuringiensis* strains it produces bipyramidal crystal protein with different sizes and consists of major proteins that are about 130 kDa, 85 kDa, 64 kDa, 50 kDa, and 20 kDa. In *Bacillus thuringiensis* subsp. *chanpaisis*, only the *cry*32 gene was detected on both chromosomal DNA and plasmid DNA and showed only toxicity to *Ae. aegypti* and *Cx. quinquefasciatus* with a LC_{50} value of 0.48×0^4 spores mL^{-1} and 1.00×10^4 spores mL^{-1}, respectively.

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