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A preliminary study of the bioactivity of vegetative proteins extracted from Malaysian *Bacillus thuringiensis* isolates

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Abstract. Vegetative proteins from Malaysian strains of *Bacillus thuringiensis israelensis* strains (Bt 11, Bt 12, Bt 15, Bt 16, Bt 17, Bt 21 and Bt 22) and *Bacillus sphaericus* H-25 strains (Bs 1 and Bs 2) were screened for haemolytic, cytotoxic and larvicidal activity. SDS-PAGE profiles of the *Bacillus thuringiensis* strains studied consistently showed major bands of 33-37 kDa and 47 kDa. Bt 16 also showed two bands of 66 kDa and 45 kDa similar to the previously reported binary vegetative protein, Vip1Ac (66 kDa) and Vip 2Ac (45 kDa). Both the *Bacillus sphaericus* strains showed a 35 kDa band that was similiar to a previously reported vegetative protein, the Mtx2 protein. Bs 2 also contains a 37 kDa band, similar to another vegetative protein, the Mtx 3 protein. With the exception of Bt 17 and Bt 21, vegetative proteins from all *Bacillus thuringiensis* and *Bacillus sphaericus* strains were highly haemolytic to human erythrocytes, causing more than 75% haemolysis at the highest concentration of 200 µg/ml. High haemolytic activity was associated with high cytotoxic activity with most of the haemolytic strains being indiscriminately cytotoxic to both CEM-SS (human T lymphoblastoid) and HeLa (human uterus cervical cancer) cell lines. Interestingly, the less haemolytic vegetative proteins from Bt 17 and Bt 21 demonstrated cytotoxic activity comparable to that of the highly haemolytic vegetative proteins. Bt 21 displayed toxicity towards both cell lines while Bt 17 was more toxic towards CEM-SS cells. Bioassay against *Aedes aegypti* and *Culex quinquefasciatus* larvae revealed that vegetative proteins from the *Bacillus thuringiensis* strains had activity against both species of larvae but vegetative proteins from *Bacillus sphaericus* were weakly larvicidal towards *Cx. quinquefasciatus* only.

INTRODUCTION

Bacillus thuringiensis (*Bt*) and *Bacillus sphaericus* (*Bs*) are entomopathogenic, Gram positive soil bacteria with two distinct growth phases – the vegetative and sporulative growth phase. During sporulation, both bacteria produce proteinaceous parasporal crystals, most of which have insecticidal activity against a wide range of hosts (Schnepf *et al.* 1998; Feitelson *et al.* 1992; de Maagd *et al*, 2003). Commercialised spore-crystal formulations of *Bt* and *Bs* are now used as bio-insecticides. However the usage of these products for over 70 years has led to insect resistance (Neppl, 2000). Hence the search for novel insecticidal proteins from both *Bt* and *Bs* has lead to the discovery of vegetative insecticidal proteins (Vip) of *Bt* (Estruch *et al.* 1996) and the mosquitocidal (Mtx) toxins of Bs (Thanabalu *et al.* 1991; Thanabalu & Porter, 1996; Liu *et al.* 1996). Both of these toxins are expressed during the vegetative phase of growth.

In 1986, a nationwide search was conducted in Malaysia to find a microbial agent to control the mosquito population, in hopes of reducing the incidence of mosquito borne diseases endemic to this region. This resulted in the isolation of *Bt* and *Bs* strains that produced crystals with a range of

mosquitocidal activity (Lee & Seleena, 1990). In accordance with the discovery of *Bt* Cyt toxins (a cytolytic group of proteins) (Guerchicoff *et al.*, 2001) and parasporins (anti-cancer proteins) (Mizuki *et al.* 1999; Kim *et al.* 2000; Mizuki *et al.* 2000), a study was initiated to investigate the haemolytic and cytotoxic activity profile of the parasporal crystals of 19 *Bt* and 2 *Bs* strains isolated from Malaysia. The results obtained thus far indicated that strains with selective haemolytic and cytotoxic activity exist among the Malaysian strains of *Bt* and *Bs* (Ting, 2003 ; Yeow, 2006).

This study focuses on isolating and determining the bioactivity of vegetative proteins from uncharacterised Malaysian *Bt* (7 strains) and *Bs* (2 strains). The proteins were subjected to; SDS-PAGE electrophoresis for estimation of molecular mass, red blood cell lysis assay (human and rabbit) to determine haemolytic activity, MTT assay to determine cytotoxic effect against CEM-SS (human T lymphoblastoid) and HeLa (human uterus cervical cancer) cell lines and bioassay to determine larvicidal activity against *Aedes aegypti* and *Culex quinquefasciatus*.

The results of this study indicate that the bioactivity of vegetative *Bt* proteins are as diverse as the parasporal *Bt* proteins.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *Bt* and *Bs* strains were grown in NB [Peptone (5g), meat extract (3g) (MERCK), $MnCl_2$ (10mg), $CaCl_2$ (80mg), $MgCl_2$ (70mg), dH_20 (1 L), pH 7] at 30°C with constant shaking at 150 rpm for 20 hours. Growth curve studies were performed on all strains. The *Bt* and *Bs* strains were grown at similar conditions. Aliquots of the culture were taken at various time intervals and the absorbance was measured at 595nm to determine growth.

Purification of Vegetative Proteins

The vegetative proteins were harvested using the method and principles described by Estruch *et al.* (1996) with some modifications. This method included precipitating the vegetative proteins from the NB supernatant using 70% ammonium sulphate. The precipitated proteins were re-suspended in an appropriate volume of 20mM Tris/HCl (pH7.5) buffer and dialyzed overnight at 4°C. Protease Inhibitor (Sigma) was added into the protein-buffer suspension to inhibit any bacterial proteases present. A centrifugal filter device (Amicon®Ultra-4, 10,000NMWL) was used to further de-salt the protein suspension and purify the proteins by removing any low molecular weight substrate present as well as to concentrate the vegetative proteins. All bacterial work was done using sterile techniques under laminar flow and all buffer solutions were autoclaved before addition of the vegetative proteins.

Determination of Protein Concentration and SDS-PAGE analysis of Molecular Mass

The Bradford protein assay (Bradford, 1976) was used to determine the concentration of the proteins. The vegetative proteins were then subjected to SDS-PAGE analysis according to the modified method of Laemmli (1970) as described by Thomas & Ellar (1983). All gels consisted of a 4% (w/v) polyacrylamide stacking gel and 10% (w/v) polyacrylamide resolving gel.

Haemolytic Assay

Haemolytic activity of the various *Bt* and *Bs* strains was assayed by the quantity of haemoglobin released from the cytoplasm of erythrocytes using a modified method of Yu *et al.* (1991). Various concentrations of vegetative proteins were incubated with 2% (v/v) human or rabbit erythrocytes for 1 hour at 27°C. The absorbance of the resultant supernatant at 550nm was measured and compared to the absorbance of 100% lysis (achieved by sonication) to determine the percentage of haemolysis induced by the vegetative proteins. Erythrocytes incubated in protein buffers and protease inhibitors, excluding the vegetative proteins were used as negative controls. All experiments were performed in triplicates.

Cytotoxic Assay

Various concentrations of vegetative proteins were incubated with 1 X 10^6 cells/ml of CEM-SS or HeLa cells in a 96-well micro plate. The plate was incubated for 72 hours at 37°C, 70% humidity and 5% CO_2. After the 72 hour period the MTT assay was performed as described by Shier (1991). The viability of cells treated with vegetative proteins was compared with the negative control to determine the percentage of cell viability. Cells incubated with protein buffers and protease inhibitors, excluding the vegetative proteins were used as the negative controls. All experiments were performed in triplicates.

Bioassay

Late third/early fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* were used in this study. The *Ae. aegypti* strain originated in Selangor, while *Cx. quinquefasciatus* came from Penang Island. Up to now in the environment, no *Ae. aegypti* or *Cx. quinquefasciatus* larvae are known to be resistant to *Bt* and *Bs* as such, these 2 mosquito larvae species are assumed to be not resistant nor tolerant to *Bt* or *Bs* toxins.

Five larvae were incubated with various concentrations of vegetative proteins for 48 hours at room temperature. At the end of 48 hours, the mortality of the larvae was determined by counting the number of live larvae. The data were then pooled and analysed using a computer programmed with probit analysis and the LC_{50} values were deduced. Larvae incubated with protein buffers and protease inhibitors, excluding the vegetative proteins were used as the negative controls. Five replicates were conducted for each bioassay.

RESULTS

Vegetative Proteins

The *Bt* and *Bs* strains were grown in NB for 20 hours before harvesting and purifying the vegetative proteins. This period of 20 hours was deemed suitable as a bacterial growth curve study (data not shown) indicated that vegetative (exponential) growth occurred between the 12th and 24th hour for Bt strains and between the 8th hour and 24th hour for Bs strains. The study done by Estruch *et al.* (1996) also proved that vegetative proteins were expressed in the exponential phase.

SDS-PAGE Analysis

Fig 1A shows the SDS-PAGE profiles of the vegetative proteins from Bs 1, Bs 2, Bt 11 and Bt 12 while Fig 1B shows the SDS-PAGE profiles of the vegetative proteins from Bt 15, Bt16, Bt17, Bt 21 and Bt 22. The major polypeptide bands seen amongst the Bt strains are bands of molecular mass 33 – 37 kDa and 47 kDa. In addition, a 25 kDa band was observed in strains Bt 11, Bt 16, Bt 21and Bt 22. With regards to Bs, both strains studied show markedly different SDS-PAGE profiles. Bs 1 shows a distinct band of 140 kDa and two other major bands of 33 – 37 kDa and 50 kDa. Bs 2 shows several major bands of 30 kDa, 32 kDa, 37 kDa, 47 kDa, 50 kDa and 75 kDa.

Haemolytic Assay

The percentage of haemolysis was calculated according to the following formula:

$$\frac{\textit{Absorbance measured}}{\textit{Absorbance of total haemolysis}} \textit{ X 100\% = Percentage of haemolysis}$$

Fig 2 shows the haemolytic activity of Bt 11, Bt 12, Bt 15, Bt 16, Bt 17, Bt 21, Bt 22, Bs 1 and Bs 2 on human erythrocytes while Fig 3 shows the haemolytic activity of the same strains on rabbit erythrocytes.

With regards to the *Bt* vegetative proteins, high haemolytic activity against human erythrocytes was observed in most strains as exemplified by Bt 11, Bt 12, Bt 15, Bt 16, and Bt 22. Among these strains, only Bt 11, Bt 16 and Bt 22 showed notable selectivity between human and rabbit erythrocytes, having lower haemolytic activity against rabbit erythrocytes. Bt 17 and Bt 21 demonstrated the lowest haemolytic activity against human erythrocytes with Bt 21 being less haemolytic that Bt 17. Again, both strains were less haemolytic towards rabbit erythrocytes.

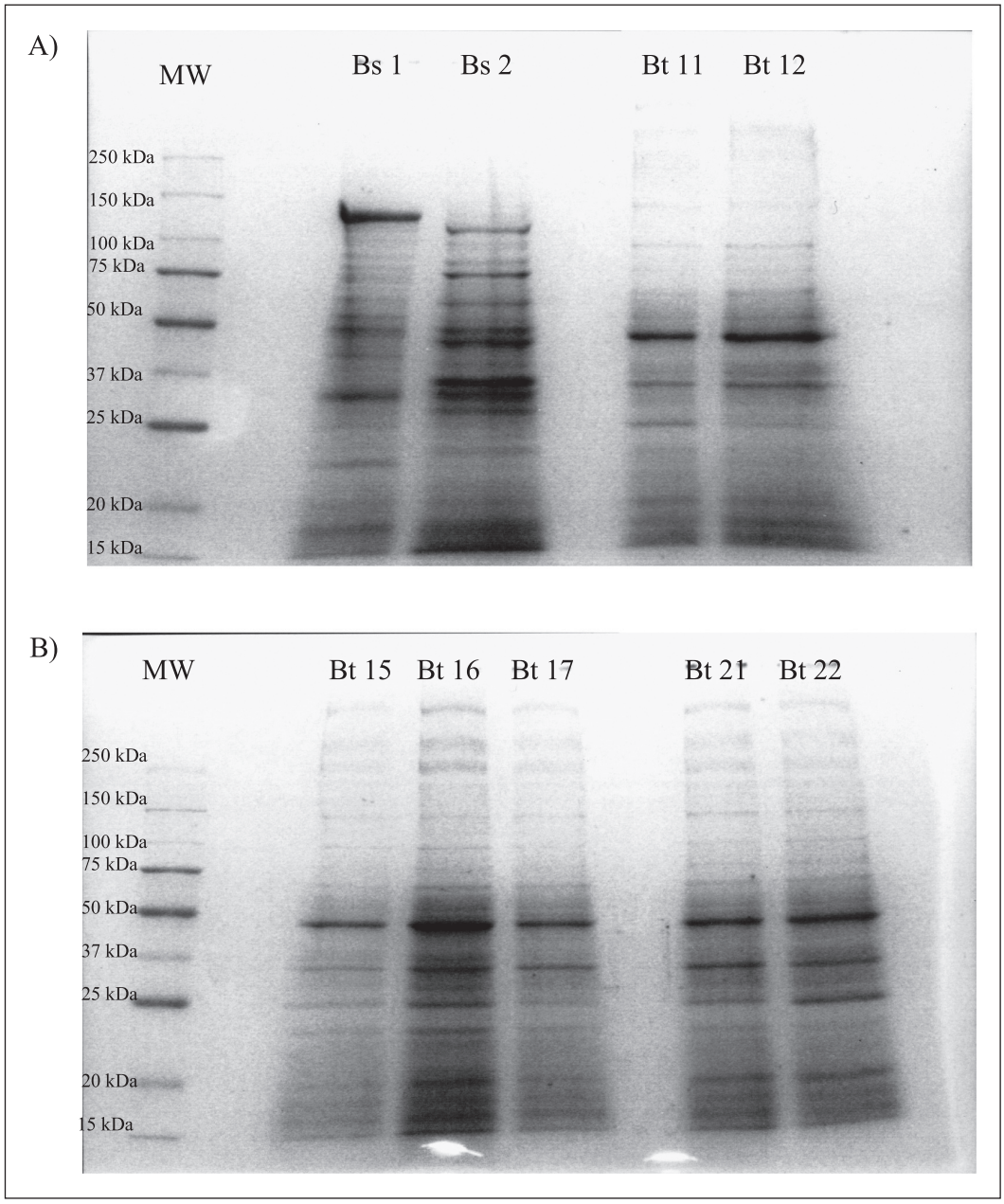

Figure 1. SDS-PAGE analysis of Bs and Bt vegetative proteins.
Coomassie blue stained SDS-PAGE gel (10% polyacrylamide gel) of various Bs (Bs1 and 2) and Bt strains (Bt11, Bt12, Bt15, Bt16, Bt17, Bt21 and Bt22)
MW: Protein marker

The Bs vegetative proteins showed indiscriminate, high haemolytic activity towards human and rabbit erythrocytes.

Cytotoxic Assay

The percentage of cell viability was calculated according to the following formula:

$$\frac{\textit{Absorbance measured}}{\textit{Absorbance of negative control}} \textit{ X 100\% = Percentage of cell viability}$$

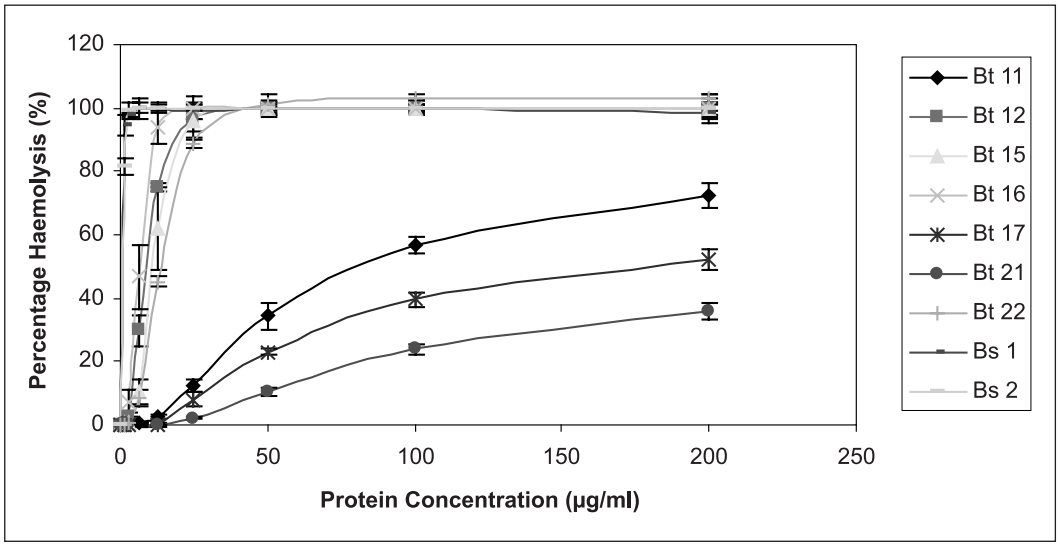

Figure 2. Haemolytic activity of Bt and Bs strains on human erythrocytes.
Vegetative proteins were incubated in 2% human erythrocytes for 1 hour at 27°C. Reaction was stopped by centrifugation at 3000 x g for 3 minutes at 4°C and the supernatant collected and read at 550 nm. Each data point is an average of triplicates, reflecting haemolysis of human erythrocytes.

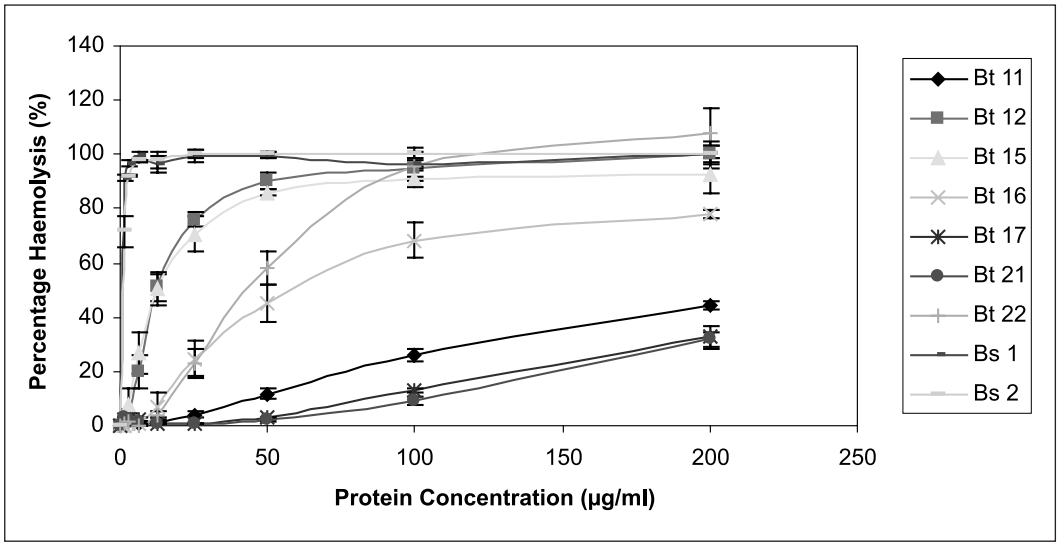

Figure 3. Haemolytic activity of Bt and Bs strains on rabbit erythrocytes.
Vegetative proteins were incubated in 2% rabbit erythrocytes for 1 hour at 27°C. Reaction was stopped by centrifugation at 3000 x g for 3 minutes at 4°C and the supernatant collected and read at 550 nm. Each data point is an average of triplicates, reflecting haemolysis of rabbit erythrocytes.

Fig 4 shows the cytotoxic activity of Bt 11, Bt 12, Bt 15, Bt 16, Bt 17, Bt 21, Bt 22, Bs 1 and Bs 2 on CEM-SS cell lines while Fig 5 shows the cytotoxic activity of the same strains on HeLa cell lines.

All of the haemolytic Bt strains; Bt 11, Bt12, Bt 15, Bt 16 and Bt 22 showed cytotoxic activity against CEM-SS and HeLa cells lines. At the highest concentration of 8 µg/ml, all haemolytic Bt strains caused at least 70% cell death to both cell lines. The Bt strains with lower haemolytic activity also showed cytotoxic activity against cancer cell lines. Vegetative proteins from Bt 17 were toxic to

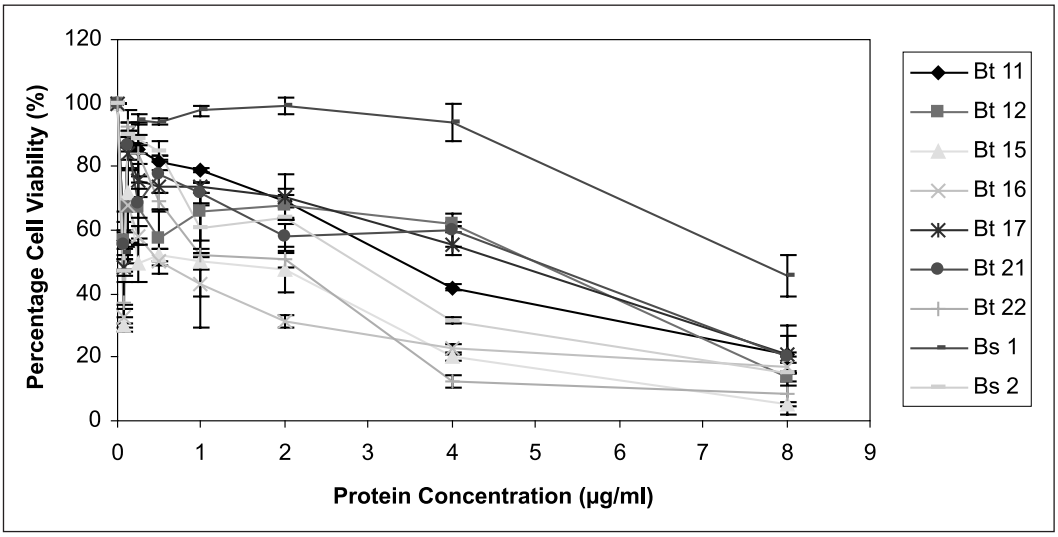

Figure 4. Cytotoxic activity of Bt and Bs strains CEM-SS cell lines.
CEM-SS cells were treated with various concentrations of vegetative proteins for 72 hours. Each data point is an average of triplicate absorbance readings at 550nm with a reference wavelength of 620 nm; determined using the MTT assay.

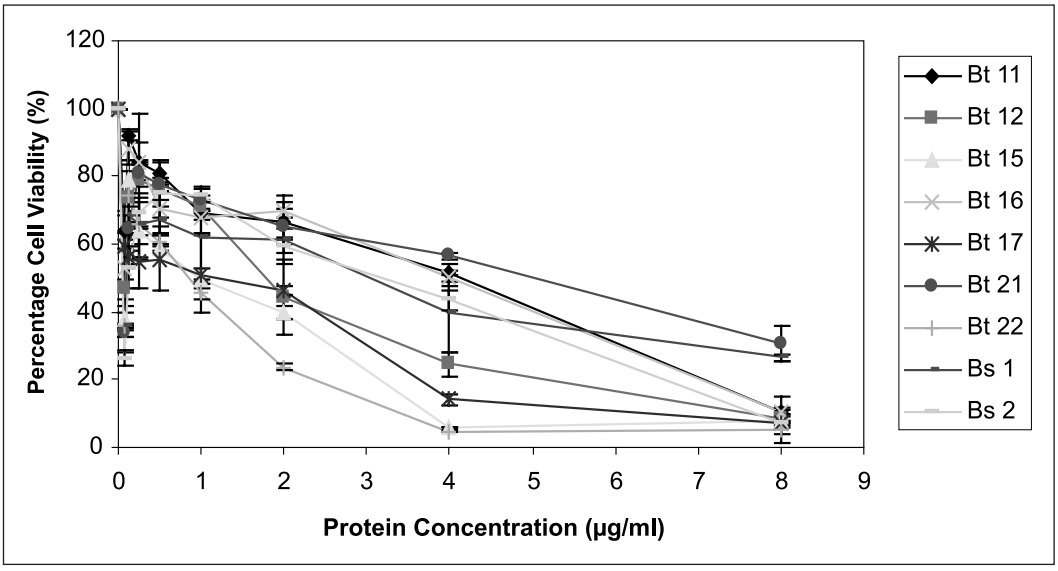

Figure 5. Cytotoxic activity of Bt and Bs strains on HeLa cell lines.
HeLa cells were treated with various concentrations of vegetative proteins for 72 hours. Each data point is an average of triplicate absorbance readings at 550nm with a reference wavelength of 620 nm; determined using the MTT assay.

both cell lines but had more potent activity against HeLa cells. Interestingly, vegetative proteins from Bt 21, the least haemolytic strain, were indiscriminately toxic towards CEM-SS and HeLa cells.

Both Bs strains were active against HeLa cell lines. However, the highly haemolytic vegetative proteins from Bs 1 showed the lowest degree of cytotoxicity (among all strains tested) towards CEM-SS cell lines. Bs

2 was equally active against CEM-SS and HeLa cell lines.

Bioassay

Table 1 shows the LC_{50} values for the vegetative proteins that confer larvicidal activity against *Ae. aegypti* and *C. quinquefasciatus*, as computed using probit analysis.

All Bt strains had notable activity against *Ae. aegypti* and *Cx. quinquefasciatus*. Vegetative proteins from Bt 15 had the highest activity against *Ae. aegypti* with a LC_{50} of 4.91 µg/ml. Vegetative proteins from Bt 22 were highly potent against *Cx. quinquefasciatus* larvae with a low LC_{50} of 2.09 µg/ml, but these proteins had the weakest activity against *Ae. aegypti* (when compared to all other strains tested). Interestingly, Bt 21, the strain with the least haemolytic activity showed potent larvicidal activity against both *Ae. aegypti* and *Cx. quinquefasciatus* larvae with a LC_{50} of 8.01 µg/ml and 3.45 µg/ml respectively.

Vegetative proteins from both Bs strains tested were selectively larvicidal against *Cx. quinquefasciatus* larvae but not against *Ae. aegypti* larvae. Despite that, the proteins were not very potent against *Cx. quinquefasciatus* when compared to the vegetative proteins of Bt.

Table 1. LC_{50} values for various Bs and Bt vegetative proteins

Strains	LC_{50} of Vegetative Proteins	
	Ae. aegypti **(95% Confidence Interval)**	***Cx. quinquefasciatus*** **(95% Confidence Interval)**
Bs 1	non larvicidal at 20µg/ml	20.87 µg/ml 13.02<LC_{50} <52.53
Bs 2	non larvicidal at 20µg/ml	40.37 µg/ml 20.43<LC_{50} <365.57
Bt 11	16.40 µg/ml 10.71<LC_{50} <34.81	38.04 µg/ml 17.39<LC_{50} <319.46
Bt 12	13.71 µg/ml 11.17< LC_{50} <17.87	17.37 µg/ml 11.26< LC_{50} <37.94
Bt 15	4.91µg/ml 3.83< LC_{50} <6.37	9.61µg/ml 6.36< LC_{50} <17.37
Bt 16	14.79 µg/ml 11.66< LC_{50} <21.06	14.90 µg/ml 9.65< LC_{50} <31.68
Bt 17	15.56 µg/ml 12.09< LC_{50} <23.20	17.28 µg/ml 8.15< LC_{50} <76.19
Bt 21	8.01 µg/ml 6.21< LC_{50} <10.70	3.45 µg/ml 2.33< LC_{50} <5.44
Bt 22	22.05 µg/ml 14.27< LC_{50} <56.10	2.09 µg/ml 1.36< LC_{50} <3.28

Vegetative proteins were tested on third/fourth instar larvae of *Ae. aegypti* and *Cx. quinquefasciatus* mosquito for 48 hours. Readings were taken based on the mortality of third/fourth instar larvae after 48 hours. Probit analysis was used to deduce LC_{50}

DISCUSSION

To date, 54 Vips with distinct amino acid sequences have been discovered (Crickmore *et al.* 2005). These Vips have been broadly grouped into two classes; the Vip1/Vip2 binary toxins and the Vip3 toxins (Yu *et al.* 1997; Crickmore *et al.* 2005; Leuber *et al.* 2006). Both groups of toxins have different modes of action and insect specificities.

SDS-PAGE analysis of vegetative proteins from the Bt strains showed many polypeptide bands with a wide range of molecular masses. A recent study conducted by Leuber *et al.* (2006) on *Bacillus thuringiensis* HD201 reported the presence of a binary Vip toxin: Vip1Ac with the molecular mass of 66 kDa and Vip2Ac with the molecular mass of 45kDa. This is interesting as Bt 16 shows the presence of a 65 kDa band and a 47 kDa band which may correspond to Vip1Ac and Vip2Ac respectively. Further N-terminal sequencing and immunogenic studies will have to be done to determine if those two bands in Bt 16 are indeed Vip1Ac and Vip2Ac.

Three distinct vegetative proteins have been discovered in Bs strains thus far; Mtx1 (100 kDa), Mtx2 (31.8 kDa) and Mtx3 (35.8 kDa) (Thanabalu *et al.* 1991; Thanabalu & Porter, 1996; Liu *et al.* 1996). Interestingly, both Bs 1 and Bs 2 have a 32 kDa band and this may very well correspond to the Mtx2 toxin. Bs 2 also shows a 37 kDa band and this may be the Mtx 3 protein. Again more definitive studies need to be done to determine if these proteins are indeed Mtx 2 and Mtx 3.

It is noted based on previous studies on parasporal inclusions of same Bt strains (Yeow, 2006) that the vegetative and parasporal protein profiles show similarities (data not shown). For instance, a 24 kDa band was observed in the parasporal protein profile of all Bt strains studied except Bt 22. These bands are comparable to the 25 kDa band seen in the vegetative protein profile of selected Bt strains in this study. The overlapping protein profiles indicate that either the machinery responsible for synthesizing the parasporal proteins may have started prior to sporulation (de Maagd *et al.* 2003) or that the vegetative proteins remain at high concentrations even during the sporulation stage as shown by Estruch *et al.* (1996). Taking into account these findings, the wide spectrum haemolytic and cytotoxic activity of these strains and the fact that the Bt species used in this study are subspecies of Bt subspecies israelensis (Bti), suggests that this 25kDa protein may be the Cyt protein (also 25 kDa) that is produced during the sporulation stage in most Bti species. The Cyt protein has broad range cytolytic activity against many vertebrate cells including human erythrocytes (Guerchicoff *et al.* 2001; de Maagd *et al.* 2003).

Amongst the Bt strains, only Bt 11, Bt 16, Bt 17, Bt 21 and Bt 22 showed notable selectivity, having lower haemolytic activity against rabbit erythrocytes. This selectivity suggests that the haemolytic activity of these vegetative proteins maybe partially due to a receptor binding mechanism. This is consistent with the study done by Nadarajah *et al.* (2006), whereby parasporal proteins from Malaysian Bt strains were found to have novel lectin activity. Haemolytic inhibition studies done on the parasporal proteins showed that the parasporal proteins recognise glycoprotein receptors present on the plasma membrane of erythrocytes and this initiates haemolytic activity. It is possible that the Bt vegetative proteins exert their haemolytic effect via a similar mechanism. The glycoprotein chains present on human and rabbit erythrocytes are different and this explains the selectivity of the Bt vegetative proteins.

The non-selective haemolytic activity of the remaining Bt (Bt 12 and Bt 15) and Bs (Bs 1 and Bs 2) strains suggests that these proteins may exert their effect through a non-specific cytolytic mechanism, perhaps by disrupting the membrane lipid arrangement (Butko, 2003).

This study focused on obtaining the cytotoxic activity profile of the vegetative proteins with lower haemolytic activity (human erythrocytes) to assess the potential of these proteins as therapeutic agents. Interestingly the vegetative proteins of Bt 21 demonstrated toxic activity on CEM-SS and

HeLa cell lines that is comparable to the haemolytic vegetative proteins. The non-selectivity of Bt 21 suggests that the protein exerts its cytotoxic activity via a target that is common to both CEM-SS and HeLa cells. The vegetative proteins of Bt 17 , unlike Bt 21, is selective for HeLa cells and has the highest cytotoxicity for HeLa cells when compared with the vegetative proteins of the remaining Bt and Bs strains.

Most of the haemolytic vegetative proteins from Bt did not show selectivity towards any cancer cell line. However, Bs 1 demonstrated unusually low cytotoxic activity towards CEM-SS cell lines. This shows that despite being indiscriminately haemolytic, Bs 1 is still selective in its cytotoxic activity. When considering the potential of these vegetative proteins as therapeutic agents, it is important to note that at the highest concentration tested in the cytotoxic assay (8 µg/ml) all vegetative proteins except Bs 1 caused more than 30% cell death in both cell lines. At this low concentration, the haemolytic activity of the vegetative proteins is negligible. Hence the haemolytic activity of the proteins should not impede future research to unearth the full potential of these proteins as therapeutic agents.

Despite having larvicidal activity against both *A. aegypti* and *C. quinquefasciatus* larvae, the vegetative proteins of Bt were less larvicidal than the parasporal proteins of the same strains as reported by the Institute of Medical Research (IMR), Malaysia (Lee, 1996). The lower potency of vegetative proteins when compared to parasporal proteins of the same strains is not surprising as vegetative proteins are probably susceptible to rapid degradation under environmental conditions compared to the parasporal proteins. The Vip proteins have only been reported to be active against coleopteran (Vip1 and Vip2) and lepidopteran (Vip3) larvae (Estruch *et al.* 1996; de Maagd *et al.* 2003; Leuber *et al.* 2006). There are no reports so far on the effect of the Vip proteins on mosquito larvae. This study indicated that the Vip proteins may be active against Dipteran species as well. However, Bt 16 despite showing the possible presence of the binary Vip toxin reported by Leuber *et al.* (2006), is not very potent as a larvicidal agent when compared to some of the other Bt strains studied (e.g. Bt 15 and Bt 21). This suggests that the Coleopteran specific binary Vip toxins may not be effective against Dipterans. Future studies can include investigations of the activity of the binary Vip toxins against different types of mosquito species.

The LC_{50} values for *A. aegypti* and *Cx. quinquefasciatus* for most strains of Bt (except Bt 22) have overlapping 95% confidence intervals indicating that there is no significant difference between the toxicity of each strain to *Ae. aegypti* and *Cx. quinquefasciatus* larvae. This in consistent with the reported larvical activity spectrum of the parasporal proteins of Bt; highly active against *Aedes* and *Culex* species and less active against *Anopheles* species (Schnepf *et al.* 1998).

The binary toxin of Bs has been reported to be more toxic towards *Culex* and *Anopheles* species and less toxic towards *Aedes* species (Baumann *et al.* 1991). The data obtained in this study show the same pattern as the vegetative proteins from both Bs 1 and Bs 2 have larvicidal acticity against *Cx. quinquefasciatus* but not *Ae. aegypti.* The study done by IMR also shows that the parasporal proteins of Malaysian Bs isolates have low toxicity against *Ae. aegypti* and again, the parasporal proteins are more potent larvicidal agents when compared to the vegetative proteins of the same strain (Lee, 1996).

This study concludes the vegetative proteins of the selected Malaysian Bt and Bs strains show haemolytic, cytotoxic, and larvicidal activity. The vegetative proteins are distinct in their bioactivity, displaying both selective and non-selective haemolytic, cytotoxic and larvicidal activity. There exist potential for these vegetative proteins to be exploited as therapeutic and vector control agents. As this study is a preliminary analysis of vegetative proteins isolated from Malaysian Bt strains, vegetative proteins with potential therapeutic and agricultural applications needs to be purified further to identify the individual active compounds.

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REFERENCES

Baumann, P., Clark, M.A., Baumann, L. & Broadwell, A.H. (1991). *Bacillus sphaericus* as a mosquito pathogen: properties of the organism and its toxins. *Microbiology and Molecular Biology Reviews* **55**(3): 425-436.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**: 248-254.

Butko, P. (2003) Cytolytic Toxin Cyt1A and Its Mechanism of Membrane Damage: Data and Hypotheses. *Applied and Environmental Microbiology*. **69**(5): 2415–2422.

Crickmore, N., Zeigler, D.R., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Bravo, A. & Dean, D.H. (2005). *Bacillus thuringiensis* toxin nomenclature (Homepage on the Internet). Available from: http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N. & Schnepf, E. (2003). Structure, diversity and evolution of protein toxins from spore-forming entomopathogenic bacteria. *Annual Review of Genetics*. **37**: 409-33.

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A. & Koziel, M.G. (1996). Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proceedings of the National Academy of Sciences U S A*. **93** (11): 5389–5394**.**

Feitelson, J.S., Payne, J., Kim, L. (1992). *Bacillus thuringiensis:* Insects and Beyond. *Bio/Technology*. **10**: 271 – 275.

Guerchicoff, A., Delécluse, A. & Rubinstein, C.P. (2001). The *Bacillus thuringiensis cyt* Genes for Hemolytic Endotoxins Constitute a Gene Family. *Applied and Environmental Microbiology*. **67**(3): 1090–1096.

Kim, H.S., Yamashita, S., Akao, T., Saitoh, H., Higuchi, K., Park, Y.S., Mizuki, E. & Ohba, M. (2000). *In vitro* cytotoxicity of non-cyt inclusion proteins of a *Bacillus thuringiensis* isolate against human cells, including cancer cells. *Journal of Applied Microbiology*. **89** (1): 16-23.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**: 680-685.

Lee, H.L. (1996). Isolation, Identification and Evaluation of Indigenous Microbial Control Agents against Mosquitoes of Public Health Importance in Malaysia. (M Sc Thesis).Universiti Sains Malaysia.

Lee, H.L. & Seleena, P. (1990). Isolation of indigenous larvicidal microbial control agents of mosquitoes: The Malaysian experience. *Southeast Asian Journal of Tropical Medicine and Public Health* **21** (2): 281-287.

Leuber, M., Orlik, F., Schiffler, B., Sickmann, A. & Benz, R. (2006). Vegetative insecticidal protein (Vip1Ac) of *Bacillus thuringiensis* HD201: evidence for oligomer and channel formation. *Biochemistry*. **45** (1): 283-288.

Liu, J.W., Porter, A.G., Wee, B.Y. & Thanabalu, T. (1996). New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. *Applied and Environmental Microbiology*. **62** (6): 2174–2176.

Mizuki, E., Ohba, M., Akao, T., Yamashita, S., Saitoh, H. & Park, Y.S. (1999). Unique activity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions: *in vitro* cell-killing action on human cancer cells. *Journal of Applied Microbiology*. **86** (3): 477-486.

Mizuki, E., Park, Y.S., Saitoh, H., Yamashita, S., Akao, T., Higuchi, K. & Ohba, M. (2000). Parasporin, a human leukaemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. *Clinical and Diagnostic Laboratory Immunologyl.* **7** (4): 625-634.

Nadarajah, V.D., Chai, S.H., Mohammed, S.M., Chan, K.K. & Karisnan, K. (2006). Malaysian mosquitocidal soil bacterium (*Bacillus thuringiensis*) strains with selective haemolytic and lectin activity against human and rat erythrocytes. *Southeast Asian Journal of Tropical Medicine and Public Health*. **37** (1).

Neppl, C.C. (2000). Management of Resistance to *Bacillus thuringiensis* Toxins. (B.A. Thesis), Environmental Studies, University of Chicago, U.S.A.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R. & Dean, D.H. (1998). *Bacillus thuringiensis* and Its Pesticidal Crystal Proteins. *Microbiology and Molecular Biology Reviews*. **62** (3): 775-806.

Shier, W.T. (1991). Mammalian cell culture on $5 a day: A laboratory manual of low cost methods. University of the Philipines. Los Banos. pp 64-71.

Thanabalu, T., Hindley, J., Jackson-Yap, J. & Berry, C. (1991). Cloning, sequencing, and expression of a gene encoding a 100-kilodalton mosquitocidal toxin from *Bacillus sphaericus* SSII-1. *Journal of Bacteriology*. **173**(9): 2776–2785.

Thanabalu, T. & Porter, A.G. (1996). A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. *Gene*. **170** (1): 85-9.

Thomas, W.E. & Ellar, D.J. (1983). *Bacillus thuringiensis* var *israelensis* crystal ä-endotoxin: effects on insect and mammalian cells *in vitro* and *in vivo*. *Journal of Cell Science*. **60**:181-197.

Ting, Y.S. (2003). Isolation and Characterisation of *Bacillus thuringiensis* species Parasporal Inclusions for *in vitro* cell killing action on Leukaemic cells. (B Med Sc Thesis). International Medical University, Malaysia.

Yeow, J.S. (2006). Haemolytic and Cytotoxic Characterisation of Biologically Active Proteins Isolated from Parasporal Inclusions expressed in Malaysian Isolates of *Bacillus thuringiensis* and *Bacillus sphaericus*. (B Med Sc Thesis). International Medical University, Malaysia.

Yu, G.G., Mullins, M.A., Warren, G.W., Koziel, M.G. & Estruch, J.J. (1997). The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Applied and Environmental Microbiology*. **63**(2): 532–536.

Yu, Y.M. & Ohba, M., Gill, S.S. (1991). Characterization of mosquitocidal activity of *Bacillus thuringiensis* subspecies *fukuokaensis* crystal proteins. *Applied and Environmental Microbiology*. **57**: 1075-1081.