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Bacillus thuringiensis subsp. Entomocidus HD9 Cry1Ba4 Insecticidal Crystal Protein: *In-silico* Mutation, Cloning, Expression, Mutation and Domain I Functional Study

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ABSTRACT

The 3D structure of the insecticidal protein Cry1Ba4 produced by *B. thuringiensis* subsp. Entomocidus HD-9 was determined using homology modelling. From the model built, we have been able to identify the possible sites for structure modification by site-directed mutagenesis. The mutation was introduced at the conserved region of α-helix 7 by substituting the hydrophobic motif that comprises alanine 216, leucine 217 and phenylalanine 218 with arginine. Wild and mutant Cry1Ba4 genes were cloned into pET200/D-TOPO and expressed in the expression host. The result suggests that mutant Cry1Ba4 protein was less toxic to the larvae *Plutella xylostella* compared to the wild-type. In conclusion, alteration in the structure of Domain I had left an impact on the toxicity of Cry1Ba4 against *P. xylostella*.

INTRODUCTION

The parasporal crystal proteins (Cry toxins) produced in *Bacillus thuringiensis* possess high specificities against insects that infect crops and trees. Cry proteins are soluble under an alkaline condition in the insect midgut and are processed into an active toxin by digestive system proteases. Schnepf *et al*. have conducted a study reporting that the toxin binds to a specific receptor on the membrane surface of epithelial gut cells resulting in a permeable pore formation and finally the death of insect [1].

Bacillus thuringiensis is a Gram-positive endospore forming bacterium characterised by the production of parasporal crystalline protein inclusions (which contain ∂-endotoxins) during the stationary phase. ∂ endotoxins are highly toxic and specific to the insects of Coleopteran, Lepidopteran and Dipteran orders. Once in the insect midgut, they are activated by gut proteases, which will after that bind specific receptors on the cells lining the larval midgut [2,1]. This interaction promotes their insertion into the membrane, which further forms ion selective channels by the oligomerisation of toxin monomers [3, 4], and causes the insect to die from a loss in osmotic pressure regulation [5]. ∂-endotoxins, also known as Cry proteins, are classified according to their degree of evolutionary divergence into 22 groups [6].

Cry1Ba4 protein was selected as the target protein in studying the structure and function of its domain I. First of all, 3D structure of Cry1Ba4 protein was determined by homology modelling. The protein model generated by Modeller6V2 was used to identify the possible sites for site-directed mutagenesis in domain I of Cry1Ba4 protein. In this study, the hydrophobic motif of α-helix 7 in Cry1Ba4 toxin of *B. thuringiensis* was substituted with weak hydrophobic amino-acids by site-directed mutagenesis. The effect of mutated toxin on *Plutella xylostella* larvae of an important pest on many crop plants, was also examined. In order to maintain the integrity of the study, automated DNA sequencing was performed to ensure whether or not the specific sites have been successfully mutated. Expression of protein was tested for verifying the wild type Cry1Ba4 gene and the mutant Cry1Ba4 gene. Western blot was carried out in the lab to detect the presence of protein according to the method developed by Towbin et al. [7]. Lastly, the toxicity effect of the synthesised Cry1Ba4 proteins (wild type

and mutant) on *P. xylostella* were detected and measured through bioassay.

MATERIALS AND METHODS

Model Building.

Homology models of the Cry1Ba4 protein were constructed using MODELLER (Accelrys) [8, 9]. From the alignment, spatial restraints including distance restraints and torsion angle restraints were derived and utilised for constructing the 3D-model of the protein. Input files were made according to the PIR database format; this is the preferred format for comparative modelling. Two Input files were separately prepared and compared with the Cry1Ba4 and variant. All models were further optimised using the internal optimizer of MODELLER.

Preparation of Plasmid

The 8kb CryBa4 gene was ligated into the pGEM-T vector, which was transformed into *E. coli* DH5α. These cell cultures were plated on the LB media and grown at 37°C with shaking. The single colony of the vector was inoculated into 5 ml LB media with ampicillin (50 µg/ml). The inoculums were incubated overnight at 37°C with shaking. Extraction of plasmid was carried out on the culture (*E. coli* in LB media) with 16 hours incubation period. The extracted DNA plasmids were kept at -20°C. After that, analysis of DNA was performed with agarose gel electrophoresis.

Gene Sequencing

A total of 10 µl master mix (comprising BigDye, 5X sequencing buffer, primer, template and ddH2O) was prepared according to the Standard Operating Procedure (SOP) of Institute Genome Malaysia. Cycle sequencing was carried out for 99 cycles by using Thermocycler. The recombinant clone was sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit and the sequencing machine known as ABI PRISM™377 DNA Sequencer (Applied Biosystem Inc., USA). After that, these sequences were analysed by using the Blastn programme (http://ncbi.nih.gov/BLAST/).

Digestion of plasmid DNA

Positive clones (carried pFC17) were digested with *Hind*III and *Eco*RI. Restriction map was constructed utilising Restriction Mapper. Double digestion was performed by the mixing of distilled water, multicore buffer, plasmid, *Hind*III and *Eco*RI.

Formation of Mutant Recombinant

Polymerase Chain Reaction (PCR)

Gene fragment of Protoxin Cry1Ba4 was amplified using PCR. The mixture of reaction consists of 10X buffer (Promega, USA), $MgCl_2$ (Promega, USA), dNTP (Promega, USA), *Pfu* DNA polymerase, forward and backward primers, template DNA and distilled water. Recombinant plasmid pFC17 was used as a template. Additionally, PCR machine AUTOSEQ, Tetrad PCR Machine (M J Research, USA) is used for this study.

Mutagenesis using PCR

Site-directed mutagenesis was carried out using the technique of overlapping PCR. Two set of different primers that have produced two fragments. In the process of mutagenesis, three codons (encoded for alanine, leucine and phenylalanine) were substituted with arginine. The size of both fragments was determined by Primer Premier 5 (Premier Biosoft International, USA) to be 802 bp and 2892 bp, respectively (**Fig.** 1).

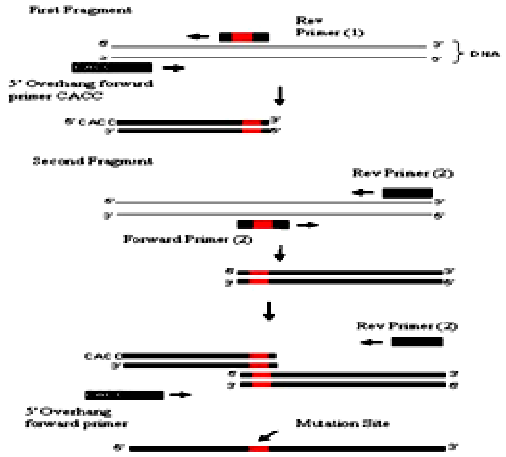

Fig. 1. Site directed mutagenesis using overlapping PCR method of two fragments. 2 mutated fragments combined to produce *cry1Ba4* variant gene which brought mutation.

Purification of PCR product

PCR product was purified using QIAquick Gel Extraction Kit. The purification process was conducted following the manual procedures are given by the distributor.

Ligation of PCR product

Purified PCR products (neither wild type nor mutant gene) was inserted and cloned into expression vector pET200D/TOPO (**Fig**. 2) The mixture was then kept in ice prior conducting the transformation process.

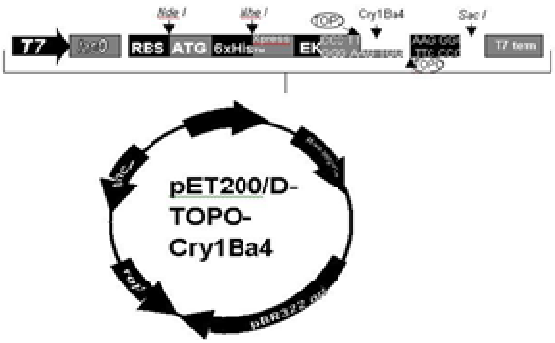

Fig. 2. Generated plasmid map with *cry1Ba4* gene fragment insertion into pET200/D-TOPO' vector.

DNA Transformation with Heat Shock

Ligation product was cloned into competent cell, which is One Shot® TOP10 (Invitrogen, USA). Heat shock was performed by immersing the tube containing the mixture in 42°C water for 30 seconds without shaking. Upon completion, the tube was further transferred to ice. After successfully cloned into the chemically competent cell, plasmid was transformed into competent cell *E. coli* BL21 Star™ (DE3) One Shot® (Invitrogen, USA) and incubated overnight.

The Characterisation of Recombinant Plasmid

Analysis of Recombinant Plasmid

The selected clones were digested by *EcoRI* and *HindIII*. Three different types of reaction were involved; single digestion (using *EcoRI)* in the first tube, single digestion (using *HindIII)* in the second tube and double digestion (*EcoRI* + *HindIII*).

After digestion has been completed, agarose gel electrophoresis was performed at 90 V by using 1.0% agarose gel, 1X TAE buffer and 1 kb DNA marker. The agarose gel was then immersed into Ethidium bromide (EtBr) before being visualised by Syngene Imager. The size of each fragment produced was determined from the result of agarose gel electrophoresis.

Automated DNA Sequencing

Automated DNA sequencing was carried out to obtain mutant Cry1Ba4 gene sequence by using Bio Basic Inc. (Ontaria, Canada).

Protein Expression, Characterisation and Purification of Recombinant Protein

Expression of Recombinant Protein

5 ml cultured bacteria was inoculated into 200 ml LB media + kanamycin (50 µg/ml) and incubated for two hours. Readings on OD_{600} were recorded. The expression of wild type and mutant gene of Cry1Ba4 was analysed according to the reading taken.

Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by using either resolving gel or stacking gel adopting on the method of Laemmli [10]. Proteins were stained with the mixture of Brilliant Coomasie Blue, methanol, acetic acid and distilled water for 15 min. Soon after, a mixture comprising 20% methanol, 10% acetic acid and 70% distilled water were added.

Purification and Fold of Recombinant Protein

Novagen ProBond™ (Invitrogen, USA) was used to purify the recombinant protein. The protocol for purification has been provided along with the system.

Western Blot

The proteins separated by SDS-PAGE were transferred to a sheet of special blotting paper called nitrocellulose (NCP) (Osmonic, USA). The blot was incubated with a generic protein (including milk protein) to bind to any sticky places remained on the nitrocellulose. An antibody capable of binding to its specific protein was then added to the solution. Monoclonal IgG that is anti-HisGly and conjugated with HRP were used in the study. The location of the antibody was revealed by incubating it with a colourless substrate that the attached enzyme converted to a coloured product that can be seen and photographed.

Bioassay

The concentration of protein was determined following the Bradford method [11]. Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G-250 into 88% Orthophosphoric acid. The stock was obtained and mixed with Bradford reagent and water. A total of 4 controls were used; I) LB (empty), II) LB (with E. coli BL21 Star™ (DE3), III) Cabbage without any toxic, IV) Wild type of Cry1Ba4 proteins. There are 270 *P. xylostella* were used in the assay. Among the wild type and mutant of Cry1Ba4 protein, 30 of them were replicated for each sample. The concentration of protein from each sample was equaled to 34 mg/ml. Two bioassay plates (containing multiple zones) were required for each sample. Each bioassay plate consists of 25 zones and with a single *P. xylostella* placed in each zone. The number of death was calculated for each 24 hours, which was carried out for three days. The average was then calculated and recorded.

RESULTS AND DISCUSSION

Domain I

Three-dimensional modelling results were analysed by using Swiss PdbViewer software as shown in **Fig**. 3. The result shows that the first domain constitutes the sheaf of eight anti parallel α –helical structures where α5-helix structure is centered in the middle and surrounded by other helixes. Other α-helix structures around helix α5 are situated inclined in a similar direction of angular +15 º to +25 º from the middle. α2b-helix contacted with helix α2a by one short open loop creating long helix structure with bending centered. Five more α-helix structures, α3 to α7 have five to nine turns with the size of more than 30 Å. The longest helix structure was α6 which possesses nine helix turns consists of 32 residues with a size of 45 Å. Furthermore, the helix structures attached outside the bundle were found demonstrating amphiphatic character where some areas are polarised while some are not, which produces hydrophilic and hydrophobic phenomena. When scrutinised, it was found that α5-helix own as much as 17 amino acids that behave hydrophobically from the total amount of 29 amino acid residues. The nature of this amphiphatic character explains why α5-helix is being embedded and surrounded by other helix structures. Amino acid residues that behave hydrophobically will incline to face towards the core, while hydrophilic residue will face out from it [12].

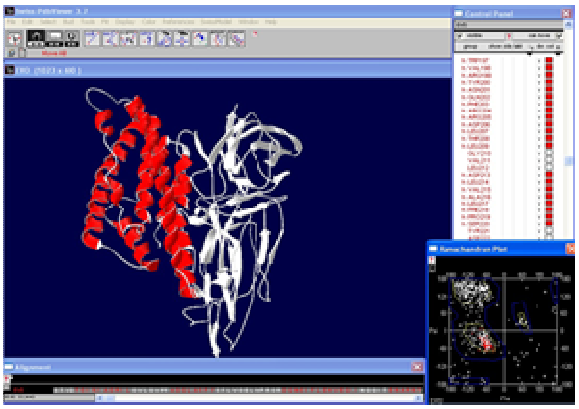

Fig. 3. Analysing the Cry1Ba4 proyein 3 dimensional struture by using SPDV Viewer programe Cry1Ba4 toxin's wild structure compared Cry1Ba4's variant.

There was a low polarised segment discovered having a coil hairpin shaped, which connects between α4-helix and α5-helix to forms an open coil structure. It is possible for this helix to have some mobility by considering that one of the cutting sites by gut proteases is located between Ser56 and Ile58, close to the middle of this helix as suggested by Segura *et al*. [13]. The charge distribution pattern in the Cry11Bb theoretical model corresponds to a negatively charged patch along ß4 and ß13 of domains II and III, respectively. The Cry1Ba4 domain I model was found to be well correlated with the data from Gazit *et al*. [14] suggesting that the insertion of α4 and α 5 into the membrane acts in an antiparallel manner as a helical hairpin. It is possible that according to surface electrostatic potential of helices 4 and 5, there is a neutral region in the middle of the helices, which probably indicates that both helices cross the membrane with their polar sides exposed to the solvent if the umbrella model is correct, as suggested by the study on mutagenesis done by Kumar & Aronson with the Cry1Ac toxin [15]. This is also the most conserved region among the Cry toxins. Kumar & Aronson demonstrated that mutations in the base of helix 3 and the loop between α3 and α 4 that cause

alterations in the balance of negatively charged residues can also result in a loss of toxicity by Cry1Ac [15]. Meanwhile, the mutations in helices α 2, α 6 and the surface residues of α 3 displayed no significant effect on toxicity with helices α 4 and α 5 seemed to be very sensitive to mutations.

Once the 3D model has been built, it was discovered that Cry1Ba4 toxin is a kind of protein globular which contains three major domains. These domains are linked by a single link comprising of polypeptide coil and no overlapping link between those domains. Domain I extended the residue No. 4 until 220 and constituted eight α-helix structures. In addition, residue No. 228 to 443 formed the domain II structure, which comprises three anti parallel β-sheets. Domain III was formed by two folded β-sandwich structures starting from residue No. 449 to 588. Domain II and III were placed beside domain I, overlapping with one another along α7-helix (**Fig**. 4).

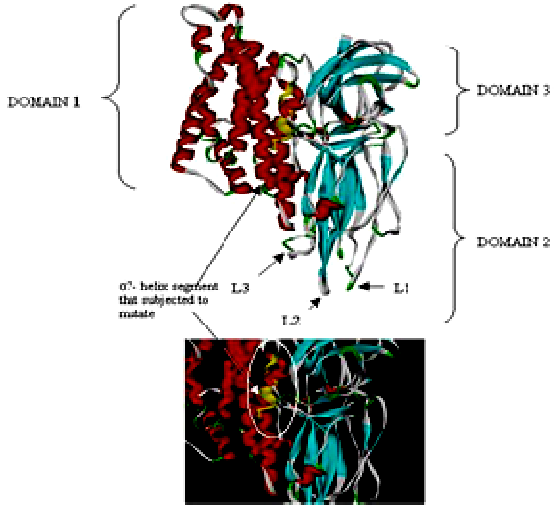

Fig. 4. Area where α7-helix protein that was mutated by computer simulation locates in no216 residue alanine, 217 leucine and 218 phenylalanine. L1, L2 and L3 are putative sites which function in fixation with *P. xylostella* insect's intestines's cell receptor during pore formation punching process.

This study suggests that the model contains as much as 588 amino acid residues that fall into three different domains. Each amino acid residue has its own unique nature due to hydrophilic and hydrophobic phenomenona, which became the main reason of conformation formed. Domain I of Cry1Ba4 protein has been built from amino acid No. 4 to 220, which was included in 8 α-helix structures. α1-helix contains 10 amino acid residues known as $_{4}$TGINIAGRIL$_{14}$, with α2-helix having 35 amino acids namely $_{22}$AGQLASFYSFL VGELWPRGRDQWEIFLE HVEQLI$_{56}$. The third structure that has included amino acids starting from No. 61 to No. 90 is rederred to as $_{61}$ENARNTALARLQGLGDSFRAYQQSLEDWLE$_{90}$.
Meanwhile, the α4- helix structure with only 17 amino acid residues is $_{102}$HTQYIALELDFLNAMPL$_{118}$ α5-helix containing 29 amino acids residue is $_{125}$EVPLLMVYAQAANLHLLL LRDASLFGSEF$_{153}$. Furthermore, the α6-helix contains consists of 32 amino acid residues and the helix α7 containing 27 amino acids are $_{157}$SQEIQRYYERQVERTRDYSDYCVEWYNT GLNS$_{188}$ and $_{194}$AASWVRYNQFRRDLTLGVLDLVA LFP$_{220}$, respectively.

The modelling of wild-type Cry1Ba4 protein crystal structure serves as the basis of comparison with the variant protein structure to assess the change in structure. Achieving apparent structural changes through mutation on a minimum number of amino acids is the main objective of any study on protein structure and its functional change. Nonetheless, this study has only focused on evaluating changes in the structure of domain I. Such mutations were made in structure α7-helix in No. 216 residue alanine, No. 217 leucine and No. 218 phenylalanine. Point mutations were made by substituting all three amino acids with strong hydrophobic behaviour to the amino acid arginine since these residues were hydrophobically weakened and polarised. This mutation area is also a conserved hydrophobic motif that acts as a sensor in binder site detection. Theoretically, its mutated protein structure will form a different conformation of wild-type. **Fig**. 4 displys the protein area aimed to be mutated from this study. Based on information obtained, it was observed that mutation has occurred in helix α7 of the highly conserved region. Mutation in the conserved region shows apparent structural change, which probably causes the change in the reaction of fixation with the receptor and the toxicity of this specific protein [16].

Analysis of Recombinant Plasmid pFC17 (carried Cry1Ba4 clone) Using Restriction Enzyme

*Hind*III and *EcoR*I were used to analyse the recombinant plasmid. The size of the vector and inserted gene are 3653 bp and 3645 bp, respectively. Therefore, the size of recombinant protein pFC17 is 7270 bp. From the restriction map, there are five fragments predicted to be produced by double digestion (*EcoRI* + *HindIII*) with the size of 21, 55, 312, 528 and 6359 bp, respectively. Results from agarose gel electrophoresis have shown that Cry1Ba4 gene was successfully inserted into pFC17. There are three fragments produced by double digestion in the agarose gel electrophoresis. Even though the bands with 55 bp and 21 bp fragments were not obviously seen, the result has been accepted since the other three bands of fragment demonstrated the sizes (~312 bp, ~528 bp and ~6359 bp) as predicted from the restriction map (**Fig**. 5).

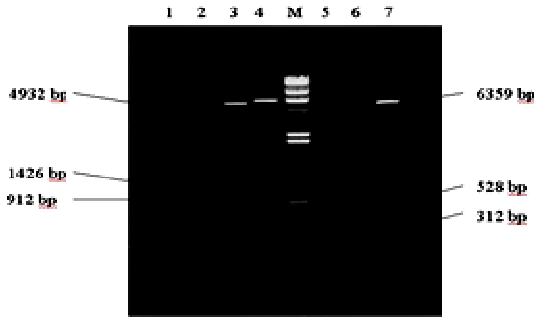

Fig. 5. Digestion of recombinant plasmid with *HindIII* and *EcoRI*. Well 1: digested with *EcoR*I; Well 4: pFC17; Well M: DNA marker 1kb; Well 7: *EcoR*I + *Hind*III (double digestion).

Gene Sequencing of Cry1Ba4 (carried by pFC17)

Automated DNA sequencing was performed to confirm the insertion of the correct target gene without any changes to the genetic code. The aim of this study is to produce mutant Cry1Ba4 at a specific site, thus blocking the presence of other mutations in the gene. Sequencing was carried out using 13 primers which have produced the sequence size of 3891 bp. Furthermore, sequence alignment by BLASTN has shown 100% identical nucleotide.

The Formation of Mutant Cry1Ba4

Electrophoresis showed that PCR product with the size of 802 bp, has been successfully amplified. The forward primer (for

first fragment) with 29 mer has been added with CACC sequence in the 5'. Meanwhile, the backward primer longs with 30 mer had caused mutation on the sequence. The first fragment (802 bp) was discovered to contain 5' CACC to ligate with the expression vector, pET200/D-TOPO (5764 bp) (**Fig**. 6A). The size of the second fragment produced by electrophoresis is ~2892 pb. Forward primer and backward primer have 28 mer and 31 mer, respectively. The forward primer of the second fragment has been discovered to cause mutation on the sequence. Site-directed mutagenesis was carried out by overlapping PCR for both fragments (**Fig**. 6A). Moreover, mutant gene was obtained by overlapping the first and second fragment. It was then purified and used as a template in the PCR process (**Fig**. 6B). PCR products (wild type and mutant type) were purified and ligated into the expression vector before being transformed into *E. coli* TOP10 (**Fig** 7).

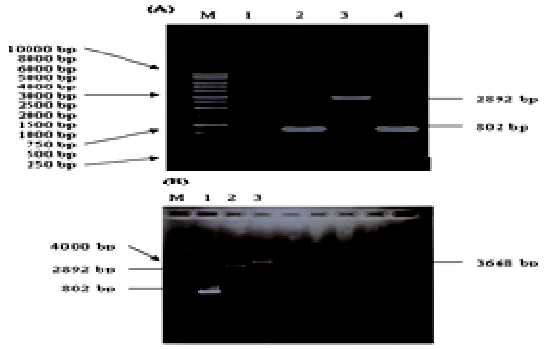

Fig. 6. (A) Well 1 and 3: second DNA fragment; Well 2 and 4: first DNA fragment. (B) Well 1: first DNA fragment; Well 2: second DNA fragment; Well 3: the ligated DNA (with mutant Cry1Ba4); Well M: DNA marker 1kb.

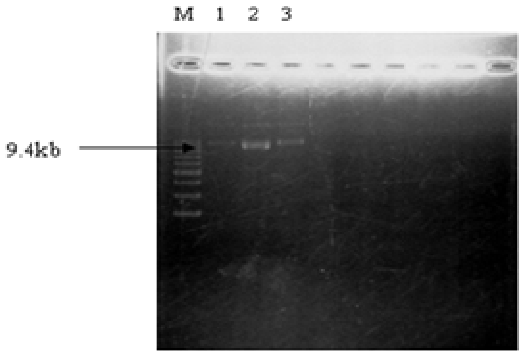

Fig. 7. Extraction of recombinant plasmid DNA from pET200/D-TOPO (carried Cry1Ba4 gene). Well 1: plasmid with Cry1Ba4 gene (wild type); Well 2 and 3: plasmid with mutant Cry1Ba4 gene; Well M: supercoiled DNA marker.

In this study, mutant Cry1Ba4 was used after being subcloned for three generation. Plasmid from the third generation was determined by using DNA sequencing, service Bio Basic Inc. (Ontario, Canada). The result from sequencing had proven that prove the DNA fragment, which is mutant, has been successfully ligated into the vector pET200/D-TOPO with the correct orientation and without other mutations on it.

Expression of Cry1Ba4 protein and Western Blot

In our research, pET200/D-TOPO was utilised as an expression vector and *E. coli* BL21 Star (DE3) was used as the host cell. The recombinants (neither wild type of Cry1Ba4 nor Cry1Ba4 mutant) were expressed in the medium of LB + kanamycin + IPTG + 1% glucose. After SDS-PAGE was carried out, it was seen that 10 hours incubation period is the optimum time where protein expression occurs at the highest rate. From the analysis of SDS-PAGE (15%), the expression of variance for Cry1Ba4 protein was observed at 8th hour and 10th hour.

The presence of target protein (120 kDa) was determined by using antibody (anti-HisGly). Expression sample in the 8th well of PAGE has shown the presence of the protein. The wild type Cry1Ba4 protein had also given similar result from the PAGE (**Fig**. 8 and 9). There are several studies demonstrating that protein expression normally favours the formation of non-soluble protein at 37°C. Hence, to obtain the soluble wild type and variance of Cry1Ba4 protein, protein expression was carried out at 25°C. Besides, 1% glucose was added into the growth medium. The addition of glucose is critical if the expressed proteins are toxic to the *E. coli*.

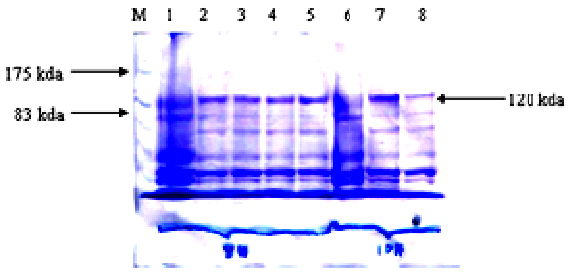

Fig. 8. Analysis of SDS-PAGE (15%). Well 1: without IPTG (at 8th hour); Well 2 to 5: with 1mM IPTG (at 8th hour); Well 6: without IPTG (at 10th hour); Well 7 to 8: with 1mM IPTG (at 10th hour); Well M: protein marker.

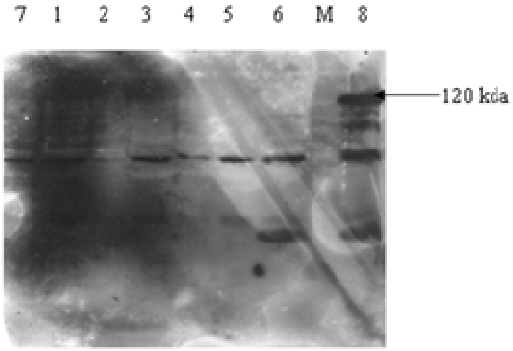

Fig. 9. Analysis of SDS-PAGE (15%) of wild type of Cry1ba4 protein. Well 1: without IPTG (at 8th hour); Well 2 to 5: with 1mM IPTG (at 8th hour); Well 6: without IPTG (at 10th hour); Well 7 to 8: with 1mM IPTG (at 10th hour); Well M: protein marker.

Toxicity of Cry1Ba4 protein on *P. xylostella*

The level of the toxicity was identified by comparing the number of dead insects, *P. xylostella* in the bioassay plate. Besides, the rate of mortality and the physical appearance of the insect were observed and recorded for both of bioassay plate (one plate contains wild type Cry1Ba4 protein and another plate contains mutant Cry1Ba4 protein). Standard Graph Bovine Serum Albumin (BSA) was plotted as a reference for the concentration of expressed protein.

Mortality test has displayed a positive and significant result (**Table** 1). The results obtained showed that the larvae in all the samples were found to be alive at the 24th hour with

25°C; most samples have constantly shown zero mortality rates at the 48th hour. For the sample of Cry1Ba4 protein (wild type), most of the larvae have changed their colour from green to dark brown. On the other hand, the original colour (green) of larvae in the sample of Cry1Ba4 (mutant) was found unchanged; most larvae have developed into a pupa in the controlled plates (**Photo** 1 A). However, the study has detected 96.67% of mortality rate in the sample of Cry1Ba4 (wild type) after 70 hours. The death larva is dark in colour. (**Photo** 1 B) The larvae in the sample of Cry1Ba4 (mutant) were expected to die in a longer period (after 72 hours). (**Photo** 1 C)

Table 1. Mortality test of Cry1Ba4 (wild type) and variance of Cry1Ba4 on *Plutella xylostella*.

Sample	Mortality Rate/30						Missing	Alive	Average mortality	%
	24		48		>48					
	I	II	I	II	I	II				
LB Broth	0		0		0		1	30	0	0
Broth LB+E.coli	0		0		0		0	30	0	0
Cabbage	0		0		0		0	30	0	0
S.litura	0		0		0		0	30	0	0
Cry1Ba4	0	0	3	1	25	29	2	0	29	96.67
Mutant Cry1Ba4	0	1	0	0	0	0	1	29	0.5	3.33

Photo 1. (A) Controlled larvae – developed to pupa. (B) I. Alive larvae in 72 hours treatment. (green) II. Mortal larva has turned its colour into dark brown. (C) The difference between controlled larvae and mutant Cry1Ba4 protein treated larvae. After 72 hours, the colour of treated larvae becomes dark brown.

CONCLUSION

In conclusion, Cry1Ba4 protein (wild type) is considered to be toxic to *P. xylostella*. However, this protein still requires more than 48 hours to kill *P. xylostella*. Mutant Cry1Ba4 protein is less toxic compared to the wild type protein. There is only one death observed in the samples of mutant Cry1Ba4 within the period of 48 hours.

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