# EXPRESSION AND PURIFICATION OF CRY8DA RECOMBINANT PROTEIN AGAINST COLEOPTERAN INSECTS OF *BACILLUS THURINGIENSIS* IN *E. COLI*

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## ABSTRACT

*Bacillus thuringiensis* (Bt) is widely used in agriculture for pest control against Lepidoptera, Diptera and Coleoptera orders. It is very difficult to control of Coleoptera pests such as larvae of Scarabaeidae family, which damage the roots of tusf grass and other horticultural and agricultural plants, because they are living in soil where sprayable insecticidal formulation is hard to reach the target insects. The Cry8-type proteins specified to larvae of scarab beetles. The Cry8-type proteins are insecticidal to larvae of scarab beetles. *Bacillus thuringiensis* Cry8D (Cry8Da, Cry8Db) insecticidal proteins are unique among Cry8 family protein in terms of its insecticidal activity against larvae and adult Scarab beetles, such as Japanese beetle (*Anomala cuprea, Popillia japonica)*. In this study, *cry8Da* gene encoding nature protein Cry8Da, which were isolated in Vietnam, was expressed in *E. coli.* The 2031 bp of *cry8Da* gene is digested from recombinant plasmid pGEM-*cry8Da* with *Bam*HI và *sac*I and ligated into pET32a(+) vector treated with the same enzymes. The recombinant plasmids pET32a(+)-*cry8Da* were expressed in BL21(DE3)LysS strain under induction of IPTG. The highest level for expression of Cry8Da recombinant *E. coli* has been obtained after induction by 1 mM IPTG at 30 $^0$C for 4 hrs. Recombinant Cry8Da protein has molecular mass about 96 kDa and purified by $Ni^{(2+)}$ affinity chromatography column, concentration 0,45 mg/ml after purification. The expressed recombinant Cry8Da protein was confirmed by Western blot using anti-Cry8Da antibody.

*Keywords: Bacillus thuringiensis*, insects, coleoptera, *cry8Da* gene, recombinant protein.

## 1. INTRODUCTION

Bacillus thuringiensis (Bt) is an biological agent to control damage of insects to crops. Bt is used for pest control by two methods: biological insecticides and transgenic Bt into crops to against pest insects. Crystal proteins from the Gram-positive spore forming bacterium Bacillus thuringiens are toxic to a wide variety of insects. The proteins toxic for lepidopteran insects belong to the Cry1, Cry2 and Cry9 groups; toxins active against coleopteran insects are the

Cry3, Cry7 and Cry8 proteins as well as the Cry1B and Cry1I proteins, which have dual activity. The Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for dipteran insects. These proteins are encoded by cry genes. Many of these genes have been cloned and expressed in different organisms, especially in plants to help plants against pests. The various plants were transferred by cry1, cry3 gene resistant lepidopteran and coleopteran insects as tomato, potato, rice, corn, canola, soybeans, peanuts, sugar, apples... [1 - 4]. Some of genes are cryptic and have not been studied for their insecticidal properties. The Cry8-type proteins are insecticidal to larvae of scarab beetles. However, Cry8Da and Cry8Db proteins have toxicity against not only larvae but also adults of scarab beetles (Anomala cuprea, Popillia japonica). The Cry8Da protein is formed from Bt. subsp. galleriae SDS-502 with molecular weight as 130 kDa and transffered into turf grass [5, 6].

In Vietnam, it will be the concern of the people to the use of widespread chemical pesticides in agricultural production and storage of agricultural products. That, it should be interested and paid attention to reducing of these problems, especially for the farmers of small producers. In recent years, some of beetles (scarabs, capricornbeetle, red flour beetle...) have had considerable damage to growing sugar cane, coconut, tobacco, glue, ginger... and reduced income for farmers. In previous papers [7, 8], we have reported on screening, toxicity, sequencing and characterization of *cry8Da* gene of *B. thuringiensis* subsp. *galleriae* isolated from Vietnam. In this paper, on recombinant protein Cry8Da, which was formed from natural *Bt* strain in Vietnam, its expression in *E. coli* BL21(DE3)LysS and purification by His-tag column will be presented.

## 2. MATERIALS AND METHODS

### 2.1. Bacterial strains and plasmids

Plasmid pGEM-*cry8Da* (5,9 kb) containing *cry8Da* gene (2031 bp) was obtained from Deparment of Genetics of Microorganisms, Institute of Biotechnology, VAST [7, 8].

pET32a(+) vector was designed for high level-expression of peptides sequences fused with 109 amino acid Trx.Tag$^{TM}$ thioredoxin protein. Cloning sites are available for producing fusion protein also containing cleavable His.Tag and S.Tag sequence for detection and purification. The fusion protein control by T7 promoter that has low background expression in the absence of T7 polimerase.

*Escherichia coli* BL21(DE3)LysS (Novagen) is the most widely user for expression recombinant protein. BL21(DE3)LysS is a strain in which background expression of the T7 promoter is dampened by the LysS gene, it is very useful for manipulation with toxic protein.

### 2.2. Constrution of expression vector

Cloning experiments, restriction-enzyme analyses, and *E. coli* transformations were carried out as described by Sambrook *et al*. (2001) [9]: The 2031 bp of *cry8Da* gene was digested from recombinant plasmid pGEM-*cry8Da* with *Bam*HI và *sac*I and ligated into pET32a(+) vector treated with the same enzymes. Recombinant plasmid pET32a-*cry8Da* was cloned into cells of *E. coli* BL21(DE3)LysS strain and incubated in medium of LB cotaining 50 μg/ml ampicillin. The individual transformed colonies were selected by PCR with cry8F-*Bam*HI / cry8R-*Sac*I specific primers and their plasmid were cut by *Bam*HI and *Sac*I.

### 2.3. Expression of *cry8Da* gene in *E. coli* BL21(DE3)LysS

A single conoly was picked in 5 ml LB medium with 50 μg/ml ampicillin (Ap) and 34 μg/ml choramphenicol (Cm), this strain was incubated 37 $^0$C overnight and then sub-cultured 1 ml into 50 ml fresh LB medium (with Ap and Cm), then induced IPTG and incubated in time for concentration of *E. coli* OD600 = 0.5 – 0.7 to express recombinant protein. To maximal expression of the fusion Cry8Da protein, some growing factors were checked including: growth temperature (25 $^0$C, 30 $^0$C and 37 $^0$C), IPTG concentration (0.1, 0.3, 0.5, 0.7, 1 and 2 mM, respectively) and incubation time (1, 2, 3, 4 and 5 hrs). Cells after expression were collected by centrifugation at 6.000 revs per minute for 5 minutes. Cell pellets were added with 5 x SDS sample buffer, denatured at 100 $^0$C for 10 minutes and loaded onto a 12.5 % SDS polyacrylamide gel. SDS-PAGE analysis was performed according to the method of Laemmli [10]. The molecular weights were estimated by comparison with the protein ladder.

### 2.4. Purification of recombinant protein Cry8Da by His-Tag column

The recombinant protein was purified by affinity chromatography using Probon Nickel-Chelating Resin (Invitrogen) under denature condition. The cells after growing and inducing IPTG were collected by centrifugation, cell pellets were suspended in Lysis Buffer. After ultra-sonification suspension was separated supernatant and pellet by centrifugation. The supernatant protein was filled into affinity chromatography column. Pure recombinant protein was recovered by elution buffer and stored at 4 $^0$C. Concentration of recombinant protein was determined by Bradford method [11].

### 2.5. Western Blot

Western blot was done according to Towbin *et al.* (1979) [12]. Protein from acrylamide gel (no staining) was transferred onto PVDF (polyvinyl idene difluoride) membrane by electro-transfer. Three-buffer system was used for conveyance. Then the membrane was blocked in 5 % skim milk at room temperature (RT) with gently shaking. The membrane was washed with TBS and TBS-T 3 times and sank in 1000 times diluted anti-Cry8Da standard antibody for 2hrs at RT. Then, it was washed and incubated with 10000 times diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1hrs at RT with gently shaking. The membrane was washed and developed with 4-chloronapthol solution for 10 minutes.

## 3. RESULTS AND DISCUSSION

### 3.1. Construction of expression vector pCAMBIA-*cry8Da*

Recombinant plasmid pET32a-*cry8Da* was constructed by inserting a 2031 bp *Bam*HI-*Sac*I DNA fragment of plasmid pGEM-*cry8Da* into the *Bam*HI-*Sac*I sites of pET32a(+). After cloning in to pET32a(+), *cry8Da* gene was checked by colony-PCR with cry8F-*Bam*HI/cry8R-*Sac*I specific primers and digested plasmid with *Bam*HI and *Sac*I restrictive enzymes. The results showed that the transformed colonies produced only one PCR product, of about 2031 bp (Figure 1A) and restrictive digestion (Figure 1B), revealed fragment with the size of approximate 2 kb corresponding to the size of *cry8Da* gene (as described by Le Thi Minh Thanh *et al.* 2008, 2009) [7, 8].

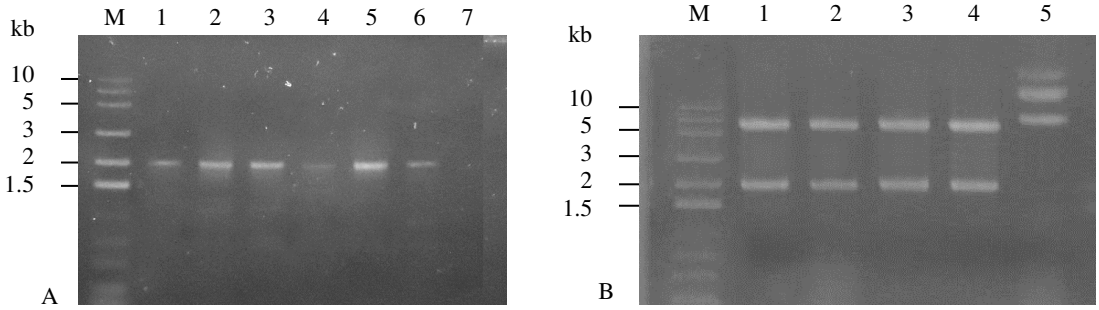


*Figure 1*. Construction of pET32a-*cry8Da* recombinant expression vector

(A) PCR to identify the direction of *cry8Da* gene in colonies containing pET32a-*cry8Da* . M: DNA ladder 10 kb; Lane 1-6: Amplification of *cry8Da* from colonies containing pET32a-*cry8Da*; Lane 7: PCR product of colony containing pET32a(+). (B) Checking expression vector pET32a-*cry8Da* using *Bam*HI and *Sac*I. M: DNA ladder 10 kb; Lane 1-4: Excised product of plasmid pET32a-*cry8Da/ Bam*HI+*Sac*I; Lane 5: Plasmid of pET32a-*cry8Da*

### 3.2. Expression of *cry8Da* gene in *E. coli* BL21(DE3)LysS

*E. coli* BL21(DE3)LysS strain was used to express recombinant plasmid pET32a-*cry8Da* because it is very useful for manipulation with toxic protein. The recombinant vector pET32a-*cry8Da* was transferred into host BL21(DE3)LysS by temperature gradient. After transformation, some single colonies were picked to check expression of *cry8Da* gene. The colonies were cultured, sub-cultured at 37 $^0$C and induced 1 mM IPTG, then cells were collected after 4 h continuous culture (OD600 = 0,7-1) and checked by SDS-PAGE analysis. The molecular weight of Cry8Da in pET32a was estimated about 96 kDa including tag-peptides. With SDS-PAGE analysis, a band with size about 96 kDa was clearly seen on gel of induced cells (Figure 2, lane 1-4). This band was not clearly appeared in no induced sample (Figure 2, lane 5). Thus, Cry8Da protein was expressed in *E. coli* BL21(DE3)LysS under the control of T7 promoter.

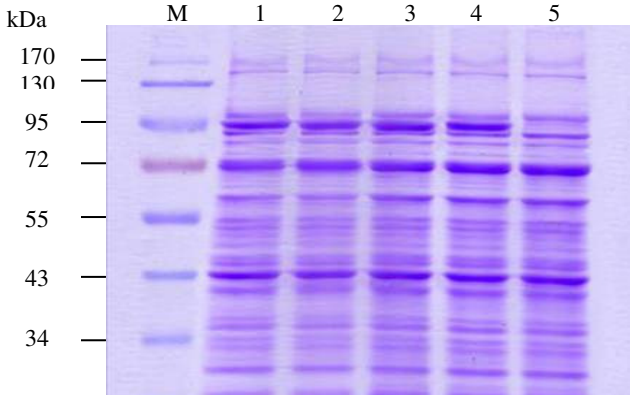


*Figure 2*. Expression of *cry8Da* gene in *E. coli* BL21(DE3)LysS on 12.5 % polyacrylamide gel. M: Protein size marker (Fermentas); Lane 1–4: Colonies containing *cry8Da* gene were induced; Lane 5: Colony containing *cry8Da* gene was not induced

### 3.3. Culture conditions for enhance expression of cry8Da gene in *E. coli*

To maximal expression of the fusion Cry8Da protein, some growing factors were checked including: growth temperature, IPTG concentration, incubation time.

*Growth temperature*. pET system was designed for bulk expression of recombinant protein in host, temperature is one important factor for expression speed. Too fast expression protein in *E. coli* may reduce expression level by hampering of rare codons in host. To avoid this situation, we performed expression Cry8Da at different temperature: 25 $^0$C, 30 $^0$C and 37 $^0$C, respectively (1 mM IPTG, 4 h). At 30 $^0$C Cry8Da expressed at higher concentration (Figure 3A, lane 3) than the others temperature.

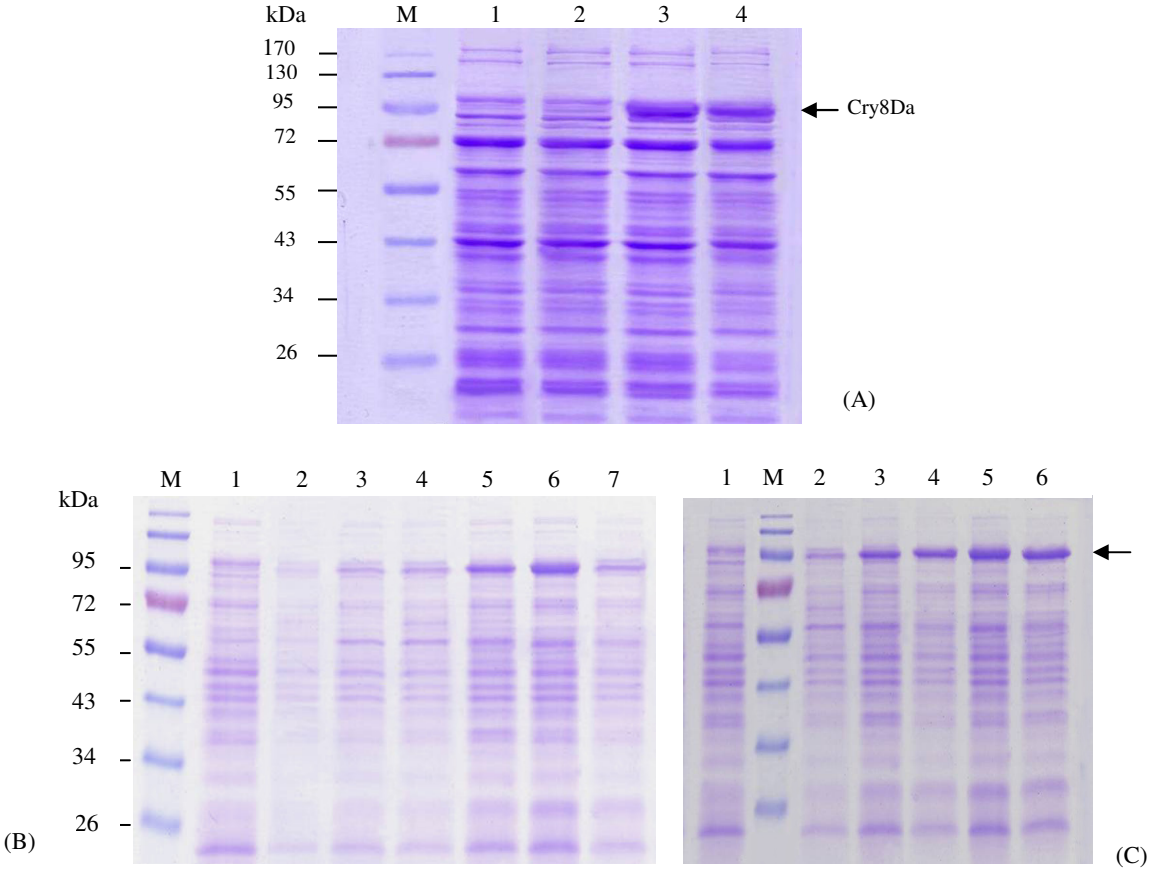


*Figure 3*. Expression of Cry8Da in BL21(DE3)LysS under different conditions: growth temperature (A), IPTG concentration (B) and incubation time (C).

(A) M: Protein size marker; Lane 1: No induced sample; Lane 2–4: Induced sample at different temperatures 25 $^0$C, 30 $^0$C and 37 $^0$C, respectively. (B) M: Protein size marker; Lane 1: No induced sample; Lane 2–5: Induced sample at different IPTG concentrations 0.1, 0.3, 0.5, 0.7, 1 and 2 mM, respectively. (C) Lane 1: No induced sample; M: Protein size marker; Lane 2–6: Induced sample collected after 1, 2, 3, 4 and 5 hrs.

*IPTG concentration*. Theoretically, expression of recombinant protein depends on IPTG concentration so this chemical usually use as a way to verify desired protein in *E. coli*. If recombinant protein expressed in *E. coli*, desired band should change density bases on IPTG until saturate expression. pET32a-*cry8Da* in BL21(DE3)LysS was cultured, sub-cultured at 30

$^0$C and induced IPTG at different concentration (0.1, 0.3, 0.5, 0.7, 1 and 2 mM, respectively), continuous cultured 4hrs long. At low IPTG induction (0.1 mM), the protein band did not finely determine (Fig. 3B, lane 2), however, in the range from 0.3 to 2.0 mM the protein band clearly appreared. Finally, saturation of IPTG concentration was defined at 1.0 mM (Figure 3B, lane 6).

*Incubation time*. Recombinant protein extracted in *E. coli* sometime digested by host protease or not stable in *E. coli* intracellular environment. Most of these recombinant proteins were degraded after long incubation. Therefore, we checked optimal time for expression of Cry8Da. The BL21(DE3)LysS containing pET32a-*cry8Da* was started to induce 1 mM at 30 $^0$C and then harvest cells at different induced-time: 1, 2, 3, 4 and 5 hrs, respectively. Cry8Da could be expressed better after 2 hrs and yields of protein did not increase than 4 hrs culture (Figure 3C, lane 5).

Thus, optimal conditions for expression of Cry8Da in BL21(DE3)LysS were cultured at 30 $^0$C, induced 1 mM IPTG and collected after 4 hrs culture.

### 3.4. Purification of Cry8Da in BL21(DE3)LysS by His-tag column

The recombinant Cry8Da expression in pET32a(+) includes His-tag, therefore we performed purifying this protein using His-tag column. The recombinant Cry8Da was purified from total protein of *E. coli* at denatured condition. pET32a-*cry8Da* in BL21(DE3)LysS was cultured at 30 $^0$C, induced 1 mM IPTG and collected after 4 hrs culture. After cell-harvest, pellet was suspended in Lysis buffer and ultra-sonicated and then loaded on His-tag column, washed to remove host protein, eluted from column and collected. Results of SDS-PAGE showed a band with size of 96 kDa on gel (Figure 4). Recombinant Cry8Da has been successfully purified and final yield was calculated approximately 0,45 mg from 1 ml of culture by Bradford method [11].

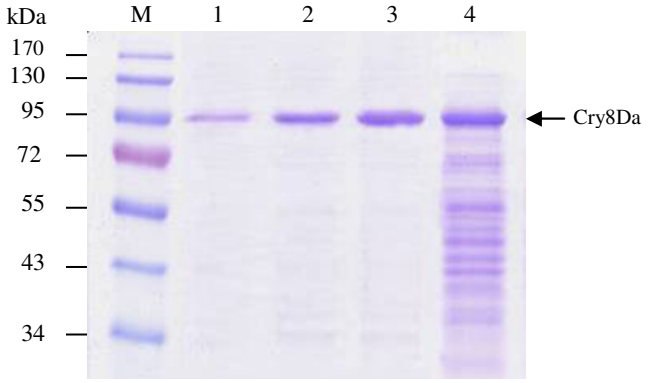


*Figure 4*. Purification of recombinant Cry8Da protein using His-tag column.
M: Protein size marker; Lane 1-3: Cry8Da after purification; Lane 4: Total protein from BL21(DE3)LysS expressed Cry8Da

### 3.5. Western blot

Polyclonal and monoclonal antibodies have been used to indentify toxic proteins in natural *Bacillus thuringiensis* by Western blot method. To check Cry8Da recombinant protein in BL21(DE3)LysS, total protein from BL21(DE3)LysS expressed Cry8Da was used as antigen and Cry8Da recombinant protein was detected by using standard antibody against Cry8Da and HRP-conjugated goat anti-rabbit IgG. Result showed that product of western blot was appeared

a main band (approximately 96 kDa) at the lanes of total protein from *E. coli* expressed Cry8Da and recombinant Cry8Da after His-tag purification (Fig. 5, lane 1 and 2).

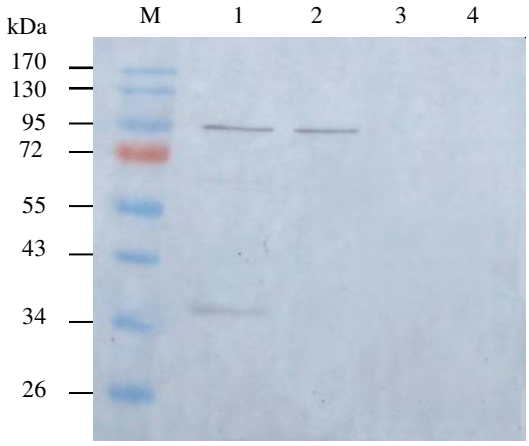


*Figure 5***.** Detection recombinant Cry8Da expressed in *E. coli* BL21(DE3)LysS by western blot. M: Protein size marker; Lane 1: Total protein from BL21(DE3)LysS expressed Cry8Da; Lane 2: Protein Cry8Da after purification; Lane 3: Total protein from BL21(DE3)LysS/pET32a-*cry8Da* no induced; Lane 4: Total protein from BL21(DE3)LysS/pET32a(+).

The result of western blot indicated that *cry8Da* gene was expressed successfully in *E. coli* BL21(DE3)LysS. Protein Cry8Da, after purification will be used in model testing for effects of protein crystals toxic to other insects in the laboratory and to produce BT insecticides and recombinant antibody against Cry8Da in rabbit as well. This antibody will be used to test GM crops by *cry* gene of *Bt*.

## 4. CONCLUSION

The *cry8Da* gene encoding nature protein Cry8Da, which were isolated in Vietnam, has been successfully constructed into vector pET32a(+).

Recombinant protein Cry8Da was expressed in *E. coli* BL21(DE3)LysS. The molecular weight of Cry8Da in pET32a was detected about 96 kDa including tag-peptides. The highest level for expression of Cry8Da recombinant *E. coli* has been obtained after induction by 1 mM IPTG at 30 $^0$C for 4 hrs. This protein was purified by using His-tag column and quantified 0,45 mg from 1 ml of culture.

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## TÓM TẮT

### BIỂU HIỆN VÀ TINH SẠCH PROTEIN TÁI TỔ HỢP CRY8DA DIỆT CÔN RÙNG BỘ CÁNH CỨNG CỦA VI KHUẨN *BACILLUS THURINGIENSIS* TRONG *E. COLI*

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Ở Việt Nam, các nghiên cứu về thuốc trừ sâu BT (*Bacillus thuringensis*) cũng như chuyển gen *Bt* vào thực vật chủ yếu tập trung vào phòng trừ sâu hại bộ cánh vảy (*cry1, vip* … diệt sâu xanh, sâu tơ, sâu đục thân…) và chế phẩm BT phòng trừ côn trùng bộ hai cánh (*cry4, cry2* diệt bọ gậy …). Các nghiên cứu về chế phẩm BT và chuyển gen *Bt (cry3, cry8…)* kháng bọ cánh cứng vào cây trồng hầu như chưa có công bố. Mặt khác, trong chuyển gen thực vật, kỹ thuật PCR thường được sử dụng để đánh giá sơ bộ sự có mặt của gen được chuyển vào cây trồng nhưng kỹ thuật này chưa thể khẳng định cây chuyển gen có khả năng biểu hiện gen chuyển thành protein có tính kháng côn trùng hay không. Trong nghiên cứu này, chúng tôi thiết kế và biểu hiện gen *cry8Da* kháng côn trùng bộ cánh cứng trong *E. coli* với mục đích tạo và tinh chế protein Cry8Da tái tổ hợp để sản xuất kháng thể nhằm kiểm tra protein diệt sâu ở các cây trồng được chuyển gen cũng như tạo chế phẩm BT diệt bọ cánh cứng. Đoạn gen *cry8Da* mã hóa protein tinh thể độc tố diệt côn trùng bộ cánh cứng được gắn vào vector biểu hiện pET32a(+) tại vị trí cắt hạn chế *Bam*HI và *Sac*I. Plasmid tái tổ hợp pET32a(+)-*cry8Da* được biểu hiện trong tế bào *E. coli* BL21(DE3)LysS. Protein Cry8Da tái tổ hợp thu được có khối lượng phân tử khoảng 96 kDa và biểu hiện tối ưu ở các điều kiện: nhiệt độ 30 $^{0}$C, nồng độ IPTG 1 mM trong 4 giờ. Protein tái tổ hợp được tinh sạch bằng cột sắc kí ái lực $Ni^{2+}$, có nồng độ trung bình 0,45 mg/ml. Sự biểu hiện protein này được khẳng định bằng Western blot với kháng thể kháng Cry8Da chuẩn.

*Từ khóa: Bacillus thuringensis*, côn trùng, bộ cánh cứng, gen *cry8Da*, protein tái tổ hợp