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Heterologous Expression and Purification of Cry1Ac22 Toxin from *Bacillus thuringiensis* W015-1

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Abstract A *cry1Ac22* gene was amplified by PCR from *Bacillus thuringiensis* strain W015-1 isolated from diapausing larvae of silkworm (*Bombyx mori*). The full-length gene was ligated into the prokaryotic expression vector *pQE30* to construct the recombinant plasmid *pQE30-Cry1Ac22*. The *pQE30-Cry1Ac22* was transformed into competent cell of *E. coli* host strain M15 and then induced by IPTG to express His-tag-Cry1Ac22 fusion protein. The results showed that the His-tag-Cry1Ac22 was highly heterologous expressed in the presence of inclusion bodies in *E. coli* cell. Inducing experiments with different IPTG concentrations and temperatures revealed that the optimum condition for the expression of the fusion protein was 1 mmol/L IPTG and 28℃. SDS-PAGE analysis demonstrated that the host with *pQE30-Cry1Ac22* generated a 133 kD His-tag-Cry1Ac22 fusion protein. The His-tag-Cry1Ac22 fusion protein was purified with affinity chromatography on a Ni^{2+}-NTA resin column. Larvacidal assays were performed and showed that the engineered bacterial lysate and purified protein exhibited high insecticidal activity against second instar larvae of *Plutella xylostella*. This study might provide a basis for the preparation of antibody and for the determination of insecticidal activity using heterologous Cry1Ac22 protein.
Keywords *Bacillus thuringiensis* W015-1, Cry1Ac22, Fusion protein, Heterologous expression, *in vitro* Purification, Larvacidal assay

Background

Bacillus thuringiensis can generate insecticidal crystal protein during sporulation. Since Schnep and Whiteley isolated the first *Bt* insecticidal crystal protein in 1981 (Schnepf et al., 1981), nearly five hundred *cry* genes have been cloned in the world, with the majority belonging to the Cry1 family of genes. The Cry1A genes are listed in nine subfamilies including *cry1Aa*、*cry1Ab*、*cry1Ac*、*cry1Ad*、*cry1Ae*、*cry1Af*、*cry1Ag*、*cry1Ah*、and *cry1Ai*. These contain a total 93 hylotypes, while there are 34 genes in the cry1Ac subfamily (Crickmore et al., 2011, http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/ Bt/).

Cry1Ac insecticidal crystal protein was first found in *Bacillus thuringiensis* spp *Kurstaki* strain HD-73, which produces bipyramidal crystal proteins during sporulation with molecular weights between 129 kD to 138 kD. *Bt* HD-73 is recognized as an important standard strain due to well documented researches. The extensive application of transgenic plants with the *Cry1Ac* gene for lepidopteran control has lead to acquired resistance in Lepidoptera to the Cry1Ac crystal protein and has attracted global attention. Therefore, it becomes a more urgent task to look for novel *Bt* strains and diverse insecticidal crystal proteins to avoid the development resistance.

Bt W015-1 strains were isolated from the guts of diapausing silkworm (Bombyx mori) by Haide Institute of Tropical Agricultural Resources (HITAR),

and its insecticidal activity was greater than that of known HD-73 strains (Xie et al., 2010).

Bt W015-1 strains can synthesize crystalline inclusions during sporulation with a molecular mass of 133 kD. The crystal protein possesses different restriction enzymatic digesting sites compared to that of *Bt* strain HD-73.

In this research we constructed the prokaryotic expression vector, pQE30-Cry1Ac22, to heterologously express in *Eescherichia coli* M15 and purified the inclusion protein His-tag-Cry1Ac22 in order to understand the characteristics and functions of the CryAc22 insecticidal crystal protein.

1 Results and analysis

1.1 The construction and identification of pQE30-Cry1Ac22 prokaryotic expression vector

We amplified the *Cry1Ac22* gene, in the length of about 3 500 bp, from the plasmid DNA of *Bt* strain W015-1 ligated to the sequencing vector named as pMD18-T-Cry1Ac22 which was identified by the restriction enzymes *Bam*HⅠ and *Sal* Ⅰ (Figure 1A).

Sequencing analysis confirmed the amplified gene to be nearly identical to the Cry1Ac22 deposited in the GenBank. We ligated the gene from the pMD18-T-Cry1Ac22 into the prokaryotic expression plasmid pQE30 to construct *E.coli* expression vector by cutting with the restriction enzyme *BamH*Ⅰ and *SaI*Ⅰ (Figure 1B). Further, the recombinant plasmid was validated by the restriction enzyme *Bam*HⅠ and named pQE30-Cry1Ac22 (Figure 1B).

The targeted gene *cry1Ac22* in the recombinant has a length of about 3.5 kb, while its vector pQE30 has a length of 3.4 kb. It is too similar in length to be distinguished by digestion with *Bam*HⅠ and *SaI*Ⅰ followed by agarose electrophoresis. Instead, we used a single restriction enzyme, *Bam*HⅠ, to cut the recombinant to obtain a single band about 6.9 kb in length.

The pQE30-Cry1Ac22 plasmids were transformed into competent cells of *E.coli* M15 and single positive colonies were identified by PCR (Figure 2). Figure 2 shows that lane 1 to lane 5 were positive transformants which confirmed that the recombinant plasmid had been transformed into host bacterial cells of *E.coli*. The transformed *Escherichia coli* harboring plasmid pQE30-Cry1Ac22 was induced with 1 mmol/L IPTG to produce the recombinant proteins and 7.5% polyacrylamide gel electrophoresis was performed on the induced lysate to identify the inclusion protein. The results of SDS-PAGE showed that a distinct band of about 133 kD in molecular weight existed in the induced bacteria lygate transformed with pQE30-Cry1Ac22, whereas the band was lacking in the control without induction (Figure 3). The results indicated that recombinant plasmid pQE30-Cry1Ac22 should produce fusion protein 6× His-Cry1Ac22 under the induction of IPTG, and that the expressed amount of the fusion protein was increased gradually with the increase of inducing time (Figure 3).

In order to stabilize harvest of the expressed fusion proteins, we optimized the conditions for expressing the fusion proteins under temperature at 21℃, 28℃ and 37℃ and concentrations of IPTG at 0.1 mmol/L, 0.5 mmol/L and 1.0 mmol/L. The optimum condition was identified as 1.0 mmol/L IPTG concent ration at

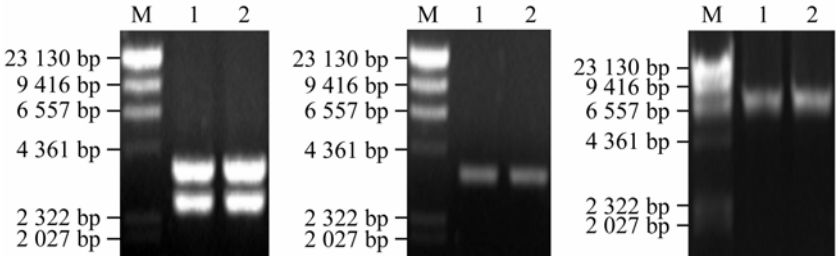

Figure 1 Construction and identification of pQE30-Cry1Ac22

Note: M: λDNA/*Hin*dIII marker; A: pMD18-T-Cry1Ac22 digested with *Bam*HⅠand *Sal*Ⅰ; 1~2: pMD18-T-Cry1Ac22; B: pQE30 digested with *Bam*HⅠand *Sal*Ⅰ; 1~2: pQE30; C: pQE30-Cry1Ac22 digested with *Bam*HⅠ; 1~2: pQE30-Cry1Ac22

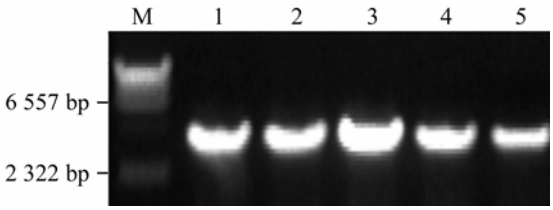

Figure 2 Identification of the positive colony by PCR
Note: 1~6: Tested transformants individually picked; M: λDNA/*Hin*dIII marker

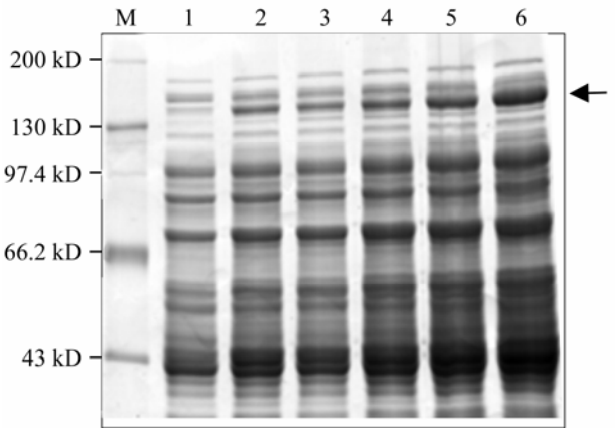

Figure 3 The expression of 6×His-Cry1Ac22 analyzed by SDS-PAGE
Note: M: PR1500 Protein Marker; 1~6: Lysate collected at the time of 0 min, 30 min, 60 min, 90 min, 120 min, 180 min after IPTG induction; Arrow indicates the expressed 6×His-Cry1Ac22 fusion proteins

28℃ based on the results of SDS-PAGE (Figure 4).

1.2 Expression of fussion protein in *E.coli* M15

We conducted an experiment for the expression of Cry1Ac22 inclusion protein under conditions of 120 r/min shaking culture at 28℃ and induced by IPTG 1.0 mmol/L for 3 h, 6 h, 9 h, 12 h to observe the difference in protein expression among the transformants (Figure 5). The results showed that the amount of the expressed proteins was gradually increased with the length of ITPG induction. The different transformants exhibited their different expression levels under the fixed culture condition induced for 12 h (Figure 6).

1.3 Purification of the fusion protein

We purified the inclusion proteins expressed in *E.coli* using the urea-denaturation procedures. Bacterial lysate (before purification) and fractionated peak eluent (after purification) which both contained target proteins were collected and analyzed by SDS PAGE. The target protein band presented in the range of about 133 kD indicating that the fusion protein would be the dominant component in the profile of whole proteins in *E.coli*. The purity of the protein fractionated with Ni^{2+}-NTA resin is about 80% (Figure 7). The concentration of the protein was detected to be 1 177 μg/mL.

1.4 Larvacide assay

Engineered bacterial lysate cultured for 20 hours and purified fusion protein were diluted and sprayed on Chinese cabbage leaves. Second instar larvae of diamondback moth, *Plutella xylostella* were introduced onto the leaves and held in an incubator at 25℃. The results showed that all larvae on treated leaves were dead after 30 hours, whereas the control larvae, normally, meaning that the engineered bacteria and its purified protein are toxic to the lepidopteran (data not shown).

2 Discussion

Making a prokaryotic fusion gene construct is a common and effective way to express an exogenous gene in *E.coli*. The prokaryotic expression vector pQE30 we used in this research is the most popular plasmid vector, having the characteristics of small molecular size (3.4 kb) and easy manipulation. It also has excellent features in its plasmid structures such as the replicon and ampicillin encoding gene from pBR322, the intense T5 promoter and the purifying tag that encodes six histidines (The QIA expressionist. 2001).

E.coli M15 is the host strain that specificly matche with the pQE30 vector to express foreign protein. Using this strain can express exogenous gene to generate 6×His tagging inclusion protein. Fusion protein expressed in *E.coli* M15 usually has good stability and is not easily degraded by bacterial protease and the expressed inclusion proteins are easily fractionated by Ni^{2+}-NTA resin affinity chromatography (Huang et al., 2008).

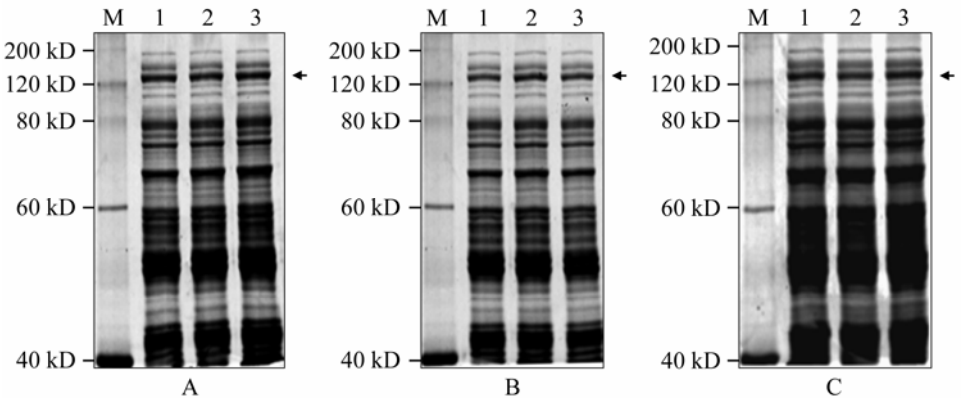

Figure 4 Effect of different IPTG concentrations and temperatures on the expression of fusion protein
Note: M: Protein Marker; A: induced by IPTG under 20℃ for 1 h; B: Induced by IPTG under 28℃ for 1 h; C: Induced by IPTG under 37℃ for 1 h; 1~3: IPTG concentration are 0.1 mmol/L, 0.5 mmol/L, 1 mmol/L, respectively; Arrow indicates the fusion proteins

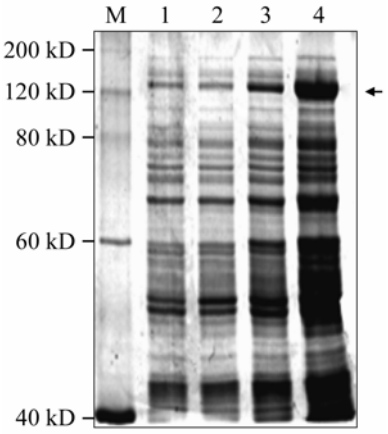

Figure 5 The large scale expression of Cry1Ac22 inclusion
Note: M: Protein Marker; 1~4: Induced by 1 mmol/L IPTG under the IPTG induction for 1 h, 3 h, 6 h, 12 h at 28℃; Arrow indicates the fusion proteins

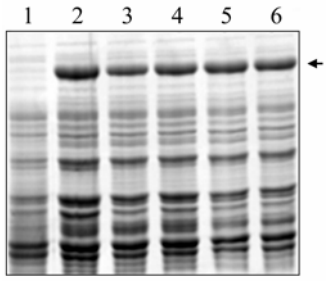

Figure 6 The fusion protein expression of different transformants induced by 1 mmol/L IPTG for 12 h at 28℃
Note: 1~6: Different transformants; Arrow indicates the fusion proteins

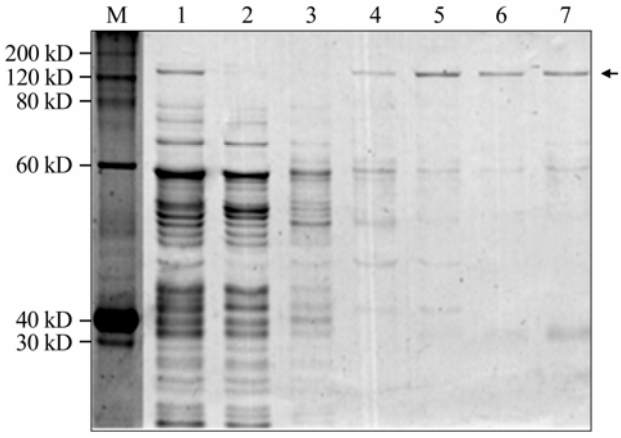

Figure 7 Batch purification of the fusion protein
Note: M: Protein marker; 1: Crude cell lysate; 2: The flow-through of crude cell lysate; 3: 8 mol/L urea wash; 4~7: The first, second, third and forth elutes after elution with 8 mol/L urea elution buffer D (pH 5.9); Arrow indicates the purified 6xHis-Cry1Ac22 fusion proteins

Inclusion body is the major form of foreign protein expressed in the bacteria, whose protein agglutinates in the host cell to develop active granules in sizes of 0.5~1 micron (μ). Inclusion body is insoluble in water while easy dissolved in some denaturants such as urea and hydrochloric acid (Haacke et al., 2008). The formation of inclusion badies will facilitate the high expression of foreign protein and prevent protein degradation by proteinase as well as avoiding poisoning the host cell from foreign protein (Hao et al., 1996).

In the present research, Cry1Ac22 fusion protein

expressed in *E.coli* exists in the form of inclusion bodies. These are a kind of toxic protein existing in the host cell which reduces the concentration of the foreign protein and alleviates poisoning the host cells, increases the amount of protein expression. We think that purifying Cry1Ac22 protein using urea as denaturant will little affect the structure and function, which will be helpful to further study the physic-chemical characteristics and *in vitro* toxicity.

3 Materials and Methods

3.1 Strains, plasmids and chemicalls

Details of the strains and plasmids used in this experiment are listed in Table 1. *Taq* DNA polyrmerase, dNTP, DNA ladder marker and λDNA /*Hin*dIII marker were bought from DEMei Biotechnology Co. Ltd; *Sal* Ⅰ and *Bam*H Ⅰ were from TaKaRa Firm. Ni^{2+}-NTAn resin is the production of Novagen Company.

3.2 Cry1Ac22 PCR amplification

A pair of primers was designed for amplifying *Cry1Ac22* gene based on the sequence deposited in GenBank with accession no Eu282379, while *Bam*H Ⅰ and *Sal* Ⅰ restriction enzymic cutting sites were introduced into the termination of the primers. The forward primer is as GGA TCC ATG GAT AAC AAT CCG AAC ATC, and the reverse primer as GTC GAC TGA GTT TGC ATG AGA CTA TTC. The underlined letters refer to the restriction enzymic cutting sites of *Bam*H Ⅰ and *Sal* Ⅰ. The target gene was PCR amplified using plasmid DNA from *Bt* W015-1 under the conditions of PCR amplification as follows: Pre-denaturation at 95℃ for 5 min, followed by 30 cycles (94℃ 30 s, 54℃ 30 s and 72℃ 30 s), and finished at 72℃ for 5 min.

3.3 Construction procedures of recombinant expression vector

The enzymic cutting vector pQE30 and amplified Cry1Ac22 were mixed at a ratio of 3 moles to 1 mole in the ligation reaction mixture and deionized water was added to a final volume 20 μL. Prior to transformation, the mixture was held overnight in an ice bath at 16℃ to make pQE30-Cry1Ac22. 4 μL ligation product were placed in an ice bath with defrosting M15 competent cells for 30 min, prior to heat shock at 42℃ for 90 s, and then 400 μL LB medium were added and the culture was shaken for 1 hour at 37℃. 100 μL of the transformed liquid was placed on LB screening plates with 100 μg/mL ampicillin and 50 μg/mL kanamycin at 37℃ for overnight culture. Single colonies were picked and used for PCR to identification of positive recombinant clones.

3.4 Expression of fusion protein His-tag-Cry1Ac22 induced and optimized

A positive single bacterial colony was incubated in 5 mL LB liquid culture medium which was shaken overnight at 37℃, and then diluted with fresh medium at a ratio of 1:50 to increase the culture. Recombinant protein was then induced by adding 1.0 mmol/L IPTG at 37℃ when the OD_{600} reached about 0.8. 1 mL samples of culture medium were collected every 30 min.

Table 1 Strains and plasmids used in this research

Strains and plasmids	Characterization	Origin	References
pMD18-T	Amp^R	This lab	
pQE30	Amp^R, T5 expression vector	This lab	
E. coli M15	Amp^R , Nal^s, Str^s, Rif^s, Thi^-,Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+ with $Kana^R$	This lab	
E. coli JM109	*E. coli* recA1, supE44, endA1, hsdR17, gyr A96, relA1 thi△(lac-proAB)	This lab	Sambrook et al., 2002
Bt W015-1	Wild strain of *B. thuringiensis*	This lab	Xie et al., 2010

The induced strains were collected by centrifugation at 4 000 r/min for 20 min and, after discarding the supernatant, the pellet was frozen at -80℃ for 2 hours, before completely suspending by first adding 5 mL suspension liquid and continuing to add suspension liquid to a final volume of 15 mL. Lysozyme was then added at 1 mg/mL to digest while shaking for 30 min in the ice bath.

Conditions for optimun expression were carried out under the IPTG concentrations of 0.1 mmol/L, 0.5 mmol/L, 1.0 mmol/L and temperatures at 20℃, 28℃, 37℃. Lysates were collected by centrifugation at 10 000 r/min at 4℃ for 40 min and 1 mL supernatant used for preparing samples for SDS-PAGE detection (Bradford, 1976).

3.5 The purification of His-tag-Cry1Ac22 fusion protein

Affinity chromatography with Ni^{2+}-NTA Resin Column was used to purify the His-tag-Cry1Ac22 fusion protein. Supernatants were collected by super speed centrifugation and the pellet was transferred onto the Ni^{2+}-NTA Resin Column for 2 h. Collect Recombinant bacterium cells were collected and cooled in an ice bath for 15 min and then Buffer B was added to re-suspend the collected pellet. The suspension was stirred for at least 15 min, (maxmum 60 min) at room temperature until the lytic reaction was finished. Supernatants were collected by super speed centrifugation at 10 000 g for 20 to 30 min, then transferred onto the Ni^{2+}-NTA Resin Column. Buffer C was initially used to wash the column while collecting the fractionation liquid for SDS-PAGE analysis. This was followed by four washings with Buffer D and a final four washings with Buffer E to collect the respective liquid fractions for analysis by SDS-PAGE.

3.6 Bioassary

Second instar larvae of *Plutella xylostella* were provided by HITAR (Haide Institute of Tropical Agricultural Resources). Larvacidal assays followed the procedures of Xie et al (2010).

Authors' contributions

ZML and SKL are the persons who executed this research and prepared the manuscript experiment; YZL partly managed the project and was involved in data analysis. XJF is the principal investigator for this project in charge of conducting experimental design, data analysis and preparing the manuscript. All authors have read and consent to the final version of this paper.

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