Characterization and Analysis of Sip1Aa Protein Expressed by cry1Ac Promoter

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Abstract

In this study, based on the pUC19 vector and using overlapping PCR technology, the researchers constructed an expression vector of the Sip1Aa protein guided by a *cry1Ac* promoter. The expression situation, insecticidal activity and solubility were researched, and the expression of the Sip1Aa protein, as guided by a T7 promoter, was compared. Additionally, the fermentation conditions were explored on a preliminary basis and a histidine label was added to the recombinant plasmid via reverse PCR for subsequent purification of recombinant proteins. The results showed that both the *cry1Ac* and T7 promoters could guide Sip1Aa to express as a soluble protein of 37.6 kDa, and there was no significant difference in insecticidal activity against *Colaphellus bowringi* Baly, with LC50 values of 1.637 mg/mL and 1.683 μg/mL, respectively. The soluble component of the Sip1Aa protein, when guided by the *cry1Ac* promoter, was significantly higher than when it was guided by the T7 promoter. The expression of the *cry1Ac* guider-promoted Sip1Aa protein was more suitable at 37℃ and 16 h. The recombinant protein was purified after an exogenous histidine sequence was added. This provides a new research method and idea to solve the problem of the Sip1Aa protein usually producing a large number of inclusion bodies when expressed in *E. coli*, and provides new ideas for the study of *sip* gene rapid expression, functional verification and insecticidal mechanisms.

Introduction

Bacillus thuringiensis (Bt), which widely exists in nature as a gram-positive bacteria (Lee, Shisa, Wasano, Ohgushi, & Microbiology; Ohba et al., 2002; Raymond, Johnston, Nielsen-LeRoux, Lereclus, & Microbiology; Swiecicka, Fiedoruk, & Bednarz, 2002; Tokio et al.), can produce a variety of insecticidal proteins, such as Insecticidal crystal proteins (ICPs)(Palma, Mu?oz, Berry, Murillo, & Caballero), Vegetative insecticidal proteins (VIPs)(Chakroun et al., 2016) and Secreted insecticidal protein (Sip) (Donovan et al.; Estruch et al.; Milne, Liu, Gauthier, & Frankenhuyzen; Sanahuja, Banakar, Twyman, Capell, & Christou, 2011). Bt has high specific insecticidal activity against a variety of insects, nematodes and other invertebrates, and has become the most widely used microbial insecticide and transgenic insect-resistant breeding resource in the world (Jizhen et al.; Qiu, 2013; Xu et al.; Zhu et al., 2013).

At present, there are few studies on Bt Sip proteins. One such study was conducted by Sha Junxue, who cloned a novel *sip* gene containing 1095 bp and encoding 364 amino acids from Bt strain QZL38, named *sip1Ab*. The first 90 bp signal peptide was removed from *sip1Ab*, and the protein in the code region of Sip1Ab, which contained 1005 bp, encoding 334 amino acids, was further amplified. The protein was named Sip1Aa. The results showed that Sip1Aa produced a soluble protein of 37.6 kDa, and the LC50 value of Sip1Aa against *Colaphellus bowringi* Baly was 1.051 μg/mL.(Sha et al., 2018).

pET-series vectors are usually used to express Sip proteins, while the T7 promoter of such vectors has a strong effect, and the transcription initiated after recognition by T7 RNAP is very active, resulting in either excessive protein expression or improper folding of the environment, thus forming a large number of inclusion bodies(Ejima et al., 1999; Kojima, Miyoshi, & Miura; Zhang, 2012). *Bacillus thuringiensiscry1Ac*

promoter can express insecticidal proteins in large quantities and is often used to construct protein expression vectors(Ma Junlan et al., 2011), while the *cry1Ac* gene promoter is regulated by germinal transcription initiation factors σE□σK, etc., and has a high similarity with the factor of *Escherichia coli*(Sedlak, Walter, & Aronson, 2000). Studies have shown that the σ factor of *E.coli* could bind to the *cry1Ac* promoter to direct the expression of exogenous genes in *E.coli*(Schnepf, Wong, & Whiteley). Liu Ming successfully expressed the Vip3Aa protein guided by a *cry1Ac* promoter in *E.coli* with the same size and similar insecticidal activity as that expressed by the T7 promoter(Liu, Sun, & Gao, 2017).

In order to solve the problem of the Sip1Aa protein forming a large number of inclusion bodies when guided by the T7 promoter of *E.coli*, as well as increase the production of soluble components of Sip1Aa, this study took the Sip1Aa protein as the research material, with its expression guided by the *cry1Ac* gene promoter in *E.coli*. The purpose was to explore the expression method of the guided Sip protein, provide a new method for efficient expression of the Bt Sip protein specifically and offer a new idea for the functional verification and insecticidal mechanisms of Sip proteins generally.

Materials And Methods

Strains, plasmids and reagents

Bt strain SH8 with gene *cry1Ac* was isolated in our laboratory, while the pUC19 plasmid was purchased from TaKaRa (Japan). The Kod-plus DNA polymerase was purchased from TOYOBO (Japan) and the restriction endonuclease was purchased from TransGen (China). Finally, the ClonExpress II One Step Cloning Kit and Taq Plus Master Mix were purchased from Vazyme (China).

Bt genome extraction

Bt strain SH8 was cultured overnight at 30℃ on solid LB medium for 12 h, and the genome was extracted by the same method implemented by Zhang Yanrui(Zhang, 2012).

Primers and PCR reaction

The primers used in this study are shown in Table 1. “£” is the overlap of two primers, and the underlined part is the cutting site. The PCR reaction system was 50 μL, including a 1 μL template, 10 μmol/L primers (1 μL each), 5 μL dNTPs, 5 μL 10×KOD Buffer, 3 μL MgSO4 and 1 μL KOD-plus DNA polymerase. The ddH_2O was supplemented to 50 μL and the PCR products were recovered. A pUC19 vector was digested with *Hind* III and *EcoR* I enzymes and, after the gel was recovered, a homologous recombination reaction was performed according to ClonExpress II One Step Cloning Kit instructions, and the JM109 was transformed into a competent state, which was cultured at 37 ℃ for 12 h. Positive clones were identified with primers M13F and M13R. It was sequenced by Comate, and DNA sequences were analyzed by DNAMAN.

Optimization of fermentation conditions

In order to optimize the expression condition of the *cry1Ac* promoter-directed Sip1Aa protein, the fermentation broth was diluted 30 times by blank medium at 37℃ and 220 r/min, and the OD_{600} value of the absorbance of the broth was measured at each culture time (four-hour intervals). The bacterial growth curve was then plotted based on this.

Expression and Extraction of Sip1Aa guided by cry1Ac and T7 promoters

The expression of the *sip1Aa* gene was directed by either the *cry1Ac* or T7 promoter in *E. coli* BL21 (DE3). The recombinant strain was pre-cultured overnight at 37℃ and 220 rpm in a 5 mL LB medium containing either 100 μg/mL ampicillin or 50 μg/mL kanamycin. The culture was transferred to 100 mL of LB medium. When the OD_{600} reached 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to achieve the final concentration of 1.5 mM. The culture continued to grow for an additional 14 h at 16℃ and 160 rpm, with the final culture being centrifuged at 8000 rpm for 5 min at 4℃. The supernatant was discarded and the cells were resuspended in 30 mL of pre-chilled PBS buffer (20 mM sodium phosphate, 0.5 M NaCl, pH 7.4). This process was repeated twice before re-centrifuging at 8000 rpm for 5 min at 4℃. The pellet was then resuspended with 5 mL of PBS buffer (pH 7.4). The bacteria were broken by lysozyme and ultrasonic vibration in an ice-water mixture (Ampl 80%, pulse on 3 s, pulse off 3 s, 10 min total), then centrifuged at 12,000 rpm for 15 min at 4 ℃ in order to remove the insoluble material. Finally, the supernatant was filtered through a 0.22 μm filter, and the pellet was resuspended in PBS buffer (pH 7.4) and the suspension was then collected. All of the collections were analyzed by SDS-PAGE electrophoresis, with the estimation of the protein concentration was performed using BSA standards and Image J software.

Purification of pAc19Sip1Aa protein

According to Qi's method(Qi Xianghui, 2010), a histidine label was added to the plasmid pAc19sip1Aa by reverse PCR, with His-F and His-R as primers, and purified using nickel affinity chromatography. According to the method in the protein purification kit (ComWin Biotech, Beijing, China), five column volumes of deionized water was added to the filled column in order to rinse the ethanol, then the column was balanced with a binding buffer (20 mmol/L Na3PO4, 0.5 mol/L NaCl, 40 mmol/L imidazole) of 10 column volumes. At the end of equilibration, 5 mL of soluble protein was added. The column containing the 15 column volumes of binding buffer was subsequently rinsed in order to remove the impurities. The purified protein was collected by elution with an appropriate amount of elution buffer (20 mmol/L Na3PO4、0.5 mol/L NaCl、500 mmol/L imidazole) and verified using SDS-PAGE. Following elution, the column was washed with deionized water (10 column volumes), after which the column was balanced with 20% ethanol (three column volumes). The column was sealed and stored at 2~8℃.

Protein solubility analysis of Sip1Aa

According to the method used by Ma(Ma Junlan et al., 2011), and under the same conditions, the protein precipitate was suspended with 50 mmol/L Na2CO3 (pH 10.5, 3% coa-mercaptoethanol, 2 mmol/L DTT), mixed evenly, then incubated at 37 ℃ for 1 h and centrifuged at 12000 rpm for 15 min, resulting in

absorption of the supernatant and suspension of insoluble matter in sterile water of the same volume as the supernatant. SDS-PAGE was used to detect the soluble and insoluble components.

Insects and bioassays

The standard Colaphellus bowringi Baly used in this study was donated by the Institute of Plant Protection (IPP), Chinese Academy of Agricultural Sciences (CAAS). An analysis of toxicity in Colaphellus bowringi Baly was conducted on the second instar larvae with fresh cabbage using a leaf-dip bioassay(Jinbo, Haitao, Rongmei, Changlong, & Control, 2015), the process being performed in triplicate using different concentrations of crude-extracted Sip1Aa protein guided by the *cry1Ac* and T7 promoters. Empty plasmid pUC19 was used as the negative control and, for each concentration and control, 16 second instar larvae were used. The number of dead insects was recorded and insect mortality was calculated after two days of larvae exposure at 27°C, 55±5% RH, and a 14/10 h light/dark cycle. The corrected mutant-protein mortality rate of the insects was calculated according to the number of dead larvae in the control group. In addition, after purification, the soluble protein was diluted into six concentration gradients for the measurement of insecticidal activity and the LC50 value was measured using POLO-PC software. Each bioassay was repeated in triplicate.

Results

Construction of pAc19sip1Aa

The 368 bp *cry1Ac* promoter sequence was amplified using Bt strain SH8 genomic DNA as the template and c1AcPF/c1AcPSip1AaR as the primers. The sequence of 1002 bp *sip1Aa* was amplified using plasmid pET28a-*sip1Aa* as the template and Sip1Aac1AcPF/Sip1AaR as the primers. Both PCR-recovery products were used as templates, and c1AcPF/Sip1AaR were used as the primers to amplify the overlapping sequences of the *cry1Ac* promoter and *sip1Aa* (Fig 1A).

PCR products were recovered and homologously recombined into a pUC19 vector digested by *Hind* III and *EcoR* I, and transformed into *E. coli* JM109. It was verified by enzyme digestion (Fig 1B), identified by PCR (Fig 1C) and further verified by sequencing. The identified recombination was named pAc19*sip1Aa*.

Optimization of fermentation conditions

The growth curve of the strain of pAc19sip1Aa is shown in Fig 2. The OD600 value of the strain increased exponentially during the first 16 h, then reached a plateau during the period from 16 h to 48 h. After 48 h, the OD600 value of the strain started to decline.

The crude protein was extracted after the pAc19*sip1Aa* culture for 16 h, 24 h and 36 h, respectively, as shown in Fig 3. The SDS-PAGE and Image J software analyses showed that the culture time had little effect on the protein expression when the strain reached saturation at the same temperature.

Expression of Sip1Aa guided by cry1Ac and T7 promoters

The Sip1Aa protein was expressed by both the *cry1Ac* and T7 promoters at 10 μL (Fig 4). SDS-PAGE analysis showed that the *cry1Ac* promoter could direct the soluble expression of Sip1Aa in *E. coli* BL21, and the protein band size was the same as that of the T7 promoter in *E. coli* at 37.6 kDa. In terms of protein expression, Image J showed that the protein expression level of Sip1Aa, as expressed by the T7 promoter, was slightly higher than when it was expressed by the *cry1Ac* promoter.

Purification of pAc19Sip1Aa protein

The successful purification of the protein pAc19Sip1Aa indicates that the histidine tag was successfully added, and the expression of pAc19Sip1Aa after the histidine tag was added is consistent with that of the protein without histidine, indicating that the addition of exogenous histidine does not affect the protein expression

Compare the protein solubility of Sip1Aa expressed by two promoters

The Na_2CO_3 with 50 mmol/L suspended Sip1Aa inclusion body protein was expressed by both the T7 and *cry1Ac* promoters in *E. coli* and the soluble and insoluble components were analyzed by SDS-PAGE. The result, shown in Fig 5, was that the Sip1Aa protein directed by the T7 promoter was difficult to dissolve in 50 mmol/L of Na_2CO_3 solution (most of it was insoluble). However, the Sip1Aa protein expressed by the *cry1Ac* promoter was largely dissolved in 50 mmol/L of the same solution with relatively few insoluble components.

Bioassay

The soluble proteins pAc19Sip1Aa and pET28aSip1Aa, as well as the alkali-soluble protein of pAc19Sip1Aa, were quantified by Image J software and standard BSA, diluted to 20, 5 and 0.5 μg/mL, respectively, to determine the qualitative insecticidal activity of *Colaphellus bowringi* Baly. After 48 hours, the dead and live insects were counted and the adjusted mortality rate was calculated. The alkali-soluble protein pAc19Sip1Aa still had a toxic effect on the *Colaphellus bowringi* Baly, and the adjusted mortality effect was not significantly different from those of soluble proteins pAc19Sip1Aa and pET28aSip1Aa.

After purification of soluble protein pAc19Sip1Aa and pET28aSip1Aa, the quantitative insecticidal activity on *Colaphellus bowringi* Baly was determined by gradient dilution to six concentrations of 50, 20, 10, 5, 1 and 0.1 μg/mL. The LC50 value was calculated 48 h later. The results showed that the LC50 of pET28aSip1Aa was 1.683 g/mL, and the 95% confidence interval was 1.135-2.409. The LC50 of pAc19Sip1Aa-BL21 for *Colaphellus bowringi* Baly was 1.637 g/mL, with a 95% confidence interval of 0.762-3.086. There was no significant difference between the two protein at the level of 0.05.

Discussion

In this study, a *cry1Ac* promoter was used to expressed a Sip1Aa protein for the first time. In previous studies of the Sip1Aa protein, it was found that it produced a large number of inclusion bodies, which

greatly affected the production of soluble protein and increased the difficulty of protein purification(Sha et al., 2018). For the study of biological activity and insecticidal mechanism, a large amount of soluble protein was needed, so it was very important to improve the solubility of the protein. The transcription of the T7 promoter, as recognized by the T7 RNAP, was very active, resulting in excessive protein expression and the formation of a large number of inclusion bodies(Zhang, 2012). While the *cry1Ac* promoter was recognized by the σ factor of *E. coli* and started the transcription as well, the solubility of the Sip1Aa protein expressed by the two promoters (T7 and *cry1Ac*) was quite different, which may have been caused by differences in the regulatory mechanism of their expression.

In this study, a *cry1Ac* promoter was used to express protein Sip1Aa in the vector pUC19. The pUC19 vector did not have a His tag sequence, so the recombinant protein could not be purified. In accordance with Qi's method(Qi Xianghui, 2010), an exogenous histidine sequence was artificially added to the plasmid pAc19*sip1Aa*, so that the histidine tag could be expressed together with the exogenous gene to form a fusion-recombinant protein, which could then be purified using nickel affinity chromatography.

In this study, a *cry1Ac* promoter was used to express the Sip1Aa protein derived from Bt in *E.coli*. Based on this vector, a series of expression vectors guided by the *cry1Ac* promoter could be further constructed for the rapid and efficient expression of Sip and other proteins as well as the reduction of insoluble inclusion body proteins. The results of this study provide a new method for the rapid and high-quality expression of the Sip protein, and create favorable conditions for the further study of the expression, functional verification and insecticidal mechanism of this protein.

Conclusions

A *cry1Ac* promoter was successfully used to express a Sip1Aa protein in *E.coli* by overlapping PCR. The protein was the same size as the Sip1Aa protein expressed by the T7 promoter, with no significant difference in insecticidal activity to the *Colaphellus bowringi* Baly. The protein pAc19Sip1Aa was purified by adding a His label to the plasmid pAc19*sip1Aa*. Solubility analysis showed that most of the Sip1Aa protein precipitation directed by the *cry1Ac* promoter could be fully dissolved by Na_2CO_3 solution, while the Sip1Aa protein precipitation directed by the T7 promoter remained as insoluble components. The preliminary investigation of fermentation conditions showed that, when the strain reached saturation, the fermentation time had little effect on protein concentration, however, further optimization of fermentation conditions was needed to express insecticidal proteins in large quantities.

Declarations

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Conflicts of interest: The authors declare no conflict of interest.

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Authors' contributions: Jing Wang, Haitao Li, and Jiguo Gao contributed to the conception and the design of experiments. Jing Wang, Lin Wang, Mingyue Ding and Jun Cui performed experiments. Jing Wang and Haitao Li conceived the study and analyzed the results.

Informed consent: All authors read and approved the final manuscript.

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Tables

Table 1. This is a table. Tables should be placed in the main text near to the first time they are cited.

Primers	Sequences (5'-3')
c1AcPF	gaccatgattacgccaagcttGCAGGTAAATGGTTCTAACATGTATAAG
c1AcPSip1AaR	TGGTTTCTGCCATCTCGAGCGGAAGTTACCTCCATCTCTTTTA
Sip1Aac1AcPF	GGTAACTTCCGCTCGAGATGGCAGAAACCAAGTCGCCAA
Sip1AaR	aaaacgacggccagtgaattcATTTCCACTTAAAATCTTTGTTTGA
M13F	CCCAGTCACGACGTTGTAAAACG
M13R	AGCGGATAACAATTTCACACAGG
His-F	AATTCCACCACCATCACCATCATACTGGCCGTCG
His-R	CAGTATGATGGTGATGGTGGTGGAATTCATTTCCAC