ORIGINAL ARTICLE

Production and characterization of the ^{13}C/^{15}N single-labeled insecticidal protein Cry1Ab/Ac using recombinant *Escherichia coli*

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Abstract

Synthetic Cry1Ab/Ac proteins expressed by genetically modified (GM) crops have a high potential to control insect pests without utilizing large amounts of chemical insecticides. Before these crops are used in agriculture, the environmental fate and interactions in the soil must be understood. Stable isotope-labeled Cry1Ab/Ac protein is a highly useful tool for collecting such data. We developed a protocol to produce ^{13}C/^{15}N single-labeled Cry proteins. The artificially synthesized gene *Cry1Ab/Ac* of *Bt* rice Huahui No. 1, which has been certified by the Chinese government to be safe for human consumption, was subcloned into pUC57, and the expression vector pET-28a-*CryAb/Ac* was constructed and transformed into *Escherichia coli* BL21 (DE3) competent cells. Next, 0.2 mM isopropyl thiogalactoside (IPTG) was added to these cells and cultured at 37°C for 4 h to induce the synthesis and formation of inclusion bodies in M9 growth media containing either [U-^{13}C] glucose (5% ^{13}C-enriched) or [^{15}N] ammonium chloride (5% ^{15}N-enriched). Then, Cry inclusion bodies were dissolved in urea and purified by affinity chromatography under denaturing conditions, renatured by dialysis, and further detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The purities of ^{13}C/^{15}N-labeled Cry proteins reached 99% with amounts of 12.6 mg/L and 8.8 mg/L, respectively. The δ ^{13}C and ä ^{15}N values of ^{13}C-labeled Cry protein and ^{15}N-labeled Cry protein were 3,269‰ and 2,854‰, respectively. A bioassay test revealed that the labeled Cry1Ab/Ac proteins had strong insecticidal activity. The stable isotope-labeled insecticidal Cry proteins produced for the first time in this study will provide an experimental basis for future metabolic studies on Cry proteins in soil and the characteristics of nitrogen (N) and carbon (C) transformations. Our findings may also be employed as a reference for elucidating the environmental behavior and ecological effects of BT plants and expressed products.

1 | INTRODUCTION

Cry proteins are crystalline insecticidal delta-endotoxins (Bt toxins or BT) produced in the spores of soil-inhabiting bacteria of the taxon *Bacillus thuringiensis* (*Bt*). There are different Cry proteins expressed with different *B. thuringiensis* strains that vary in their specificity toward target organisms, for example, Cry1Ab proteins act against Lepidoptera and Cry3Ab proteins against Coleoptera. BT-encoding genes have widely been transferred by molecular breeding to many important crops to confer insect resistance. At present, *Cry* genes have become some of the most widely used insecticidal genes in transgenic plant breeding. In 2018, the global planting area of genetically modified (GM) crops has reached more than 191.7 million hectares, of which transgenic BT crops are the second largest in the area after herbicide-tolerant GM crops (James, 2018). The Cry1 protein exhibits highly specific toxicity to Lepidoptera and is encoded by the *Cry1ab/ac* gene, and the fusion of *Cry1ab* (GenBank Accession No. X54939) and *Cry1ac* (GenBank Accession No. Y09787) into a single gene is highly toxic to *Chilo suppressalis*, *Scirpophaga incertulas*, and *Cnaphalocrocis medinalis*, three important lepidopteran insects of rice (Cheng et al., 1998). The codon-optimized *Cry* gene has successfully been transformed into a variety of plants such as tobacco, corn, and cotton, and numerous transgenic plant varieties or germplasm resources with insecticidal traits have been obtained. With the rapid increase in the planting area of BT crops, the potential environmental concerns for applications related to BT crops have attracted the attention of researchers around the world. In terms of environmental risks, one of the main areas of concern is related to their persistence in soil and potential interactions with nontarget organisms (Arpaia et al., 2017). Addressing this issue will influence the metabolic fate of Cry proteins in different types of soil (Li, et al., 2018).

Cry proteins synthesized in the roots, stems, and leaves of BT crops are released into the soil by decaying plant residues (Ahmad et al., 2005), root exudates (Li, et al., 2018; Saxena & Stotzky, 2000), or pollen (Losey et al., 1999). For a *Cry3Bb1*-expressing Bt maize, it has been estimated that approximately 820 g of Cry3Bb1 is synthesized by roots in 1 ha of soil (Miethling-Graff et al., 2010; Nguyen & Jehle, 2007). The rhizosphere Cry protein content of transgenic *Cry1Ac* cotton from samples collected at different time points of growth is between 54.43 and 303.94 pg/g soil and significantly increases at the budding and flowering stages (Li, et al., 2018). To improve the expression level of the insecticidal gene of transgenic plants, researchers have optimized *Cry* gene codons (Jabeen et al., 2010; Li et al., 2013), which probably cause alterations in the structure and function of the Cry protein expressed by the BT plants compared with that expressed by soil indigenous *B. thuringiensis*. Therefore, the Cry protein released from BT plants is regarded as a kind of new-to-nature compound with insecticidal activity (Wang et al., 2007). Consequently, before such BT plants with engineered synthetic proteins were released into the environment for agricultural use, the environmental fate of these new-to-nature proteins must be understood, including their persistence and degradation as well as their activity against nontarget organisms. Currently, the main quantification method for Cry protein is the enzyme-linked immunosorbent assay (ELISA), which is based on the complete extraction of the Cry protein from a sample. Sims and Holden (1996) found that the Cry protein released from transgenic plants was bound tightly to the soil particles and thus difficult to isolate and purify. Therefore, ELISA data may only indicate a decrease in the amount of the initial compound without providing information whether it was degraded, mineralized, or only adsorbed onto surface soil particles including organic matter (Valldor et al., 2012). In terms of the environmental assessment of the released Cry protein, the degradation of the initial Cry protein released into the soil and the transformation pathways of the main elements C and N should be compared with poorly extractable Cry protein in soil by ELISA to determine its absorbance and persistence. Radioactively labeled Cry1Ab proteins have been used in soil microcosm incubation studies, and it was possible to quantify and distinguish between the mineralization and adsorption of the compound onto the soil (Valldor et al., 2015). However, working with radioactive compounds requires laboratory precautions and generates waste that requires careful disposal. Stable isotopic mass spectrometry can be used to trace and quantitatively monitor the transformation, partitioning, and dynamic change processes of the carbon and nitrogen of the Cry protein in different carbon and nitrogen forms. This approach effectively circumvents the disturbance of the indigenous Cry protein in the soil. However, the production of ^{13}C-labeled and ^{15}N-labeled Cry proteins has not been investigated to date.

In this study, we describe how ^{13}C/^{15}N single-labeled Cry proteins with high purity and strong insecticidal activity can be produced using *E. coli* cultivated in a common and simple laboratory growth medium with either ^{13}C-labeled glucose as the sole carbon source or ^{15}N-labeled ammonium chloride as the sole nitrogen source. This biotechnological production can provide the foundation for subsequent experimental analyses on the fate and interactions of recombinant Cry proteins (BT) of transgenic crops for environmental risk assessments.

2 | MATERIALS AND METHODS

2.1 | Strains and plasmids

E. coli (JM109 and BL21 [DE3]), prokaryotic clone vector pUC57, and expression vector pET-28a were purchased from Transgen Biotech Co., Ltd. and preserved by our laboratory. Primers for DNA

fragment identification were purchased from Sangon Biotech Co., Ltd. (Table 1).

2.2 | Reaction reagents

High-fidelity DNA polymerase, *NcoI* and *XhoI* endonuclease, T4 DNA ligase, a MinElute Gel Extraction Kit, and a Plasmid Extraction Kit were purchased from Takara Biotechnology Co., Ltd. The Ni-NTA Spin Kit for the spin purification of His-tagged proteins was purchased from Qiagen Sciences. Glucose (U-^{13}C6, 99%) for the preparation of ^{13}C-glucose substrate with an isotopic purity of 5% and ammonium chloride (^{15}N, 99%) for preparation of ^{15}N-ammonium chloride substrate with an isotopic purity of 5% were purchased from Shanghai Research Institute of Chemical Industry. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. The LB liquid medium was prepared by mixing 10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl, followed by wet heat sterilization at 121°C for 20 min. The M9 medium consisted of 12.8 g/L $Na_2HPO_4\cdot7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1 g/L NH_4Cl, 0.492 g/L $MgSO_4\cdot7H_2O$, 0.02191 g/L $CaCl_2\cdot6H_2O$, and 0.08 g/L glucose.

2.3 | Expression vector construction

2.3.1 | Chemical synthesis and cloning of the *Cry1Ab/Ac* gene

The gene sequence of *Cry1Ab/Ac* (EU816953.1) was retrieved from NCBI, and the terminating codon was removed. *NcoI* and *XhoI* restriction sites were introduced at the 5' and 3' ends, respectively. The *Cry1Ab/Ac* gene was synthesized by Sangon Biotech (Shanghai) Co., Ltd., cloned into plasmid pUC57, and transferred into competent *E. coli* JM109 cells. JM109 cells containing pUC57-*Cry1Ab/Ac* vector were grown on an LB agar plate overnight. The plasmids of positively identified colonies were extracted and further verified with primers *Cry1Ab/Ac*-F/R on a Gene Amp PCR System 9700 (Thermo Fisher Scientific Inc.). PCR was conducted using the following conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 45 s, annealing at 56°C for 45 s, and elongation at 72°C for 120 s; and a final extension at 72°C for 10 min. The reactions were performed using 30 μl of a reaction mixture containing 3 μl of 10× buffer, 3 μl of 2.5 mM dNTPs, 1 μl of each primer (5 μM), 0.3 μl of Taq DNA polymerase, 2 μl of 100 ng/μl template DNA, and deionized distilled water added up to a total volume 30 μl. The amplified fragments were sequenced by Sangon Biotech Co., Ltd.

2.3.2 | Construction and identification of the Cry1Ab/Ac expression vector

First, pUC57-*Cry1Ab/Ac* and pET28a were digested with *NcoI* and *XhoI* restriction enzymes. Then, *Cry1Ab/Ac* and pET28a were ligated by T4 DNA ligase at 22°C overnight. The prokaryotic expression vector pET28a-*Cry1Ab/Ac* was transformed into *E. coli* JM109 cells. The recombinant expression plasmids were checked by *NcoI* and *XhoI* double-restriction digestion, and positive ones were transformed into *E. coli* BL21 (DE3) to express the Cry1Ab/Ac protein.

2.3.3 | Expression of Cry1Ab/Ac fusion protein in *E. coli*

Single colonies grown on agar plates were inoculated into the M9 medium with either glucose as the sole carbon source or ammonium chloride as the sole nitrogen source at 37°C until OD_{600} = 0.6–0.8. IPTG was added to a final concentration of 0.5 mM and incubated at 37°C for 4 h. Then, the bacterial suspension was collected for identification by SDS-PAGE (8% separation gel).

2.3.4 | Induction conditions for optimization of Cry protein expression

The optimal conditions for protein expression with IPTG induction were investigated. The cells were oscillated at 37°C. Cells were cultivated in 500-ml Erlenmeyer flasks filled with 200 ml of the M9 medium at 37°C and shaken at 200 rpm. When the growth culture reached an OD_{600} of 0.6–0.8, protein expression was induced by adding IPTG to reach final concentrations of 0.2 to 1 mM, testing a temperature range of 15–37°C, and periods ranging from 4 to 16 h. The bacterial suspension was collected for SDS-PAGE.

2.3.5 | Amplified expression, purification, and dialysis renaturation of 13C/15 N single-labeled Cry proteins

Under the above-mentioned optimum conditions, the recombinant strains were inoculated in 1 L of M9 medium with either glucose as the sole carbon source ([U-^{13}C] glucose [5% ^{13}C-enriched]) or ammonium chloride as the sole nitrogen source ([^{15}N] ammonium chloride [5% ^{15}N-enriched]). The bacterial solution was collected, mixed with sterile phosphate buffer solution (PBS), and sonicated

TABLE 1 Primers used in this study

Primer	Sequence	Fragment length
Cry1Ab/Ac-F	5'-CGGGATCCATGGACAACTGCCGTCCATACA-3'	1,844 bp
Cry1Ab/Ac-R	5'-CCAAGCTTATTCAGCCTCGAGTGTTGCAGT-3'	

at 4°C for 30 cycles, with each cycle consisting of a 10-s pulse and at 10-s intervals. The precipitation (crude inclusion body) obtained by centrifugation was dissolved in 8 M urea denaturation solution. Then, the supernatant was collected by centrifugation, which was purified using Ni-NTA Spin Kit (QIAGEN China Co., Ltd.) under denaturing conditions according to the manufacturer's instructions. The amount of Cry protein relative to the total protein was assessed by SDS-PAGE.

The purified ^{13}C-labeled and ^{15}N-labeled Cry1Ab/Ac proteins were renatured by dialysis against 8 M urea, 50 mM Tris-HCl (pH 8.0), and 0.4 M L-arginine with stepwise reduction of the urea concentration (6, 4, 2, 1, and 0 M) at 4°C for 6 h. The refolded Cry1Ab/Ac in 50 mM Tris-HCl buffer (pH 8.0) was centrifuged at 14,475 g for 15 min at 4°C to remove the aggregated protein. Finally, the solution was dialyzed overnight at 4°C in PBS (pH 7.2). The relative proportions of the purified ^{13}C-labeled and ^{15}N-labeled Cry proteins were analyzed using the grayscale scanning function of the gel image analyzer (Bio-Rad).

2.3.6 | Western blot analysis and ELISA quantification of 13C/15N single-labeled Cry1Ab/Ac protein

Protein aliquots were transferred onto a nitrocellulose membrane. Cry protein hybridization signals were detected by Western blotting using His-labeled antibody (1:2,000 dilution) (Hamadou-Charfi et al., 2015). The contents of ^{13}C-labeled and ^{15}N-labeled Cry proteins were determined using an assay kit (QuantiPlate™ Kit for Cry1Ab/ Cry1Ac, EnviroLogix Inc.) (Li, et al., 2018).

2.3.7 | δ 13C and δ 15N values of 13C and 15N single-labeled Cry proteins

^{13}C-labeled and ^{15}N-labeled Cry proteins in 1× PBS buffer were lyophilized to obtain dried Cry proteins using a CoolSafe Freeze Dryer (LABOGENE Co., Ltd.). After the samples were packed with tin foil paper, the δ ^{13}C values of the ^{13}C-labeled Cry protein and the δ ^{15}N values of the ^{15}N-labeled Cry protein were determined by element analyzer–isotope ratio mass spectrometry (EA-IRMS, deltaV advantage, Thermo Fisher Scientific).

2.3.8 | Insecticidal bioassay and data analyses

The ^{13}C-labeled and ^{15}N-labeled Cry proteins were incorporated into conventional feeds of *Chilo suppressalis* with different addition gradient levels (1, 5, 10, 15, 20, and 25 µg/g) with conventional diet (Han et al., 2012) as a control. Three replicates were performed for each gradient level. Twenty larvae were incubated in each culture tube for each replicate at 25 ± 1°C (Li et al., 2017). All insects were checked for viability after 4 days. Based on the results of the insecticidal test, the semilethal concentrations (LC$_{50}$) of the ^{13}C-labeled and ^{15}N-labeled Cry proteins were calculated with Excel using Koch's method, and a 95% confidence interval was determined based on the LC$_{50}$ of different groups.

3 | RESULTS

3.1 | Cloning of recombinant plasmid

PCR-based identification for plasmid pUC57-*Cry1Ab/Ac* confirmed a 1.8-kb target band in most transformants (Figure 1). This coincided with the expected size of the *Cry1Ab/Ac* gene insertion. Positive clones were selected, sequenced, and compared to the *Cry1Ab/Ac* gene in GenBank. The results confirmed that the recombinant plasmid was successfully constructed.

3.2 | Identification of prokaryotic expression recombinant plasmid

The constructed recombinant plasmid pET28a-*Cry1Ab/Ac* digested by *NcoI* and *XhoI* double enzymes produced two fragments
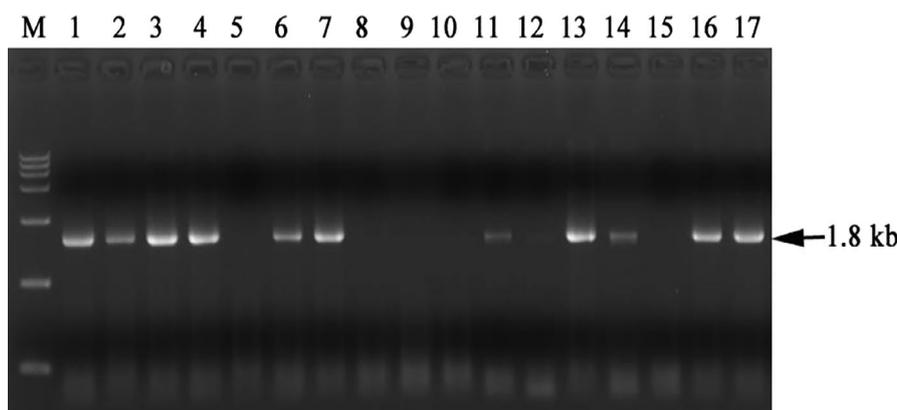
FIGURE 1 PCR identification of positive plasmid pUC57-*Cry1Ab/Ac*. M: DNA marker; 1–17: PCR products of randomly selected plasmids

of about 1.8 and 5.3 kb, sizes that were consistent with the length of *Cry1Ab/Ac* and the expression vector pET28a fragment length (Figure 2), indicating the successful construction of recombinant prokaryotic expression vector pET28a-*Cry1Ab/Ac*. Moreover, the recombinant plasmid was sequenced to identify the *Cry1Ab/Ac* gene, and clones selected with the correct sequences were used in subsequent experiments.
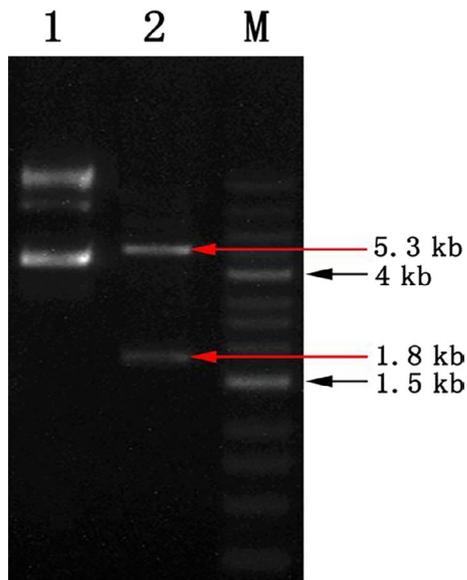
FIGURE 2 Identification of expression recombinant plasmid. 1: pET28a-*Cry1Ab/Ac*; 2: double-restriction enzyme digestion of pET28a-*Cry1Ab/Ac*; M: DNA marker

3.3 | Prokaryotic expression of the *Cry1Ab/Ac* gene

The recombinant plasmid pET28a-*Cry1Ab/Ac* was transformed into *E. coli* BL21 (DE3), and *E. coli* BL21(DE3)/pET28a-*Cry1Ab/Ac* was induced by IPTG in M9 medium with either glucose as the sole carbon source or ammonium chloride as the sole nitrogen source. The bacterial solution was added into a 2× SDS gel-loading buffer, which was boiled and centrifuged and then analyzed by SDS-PAGE. An apparent protein band was seen at the position of Cry1Ab/Ac protein (Figure 3), demonstrating that the protein was successfully expressed. The protein band markedly increased after IPTG addition, indicating that *E. coli* with the recombinant plasmid was induced using IPTG.

3.4 | Screening for the optimum conditions of induced expression

When the OD_{600} of the cell density of the recombinant strain reached 0.6–0.8, the IPTG addition amount, induction time, and induction temperature were screened, and the whole bacterial solution was analyzed with SDS-PAGE. The maximum expression amount of protein was detected at 37°C, 4 h after the addition of 0.2 mM IPTG (Figure 4). Then, the bacterial solution was sonicated and centrifuged according to the above-mentioned conditions of induced expression, and the precipitate and the supernatant were analyzed by SDS-PAGE (Figure 5), which confirmed the presence of the Cry1Ab/Ac protein in the inclusion bodies.

FIGURE 3 Expression of recombinant plasmid pET28a-*Cry1Ab/Ac*. M: protein marker; 1: samples before induction; 2–6: samples after induction
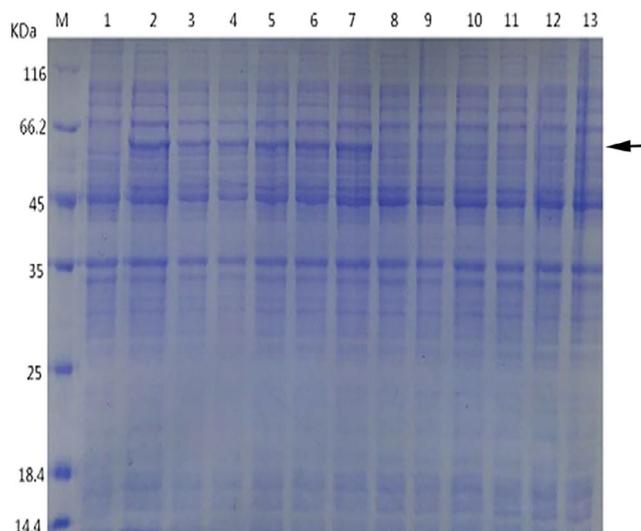
FIGURE 4 SDS-PAGE analysis of expression conditions optimization of Cry1Ab/Ac protein. M: protein marker; 1: not induction bacterial solution; 2–4: bacterial solution after induction with 1.0 mM IPTG at 37°C for 4 h; 5–7: bacterial solution after induction with 0.2 mM IPTG at 37°C for 4 h; 8–10: bacterial solution after induction with 1.0 mM IPTG at 15°C for 16 h; 11–13: bacterial solution after induction with 0.2 mM IPTG at 15°C for 16 h
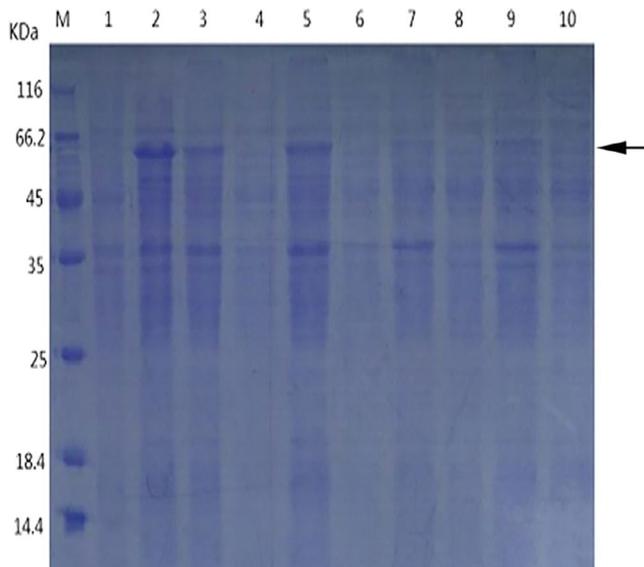
FIGURE 5 SDS-PAGE analysis of Cry1Ab/Ac protein solubility. M: protein marker; 1: not induction bacterial solution; 2: total bacterial solution after induction; 3, 4: bacterial solution precipitate and supernatant after induction with 1.0 mM IPTG at 37°C for 4 h; 5, 6: bacterial solution precipitate and supernatant after induction with 0.2 mM IPTG at 37°C for 4 h; 7, 8: bacterial solution precipitate and supernatant after induction with 1.0 mM IPTG at 15°C for 16 h; 9, 10: bacterial solution precipitate and supernatant after induction with 0.2 mM IPTG at 15°C for 16 h

3.5 | Amplification expression and purification of $^{13}C/^{15}N$ single-labeled Cry1Ab/Ac

The recombinant strains were inoculated into M9 medium either with glucose as the sole carbon source (containing ^{13}C-labeled glucose ([U-^{13}C], 5%)) or with ammonium chloride (containing ^{15}N-labeled ammonium chloride (^{15}N, 5%) as the sole nitrogen source and cultivated at 37°C using the above-mentioned optimal induction conditions. Then, the cells were incubated with 1× PBS buffer and subjected to ultrasonic treatment. Subsequently, the crude inclusion bodies obtained by centrifugation were completely solubilized in 8 mol/L urea. The supernatant collected by centrifugation was purified by Ni-NTA affinity chromatography (Figure 6).

3.6 | Western blot analysis and content determination of $^{13}C/^{15}N$ single-labeled Cry1Ab/Ac

The denatured ^{13}C-labeled and ^{15}N-labeled Cry1Ab/Ac proteins were refolded with 0.4 mol/L L-arginine in a linear 8 to 0 mol/L urea gradient refolding buffer. SDS-PAGE revealed that the expressed and purified ^{13}C-labeled and ^{15}N-labeled Cry1Ab/Ac proteins each had a single band with a molecular weight of approximately 66.2 kD (Figure 7a,c). The purity of the Cry1Ab/
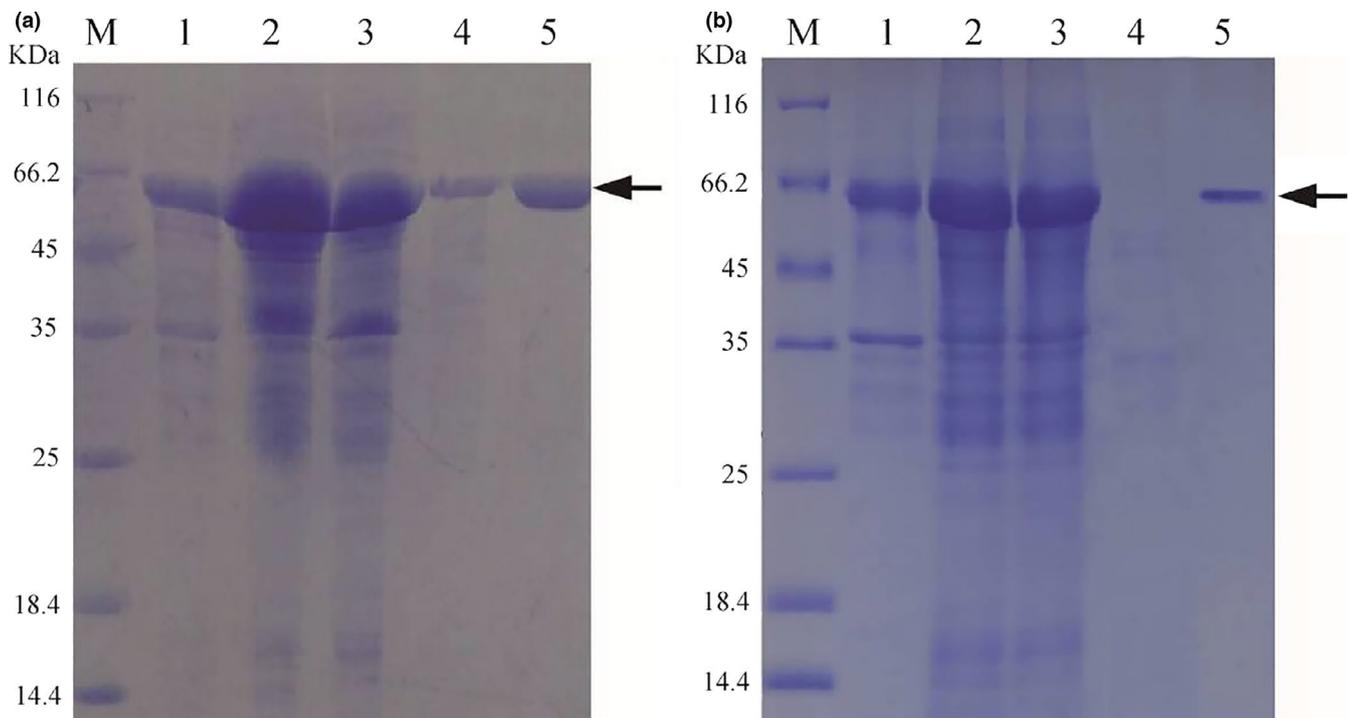
FIGURE 6 SDS-PAGE analysis of Cry1Ab/Ac protein purified by nickel column affinity chromatography (prokaryotic expression of Cry1Ab/Ac protein in M9 medium containing ^{13}C-labeled glucose (a) and ^{15}N-labeled ammonium chloride (b), respectively). M: protein marker; 1: precipitate after lysis; 2: supernatant after lysis; 3: flow-through liquid; 4: wash fraction; 5: eluate

Ac proteins obtained was above 99% with grayscale scanning. Western blotting confirmed that the stable isotope-labeled recombinant proteins were successfully expressed and purified (Figure 7b,d). ELISA analysis showed that the expression levels of ^{13}C-labeled and ^{15}N-labeled Cry proteins were 12.6 mg/L M9 medium and 8.8 mg/L M9 medium, respectively.

3.7 | Assay of δ ^{13}C and δ ^{15}N value of ^{13}C/^{15}N single-labeled Cry proteins

The δ ^{13}C value of the ^{13}C-labeled Cry protein and the δ ^{15}N value of the ^{15}N-labeled Cry protein determined by EA-IRMS were 3,269‰ and 2,854‰, respectively.

3.8 | Insecticidal bioassay of the ^{13}C/^{15}N single-labeled Cry1Ab/Ac protein

Data obtained from the *C. suppressalis* assay are presented in Table 2. The mortality of the newly hatched larvae gradually increased with

the increase in the application of insecticidal protein. When the protein content in the feed reached 25 µg/g, the mortality reached 100%. The LC$_{50}$ values of ^{13}C/^{15}N single-labeled Cry proteins were 5.41 and 5.40 µg/g, and the 95% confidence interval (CI) of LC$_{50}$ were 2.86–10.26 µg/g and 2.88–10.19 µg/g, indicating strong insecticidal activity of a stable isotope-labeled protein.

4 | DISCUSSION

This study aimed to produce ^{13}C-labeled and ^{15}N-labeled Cry1Ab/A proteins suitable for assessing the metabolic fate of Cry protein in soil. Many studies have shown that various types of insecticidal proteins are expressed by recombinant strains of *E. coli* (Bukhari & Shakoori, 2009; Cao et al., 2009; Chen et al., 2014; Okumura et al., 2006; Valldor et al., 2012). However, the supplementation of growth with media-stable isotopes for generating labeled proteins was not applied. Valldor et al. (2012) used radioactive isotope ^{14}C-labeled glycerol as a carbon source and cultured recombinant *E. coli* in small-batch fermentation to obtain ^{14}C-labeled Cry1Ab protein. Although the radioisotope technique is appealing because it is simple and can be highly sensitive, it can
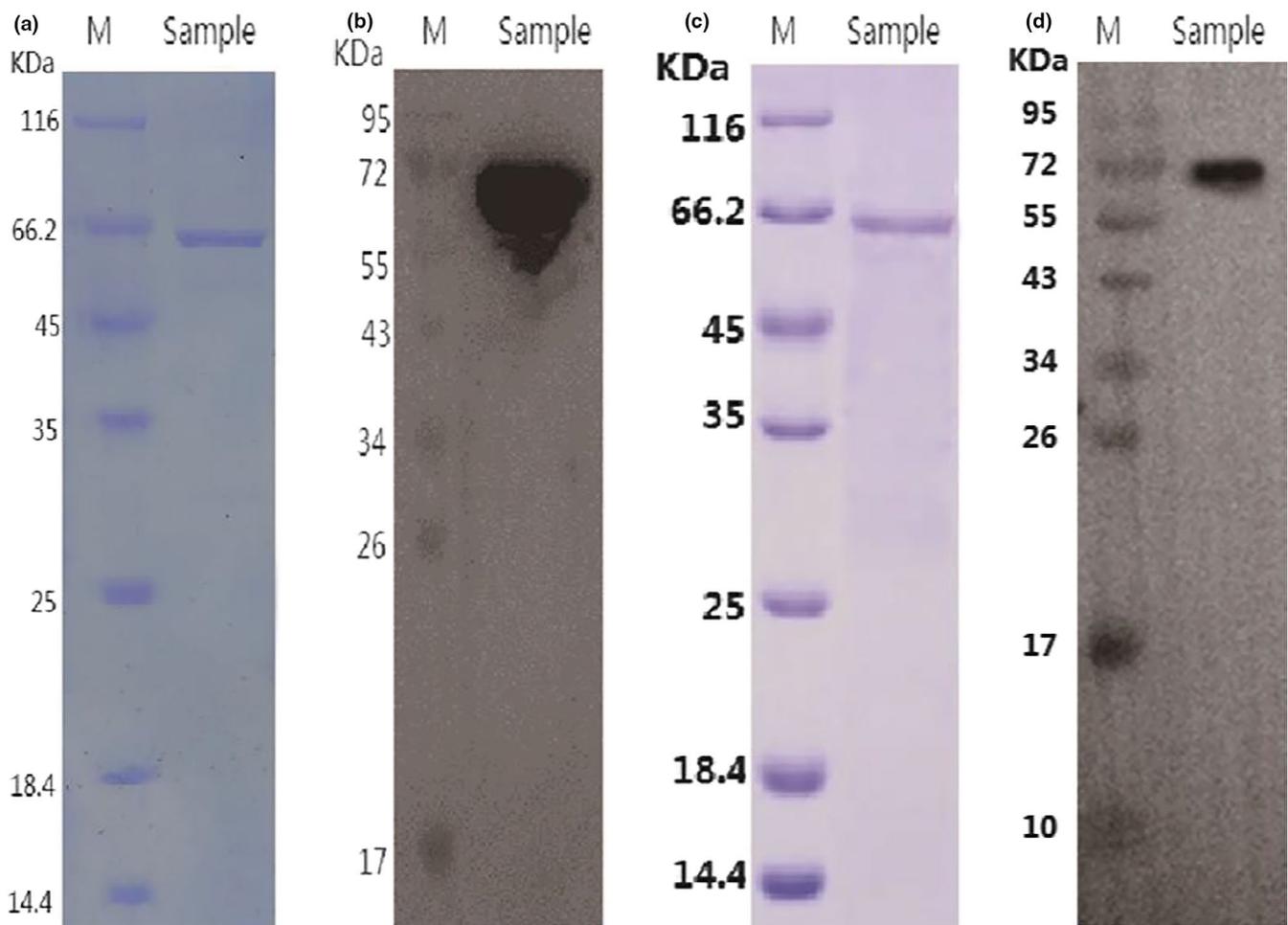
FIGURE 7 SDS-PAGE (a, c) and Western blot analysis (b, d) of Cry1Ab/Ac protein [prokaryotic expression of Cry1Ab/Ac protein in M9 medium containing ^{13}C-labeled glucose (a, b) or ^{15}N-labeled ammonium chloride (c, d)]

TABLE 2 Insecticidal analysis of Cry1Ab/Ac protein

Dosage/(μg/g)	Replicates	Number of inoculations	Average death
1 (^{13}C-labeled Cry protein)	3	20	3.3 ± 0.56
1 (^{15}N-labeled Cry protein)	3	20	2.7 ± 0.54
5 (^{13}C-labeled Cry protein)	3	20	7.6 ± 0.57
5 (^{15}N-labeled Cry protein)	3	20	8.0 ± 1.72
10 (^{13}C-labeled Cry protein)	3	20	12.3 ± 2.51
10 (^{15}N-labeled Cry protein)	3	20	12.0 ± 1.73
15 (^{13}C-labeled Cry protein)	3	20	15.3 ± 2.31
15 (^{15}N-labeled Cry protein)	3	20	16.0 ± 1.73
20 (^{13}C-labeled Cry protein)	3	20	19.3 ± 2.56
20 (^{15}N-labeled Cry protein)	3	20	19.0 ± 3.61
25 (^{13}C-labeled Cry protein)	3	20	20.0 ± 0.00
25 (^{15}N-labeled Cry protein)	3	20	20.0 ± 0.00

also be problematic with following safety regulations during laboratory work and the subsequent disposal of waste. The stable isotope labeling technique using stable isotopes as tracers utilizes a mass spectrometer to quantify the abundance of stable isotope tracers in biological samples, and it can be used to study the metabolic fate of a compound. More than 6,000 stable isotope-labeled compounds (tracers) are commercially available for use in metabolic studies. However, stable isotope-labeled Cry proteins are not currently available. Generally, stable isotope-labeled proteins can be produced by biosynthesis and chemical synthesis. Chemical synthesis based on the covalent attachment of stable isotopes, however, may modify the protein structure and thus affect its biological activity and biodegradability (Valldor et al., 2012). Therefore, in this study, it was decided to produce the ^{13}C-labeled and ^{15}N-labeled Cry proteins by a recombinant Cry1Ab/Ab protein-synthesizing E. coli strain under optimum culture conditions. At first, ^{13}C-labeled glucose ([U-^{13}C], 99%) as a carbon source and ^{15}N-labeled ammonium chloride ([^{15}N], 99%) as a nitrogen source were used to produce Cry1Ab/Ac proteins. However, the δ ^{13}C and δ ^{15}N values of the proteins exceeded the upper limit of the analytical measurement range of EA-IRMS, so the stable isotopic purities of glucose and ammonium chloride were adjusted to 5% by the addition of corresponding unlabeled materials. Consequently, the δ ^{13}C value of the ^{13}C-labeled Cry protein and the δ ^{15}N value of the ^{15}N-labeled Cry protein were 3,268.68‰ and 2,854.28‰, respectively, which could be applied to future studies of biodegradation processes and metabolic pathways of Cry protein in soil.

Previous studies showed that the IPTG addition amount, induction time, and induction temperature influenced the expression of exogenous proteins (Hayat et al., 2018; Overton, 2014). Therefore, to enhance protein expression and save experimental costs, we optimized the prokaryotic expression conditions. Our results indicated that 0.2 mM IPTG treatment for 4 h at 37°C was most effective for expression, and the main reasons may be related to the host bacteria: The optimum growth temperature of E. coli is about 37°C (Li et al., 2009). Although some studies showed that low growth temperatures enhance protein folding and solubility (Haranahalli et al., 2016; Overton, 2014), no protein was obtained at 15°C. The maximum expression amounts of the protein, which existed in the form of inclusion bodies, appeared at 37°C. Hence, 37°C was optimum for prokaryotic expression. Accordingly, in the present study, the insoluble inclusion body was denatured with urea, purified with Ni column affinity chromatography under denaturing conditions, and then renatured after dialysis with the phosphate buffer solution. There is a need to purify inclusion bodies before refolding, considering that the presence of inclusion body impurities could affect the refolding yield of recombinant proteins (Batas et al., 1999). Furthermore, stable isotope-labeled protein revealed strong insecticidal activity after it was purified.

In conclusion, this study established a method to produce ^{13}C/^{15}N single-labeled insecticidal protein Cry1Ab/Ac using a recombinant E. coli strain. Stable isotope-labeled Cry protein existing in the form of inclusion bodies was solubilized, purified, and refolded successfully, revealing strong insecticidal activity. The results of this study lay a foundation for further studies on the metabolic fate, biological function, and safety evaluation of Cry proteins.

CONFLICT OF INTERESTS

None declared.

AUTHOR CONTRIBUTION

Z Wang: Investigation (lead); Methodology (supporting). **C Hu:** Data curation (supporting); Investigation (supporting). **Yu Sun:** Conceptualization (supporting). **W Jiang:** Validation (supporting). **G Wu:** Formal analysis (supporting). **Al Pan:** Methodology (supporting). **Peng Li:** Funding acquisition (supporting); Writing-original draft (supporting). **Xue-ming Tang:** Resources (equal); Software (supporting).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

The datasets used and analyzed during the current study are available in this published article and on request from the corresponding author.

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