Anal Bioanal Chem (2016) 408:8231–8239
DOI 10.1007/s00216-016-9938-5

RESEARCH PAPER

Development of monoclonal antibody-based sensitive ELISA for the determination of Cry1Ie protein in transgenic plant

Yuwen Zhang[1,2] · Wei Zhang[2] · Yan Liu[2] · Jianhua Wang[2] · Guoying Wang[1] · Yunjun Liu[1]

Received: 6 August 2016 / Revised: 1 September 2016 / Accepted: 8 September 2016 / Published online: 22 September 2016

Abstract Cry1Ie is a kind of *Bacillus thuringiensis* (Bt) toxin protein which has a different action model than the Cry1Ab and Cry1Ac protein. The transgenic maize expressing *Cry1Ie* might be commercially used in the near future and it is urgent to develop a method to detect Cry1Ie protein in transgenic plants and their products. To develop an ELISA method, Cry1Ie protein was expressed in *Escherichia coli* strain Transetta DE3, purified with the Ni-NTA spin columns, and then validated by sequencing. Bioassay results showed that the purified Cry1Ie protein was highly toxic to the Asian corn borer. The polyclonal antibody (pAb) and the specific monoclonal antibody (mAb) $1G_42D_6$ were generated from rabbit and mice which were immunized with Cry1Ie protein, respectively. Western blotting of crude Cry1Ie protein extracts was established by employing mAb $1G_42D_6$, whereas the mAb $1G_42D_6$ negligibly recognized other Bt proteins. Sandwich ELISA against Cry1Ie protein was established by coating with pAb and detecting with mAb $1G_42D_6$. The limit of detection (LOD), the limit of quantification (LOQ), and the quantification range of the assay in different matrices of maize plant were determined as 0.27–0.51, 0.29–0.78, and 0.45–15.71 ng/mL, respectively. Recoveries of Cry1Ie protein spiked in different maize tissues ranged from 75.1 to 99.5 %. The established sandwich ELISA was verified using transgenic maize overexpressing *Cry1Ie*. The results in this study suggested that the established ELISA method is effective for detecting Cry1Ie protein in transgenic plants.

Keywords *Bacillus thuringiensis* · Cry1Ie · Sandwich ELISA · Transgenic plant

Yuwen Zhang and Wei Zhang contributed equally to this work.

Published in the topical collection *Immunoanalysis for Environmental Monitoring and Human Health* with guest editors Shirley J. Gee, Ivan R. Kennedy, Alice Lee, Hideo Ohkawa, Tippawan Prapamontol, and Ting Xu.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9938-5) contains supplementary material, which is available to authorized users.

✉ Guoying Wang
wangguoying@caas.cn

✉ Yunjun Liu
liuyunjun@caas.cn

[1] Institute of Crop Science, Chinese Academy of Agricultural Sciences, Zhongguancun South Street 12, Beijing 100081, China

[2] College of Agriculture and Biotechnology, China Agricultural University, Yuanmingyuan West Road 2, Beijing 100193, China

Introduction

During the past 20 years, the global area of genetically modified (GM) crops continued to increase, reaching 179.7 million hectares in 2015 [1]. The commercially grown GM crops are mainly insect-resistant or herbicide-tolerant transgenic crops. In the insect-resistant GM crops, the *Bacillus thuringiensis* (Bt) insecticidal proteins were produced to kill some major pests such as cotton bollworm and corn borer, but cause little or no harm to most other organisms [2]. Although more than 800 different Bt proteins have been identified and classified into 74 Cry groups and 3 Cyt groups (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/toxins2.html), only a few *Bt* genes, such as *Cry1Aa*, *Cry1Ab*, *Cry1Ac*, *Cry1F*, *Cry2Ab*, *Cry3A*, *Cry3Bb1*, *Cry34/35Ab1*, etc., were used commercially in Bt crops [3, 4]. Bt crops were first commercialized in 1996 and the widespread use of Bt crops

has led to the evolution of insects resistance which threat the sustainable usage of Bt crops [5–7]. Several strategies have been used to delay insects' resistance to Bt crops, such as high-dose/refuge strategy, pyramid strategy, and novel toxin strategy [8–10].

Cry1Ie was cloned from *B. thuringiensis* and the overexpressed product was shown to be toxic to a wide variety of insects, such as *Plutella xylostella* and *Ostrinia furnacalis* [11]. Our previous works showed that the Cry1Ie proteins expressed in *E. coli*, transgenic tobacco, and transgenic maize were toxic to the corn borer [12, 13]. It was also shown that no cross-resistance exists between Cry1Ie and Cry1Ab, Cry1Ac, or Cry1Fa, even though high cross-resistance exists between Cry1Ac and Cry1Ab, and low cross-resistance exists between Cry1Fa and Cry1Ac/Cry1Ab [14]. Due to that Cry1Ie is highly tolerant to the corn borer and there is no cross-resistance between Cry1Ie and Cry1A toxins, *Cry1Ie* might be a good candidate for the development of Bt crops. To date, some transgenic plants overexpressing the Cry1Ie protein have been developed, which might be commercially used in the near future in China [12, 15].

With the increasing commercial usage of GM crops, their biosafety issues gained more concerns from the consumers. Worldwide biosafety protocols and amendments on genetically modified organisms (GMOs) are strictly implemented. In 1992, the United Nation conference published the safe guidelines for GMOs [16]. Later, the World Trade Organization-Technical Barrier to Trade (WTO-TBT) laid down guidelines for regulations, standards testing, certification process, packaging, marking, and mandatory labeling requirements in 1995 [17]. Several countries have implemented mandatory labeling for foods derived from the GM crops [18].

For the detection, tracing, and quantification of GMO, different methods based on DNA or the novel protein are employed. At present, the most accepted techniques are the polymerase chain reaction (PCR) and the enzyme linked immunosorbent assay (ELISA). PCR has the advantage of sensitivity and high throughput, and has been successfully applied on many GM crops [19–21]. However, PCR-based methods require a well-equipped lab and need skillful investigators to operate the experimental apparatus. Furthermore, PCR-based methods are time-consuming and difficult for the quantification [22, 23]. Now, the method of immunoassay had been widely used for the detection of Bt proteins in transgenic crops. Various ELISA kits for detecting Cry1Ab, Cry1Ac, Cry2Ab, and other Bt proteins have been developed [19, 22, 23].

In order to monitor and control the usage of Cry1Ie in GM plants, a sensitive sandwich ELISA was developed in this study. We successfully analyzed the maize variety samples by this method and could quickly pick out the *Cry1Ie* transgenic maize samples among the maize variety.

Experimental

Reagents

Chemical reagents were purchased from Sigma (St. Louis, MO, USA). Cell culture medium [Dulbecco's modified Eagle's medium, DMEM] and fetal bovine serum (FBS) were purchased from Gibco BRL (Paisley, Scotland). Tryptone and yeast extract were purchased from OXOID (Basingstoke, Hampshire, England). Pre-stained protein ladders were purchased from Fermentas Life Sciences (Vilnius, Lithuania). The goat anti-mouse IgG-HRP was purchased from ZSGB-BIO Biological Technology Co., LTD. All the restriction enzymes were purchased from New England Biolabs (Beijing, China). ProteinPure^{TM} Ni-NTA Resin, competent cells of *E. coli* strain Transetta (DE3), and Easy Protein Quantitative Kit were purchased from TransGen Biotech (Beijing, China). The microtiter plates (Polystyrene, EIA/RIA 1 × 8 Stripwell^{TM} 96-well plates, No lid, Flat bottom, Certified high binding) were purchased from Costar (Corning, NY, USA).

Preparation of immunogen

The *Cry1Ie* gene (GenBank accession number AF211190) was artificially synthesized using the maize bias codons and cloned into vector pET-30a(+) which was digested with *Eco*RI and *Xho*I. Plasmid pET-30a(+)-Cry1Ie was constructed and then transformed into *E. coli* strain Transetta (DE3). Two milliliters overnight culture of a single positive colony was added into 200 mL LB medium with 100 mg/L kanamycin and 25 mg/L chloramphenicol, and cultured to $OD_{600} = 0.4–0.6$ in a thermostatic shaker (37 °C, 200 rpm), then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the cells were incubated in a thermostatic shaker (16 °C, 160 rpm) for 20 h.

Cells were harvested and resuspended with lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 15 mM imidazole, pH 8.0), disrupted by ultrasonication (3 s off, 4 s on) for 15 min on ice. The mixture was centrifuged at 12,000 rpm for 10 min, and the supernatants were filtered using MillEx® HV (Filter Unit 0.45 μm). Because the recombinants were equipped with a His-tag, the supernatant containing the toxin was passed through a miniature column (4 mL bed volume) of Ni-NTA resin that had been equilibrated with lysis buffer. Then the column was washed with 8 mL buffer A (25 mM Tris-HCl, 150 mM NaCl, 30 mM imidazole, pH 8.0), 4 mL buffer B (25 mM Tris-HCl, 150 mM NaCl, 100 mM imidazole, pH 8.0), and 10 mL buffer C (25 mM Tris-HCl, 150 mM NaCl, 250 mM imidazole, pH 8.0), respectively. The purified proteins in buffer C was then concentrated using Polyethylene Glycol 8000, dialysed with the buffer (25 mM Tris-HCl, 150 mM NaCl, pH 8.0) to remove imidazole. The concentrated and dialysed protein was named as L250, the purity of

which was characterized by running SDS-PAGE. The concentration of L250 was quantified using the Easy Protein Quantitative Kit.

Sequencing and bioassay

The purified Cry1Ie protein was sequenced on a high performance liquid chromatography (HPLC) (Waters, MA, USA) tandem a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (MS) (Thermo Fisher, MA, USA) at the biological mass spectrometry laboratory of China Agricultural University.

For the bioassay, the purified Cry1Ie protein was diluted into eight different concentrations (0.025, 0.05, 0.25, 0.5, 2.5, 5, 25, 50 mg/L), then mixed into the agar-free semi-artificial diet. The bioassays were performed in 48-well Petri dishes and each well was filled with the diet and infested with one 1st-instar Asian corn borer (*O. furnacalis*) larva. The bioassay was performed in an environmental chamber at 70–80 % relative humidity, 26–28 °C, and a photoperiod consisting of 16/8-h light/dark cycle. Seven days later, the assays were evaluated by counting the number and the weight of surviving larvae. The experiment was replicated three times. Fifty percent lethal concentrations (LC_{50}) were calculated by probit analysis.

Myeloma cell line, cell culture mediums, and experimental animals

SP2/0-Ag14 (HAT-sensitive mouse myeloma cell line) was purchased from the Chinese Institute of Veterinary Drug Control (Beijing, China). The complete culture medium for myeloma and hybridoma cell was made from DMEM with 0.2 M glutamine, 50,000 U/L penicillin, 50 mg/L streptomycin, and 10–20 % (*v/v*) fetal bovine serum. HAT (hypoxanthine, aminopterin and thymidine) selection medium was complete culture medium with 1 % HAT medium supplements.

The white New Zealand rabbit and female Balb/c mice were purchased from the Laboratory Animal Center of the Institute of Genetics (Beijing, China). All animal treatment procedures were performed strictly according to the standards described in the "Guide for the Care and Use of Laboratory Animals" (National Research Council Commission on Life Sciences, 1996 edition). The animals were raised under controlled light (12 h light/12 h darkness) and temperature (22 ± 2 °C) with food and fresh water.

Antibody production, purification, and characterization

The animal experimental procedure was approved by the Beijing Experimental Animal Management Office. One white New Zealand rabbit (3 kg) and six female Balb/c mice (6 weeks old) were immunized with the immunogen. The protocols of immunization, fusion, antibody production, purification, and characterization of monoclonal antibody (mAb) and polyclonal antibody (pAb) were described as previously [23, 24].

For the pAb preparation, a rabbit was immunized subcutaneously on the back and the inner thigh muscles of legs with 600 μL of immunogen emulsified with an equal volume of complete Freund's adjuvant. Subsequent injections were carried out for three times at a 2-week interval using incomplete Freund's adjuvant. One week after the third injection, the rabbit antisera were collected from the retrobulbar plexus and ear marginal vein for the testing for the anti-Cry1Ie antibody titer. One week after the fourth injection, the rabbit blood was collected from heart and the antisera (pAb) were separated by centrifuging and stored in−40 °C refrigerator.

For the mAb preparation, six Balb/c mice were initially injected subcutaneously and intraperitoneally with 200 μL of immunogen (purified Cry1Ie protein, 0.88 mg/mL) emulsified with an equal volume of complete Freund's adjuvant. One week after the third injection, the mouse antisera were collected from the retrobulbar plexus and ear marginal vein for the testing for the anti-Cry1Ie antibody titer. Four days after the fourth injection, the mouse with the highest titer was boosted intraperitoneally with 0.1 mL immunogen. The spleen cells harvested from that mouse were fused with SP2/0 cell line using PEG-2000 at a ratio of 10:1. The hybridoma cells were selectively cultured in the 96-well plates (Corning, NY, USA) and incubated at 37 °C in a CO_2 incubator (5 % CO_2). About 1 week after fusion, the supernatant was tested by direct ELISA. Positive hybridomas were cloned by limiting dilution. The clone (mAb $1G_42D_6$) having a high antibody titer and good sensitivity than other clones (date not shown) was expanded for production of mAb in ascites in the abdominal cavity of mice which were treated with mineral oil 1 week before. The antibody was purified by ammonium sulfate precipitation. The immunoglobulin isotype was determined using a mouse antibody isotyping kit (Pierce, Rockford, IL, USA). The specificity of mAb $1G_42D_6$ was evaluated by cross-reactivity with other Bt proteins in sandwich ELISA [23].

Sandwich ELISA

The sandwich ELISA was performed as previously described [25] with some modification. pAb was diluted for 500-folds in coating buffer (0.05 M Na_2CO_3-$NaHCO_3$, pH 9.6), and microtiter plate was coated with 100 μL diluted pAb at 37 °C for 3 h. The plate was washed with PBST buffer (0.01 M phosphate solution, 9 g/L sodium chloride, 0.1 % Tween-20, pH 7.5) for three times, then 50 μL of standard sample or analytes which were diluted in PBSTG (PBST containing 0.1 % gelatin) was added to each well, followed by 50 μL of diluted mAb $1G_42D_6$. After incubation at 37 °C for 0.5 h,

the plate was washed four times. One hundred microliters goat anti-mouse IgG-HRP with 10,000-fold dilution was added in each well. The plate was incubated at 37 °C for 0.5 h, and then washed again. One hundred microliters of substrate solution (0.01 M citric acid, 0.03 M monosodium phosphate, 0.04 % of 30 % stock solution of H_2O_2, 0.2 % o-phenylenediamine, pH 5.5) was added to each well for color development. Ten minutes later, 50 μL per well of the stop solution (2.0 M sulfuric acid) was added, and the absorbance was recorded with the microplate reader (Multiskan FC, Thermo, Finland) at 492 nm. Data were calculated with OriginPro8.5 (OriginLab) software.

For the real-world samples, the maize seed samples were collected from Zhangye corn seed production base in Gansu Province, China. The maize inbred line Z31 and transgenic maize IE034 were kept in our own laboratory. The maize seeds were germinated in an incubator for a week; 0.2 g maize seedlings was ground on ice bath and then extracted with 5.0 mL PBST at 4 °C for 3 h. The extract was centrifuged for 10 min at 8000×*g* at 4 °C. The supernatant was diluted 10-fold in PBST, and then was used for sandwich ELISA analysis.

Western blot analysis

Approximately 0.1 g leaves of transgenic maize overexpressing *Cry1Ie* [12] was ground in liquid nitrogen and the total proteins were extracted with PBS (0.01 M phosphate solution, 0.9 % sodium chloride, pH 7.5). The extract was separated by 12 % SDS-PAGE and electrotransfered to nitrocellulose transfer membrane (20 V, 45 min). Nonspecific binding was prevented by blocking with 5 % dry skimmed milk powder in PBST (PBS with 0.1 % Tween-20, pH 7.5) for 2 h at room temperature on rotary shaker. Then, the membrane was incubated with the mAb $1G_42D_6$ (diluted at a ratio of 1:2000 in PBST) for 1 h at room temperature on rotary shaker at 60 rpm. After incubation, the membrane was washed three times (5 min per time) in 50 mL PBST, and HRP-conjugated goat anti-mouse (diluted at a ratio of 1:8000 in PBST) was added. Then the membrane was incubated for another 1 h. Finally, the membrane was washed and the immunoreactive sites on the membrane were detected by SuperSignal® ELISA Pico Chemilumimescent Substrate (Thermo, Vantaa, Finland).

Fortification for recovery tests and dilution agreement tests

The samples namely 0.2 g leaf, seed, pollen, stem, tassel, root, husk leaf, and silk from non-transgenic maize Z31 were ground, respectively. The total protein in each sample was extracted as above-described and was fortified with the purified Cry1Ie protein at varying concentrations (16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 μg/g) for recovery studies [26]. Above eight kinds of maize samples were also fortified with Cry1Ie protein at a concentration of 8.0 μg/g for dilution agreement tests, and the supernatant was diluted for 10-, 20-, 40-, and 80-folds in PBST, respectively. The prepared samples were detected by sandwich ELISA according to the above-described method.

Results and discussion

Immunogen preparation

The *Cry1Ie* gene (GenBank number AF211190) was artificially synthesized using the maize bias codons and was cloned into vector pET-30a(+) which was digested with *Eco*RI and *Xho*I, to construct the plasmid pET-30a(+)-Cry1Ie which was then transformed into *E. coli* strain Transetta (DE3). After induced by adding IPTG, Cry1Ie protein was expressed mainly in the soluble supernatant instead of the inclusion bodies (Fig. 1a). Cry1Ie protein was purified with Ni-NTA resin and SDS-PAGE results showed that the purified proteins have high purity (>95 %) (Fig. 1b). HPLC-MS analysis was performed to sequence the purified protein, and the MS data was analyzed using Mascot software. The results clearly showed that the produced protein was exactly the Cry1Ie protein (see Electronic Supplementary Material (ESM) Fig. S1). Then, the concentration of Cry1Ie protein was measured as 0.88 mg/mL using the Easy Protein Quantitative Kit (TransGen Biotech, Beijing).

The native Cry1Ie protein is a crystal protein which was produced by *B. thuringiensis* during sporulation. The Cry1Ie protein obtained from the prokaryotic expression vector was shown to be toxic to a wide variety of insects [11]. Several papers have described the expression and purification of Cry1Ie protein in *E. coli* strain BL21 (DE3); however in these studies, the Cry1Ie protein was mainly expressed in the inclusion bodies [13, 27, 28]. Although several studies have shown that the inclusion bodies can be recovered in a form suitable for immunization by solubilization [29, 30], it is difficult to allow the protein to recover full bioactivity in the refolding step [31].

It was reported that the truncated Cry1Ie protein could be expressed in *E. coli* strain BL21 (DE3) mainly as soluble forms when cultured at 20 °C [28]. However, the full-length protein might be better than the truncated protein when they were used as immunogen. A procedure of inducing the full-length Cry1Ie protein was reported in the present study, in which the Cry1Ie protein was expressed as soluble form in medium. The *E. coli* strain Transetta (DE3) had contributed to the high protein level and purity in experiment [32]. The optimum working concentration of IPTG was 0.5 mM, which was used to induce the cells to express Cry1Ie protein efficiently. We performed bioassay with the purified Cry1Ie protein against Asian corn borer, and the LC_{50} was determined as

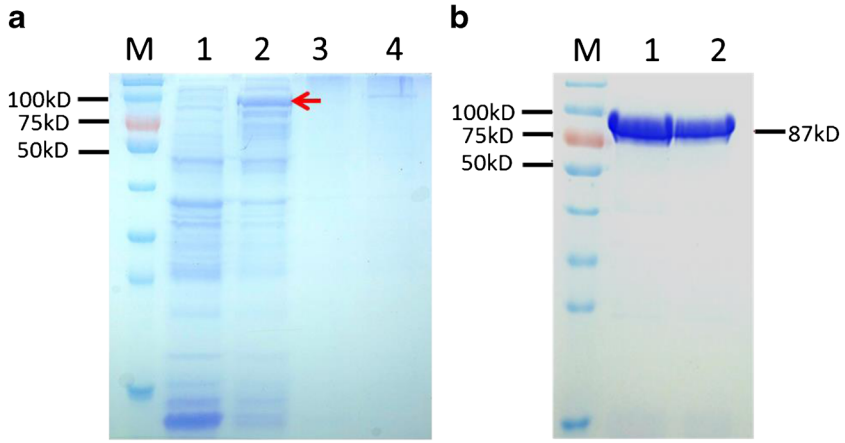

Fig. 1 Expression and purification of Cry1Ie immunogen. **a** The expression of Cry1Ie protein in *E. coli* strain Transetta (DE3). *M*, pre-stained protein ladders; *1*, the soluble supernatant of *E. coli* strain containing plasmid pET-30a(+); *2*, the soluble supernatant of *E. coli* strain containing plasmid pET-30a(+)-Cry1Ie; *3*, the precipitate of *E. coli* strain containing plasmid pET-30a(+); *4*, the precipitate of *E. coli* strain containing plasmid pET-30a(+)-Cry1Ie. **b** The purification of Cry1Ie. Cry1Ie protein was purified with Ni-NTA resin and SDS-PAGE results showed that the purified proteins have high purity (>95 %). *M*, pre-stained protein ladders; *1–2*, the purified Cry1Ie protein

5.395 μg/g (95 % confidence interval, 2.51–8.75) (see ESM Table S1), indicating that the purified Cry1Ie protein has high biological activity. In the experiment to determine LC_{50}, we observed the correlation between protein concentration and biological activity (data not shown). The high purity, exact sequence, and good biological activity of the purified Cry1Ie protein made it suitable to be used as immunogen to develop pAb and mAb.

Antibody generation and characterization

After the fourth immunization, the mouse with the highest titer (the serum dilution that gave an absorbance of 1.0 in the noncompetitive assay conditions) of 6.4×10^4 was used to develop monoclonal antibody. The hybridomas, named as $1G_42D_6$, gave the highest antibody titer of 3.2×10^4 among four hybridomas lines in sandwich ELISA, which was expanded and used to produce ascites. The mAb $1G_42D_6$ is a IgG1 isotype with κ light chains and has no cross-reactivity with Cry1Ac, Cry1Ab, Cry1F, PAT, EPSPS, bovine serum albumin (BSA), and ovalbumin (OVA) (data not shown). Walschus et al. [22] also showed that the developed mAb against Cry1Ab had no cross-reactivity to Cry1Ac, Cry1C,

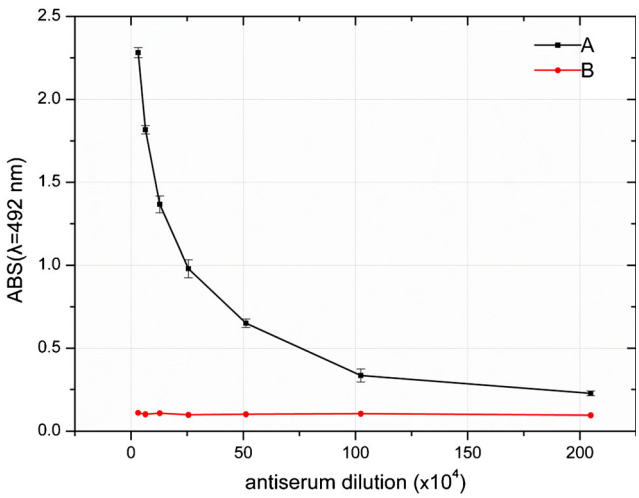

Fig. 2 Antisera titer of pAb against Cry1Ie protein determined in ELISA. (*a*) positive rabbit antiserum; (*b*), negative rabbit antiserum (control group)

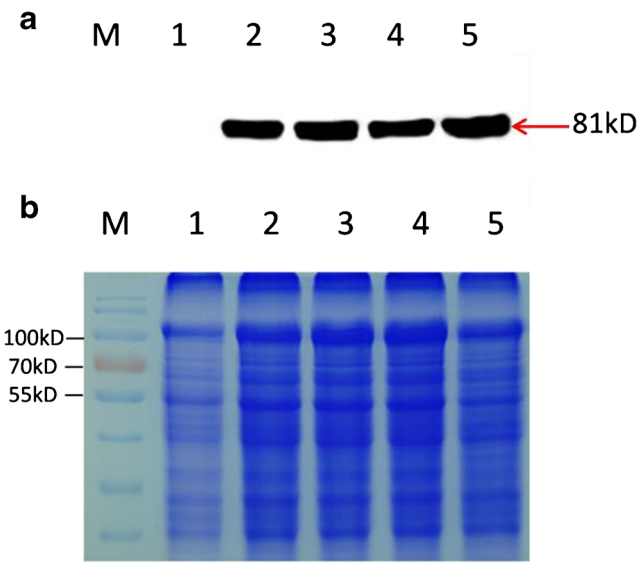

Fig. 3 Western blot analysis of transgenic maize IE034 using antibody mAb $1G_42D_6$ (**a**, Western blot; **b**, SDS-PAGE). *M*, pre-stained protein ladders; *1*, non-transgenic maize Z31; *2–5*, individual plants of transgenic maize IE034

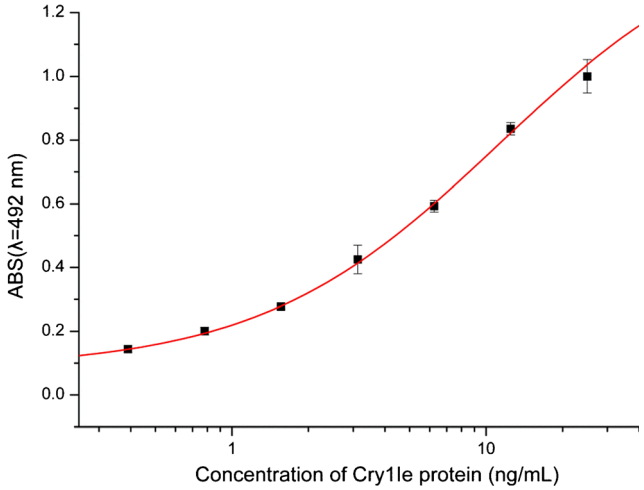

Fig. 4 The standard curve of Cry1Ie protein detected by sandwich ELISA. Each value represents the mean of three replicates

Cry2A, and Cry3A, and only had 18.5 % cross-reactivity to Cry9C. In this study, the pAb (antisera) was collected from the rabbit after the fourth injection. The titer of the pAb was measured as about 2.5×10^5 (Fig. 2), and the pAb was used as capture antibodies.

The total proteins from four individual plants of transgenic maize IE034 overexpressing *Cry1Ie* [12] were extracted to assess the specificity of mAb $1G_42D_6$ by Western blot analysis. The maize inbred line Z31 was selected as a negative control. For the samples from transgenic maize IE034, only 81 kDa hybriding bands were observed, whereas no band was detected from the sample Z31 (Fig. 3). This confirmed that the mAb $1G_42D_6$ can specifically recognize the Cry1Ie protein and there is no reactivity with other proteins from the maize.

Optimization of sandwich ELISA and the standard curves

The optimal concentrations of mAb $1G_42D_6$ and Cry1Ie protein were screened by checkerboard titration. The pAb was used as capture antibodies and coated on microplate at a dilution ratio of 1:1000 to ensure enough antibodies adsorbed on microplate wells. The purified mAb $1G_42D_6$ (1.0 mg/mL) and the goat anti-mouse IgG-HRP were added at a dilution ratio of 1:20,000 and 1:10,000, respectively. A series of concentration of Cry1Ie protein (25.0, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0 ng/g) was used as standard sample. Figure 4 showed a standard curve of Cry1Ie protein detected by the sandwich ELISA. The working range was between 0.83 and 11.74 ng/mL and the limit of detection (LOD) calculated as 3 standard deviations of background signals was 0.56 ng/mL.

Validation of the ELISA method

To further validate the established ELISA method, a few key assay parameters were measured. Protein extracts from leaf, seed, pollen, stem, tassel, root, husk leaf, and silk of non-transgenic maize Z31 were used as the matrices. LOD, the limit of quantification (LOQ), and the quantification range of the assay in different matrices of non-transgenic maize plant were determined as 0.27–0.51, 0.29–0.78, and 0.45–15.71 ng/mL, respectively (Table 1). It was speculated that matrix effects might be due to the nonspecific interactions of the antibodies with proteins, surfactants, or phenolic compounds from the samples [33]. Pollen is rich of phenolic compounds [34], which might cause the high LOD and LOQ when pollen sample was used as matric in this study. The LOD of the ELISA in this study is 0.27–0.51 ng/mL, which is similar to the LOD (0.125 ng/mL) of the commercial sandwich immunoassay Abraxis Bt-Cry1Ab/Ac ELISA kit (#PN 51001, Warminster, PA, USA) (http://www.abraxiskits.com/moreinfo/PN510001USER.pdf), indicating that the ELISA in this study has the potential for the commercial usage.

The recovery tests were performed with non-transgenic maize Z31 samples containing different concentrations of Cry1Ie protein (16.0, 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 μg/g). The concentration of Cry1Ie protein in each sample was designed according to the results of the different positive real-world samples. Each concentration in triplicate was analyzed by the sandwich ELISA. The results showed that the recoveries of Cry1Ie protein spiked in maize samples averaged from 75.1 to 99.5 % (Table 2). The non-transgenic maize Z31 samples were fortified with Cry1Ie protein at a concentration of 8.0 μg/g. After extracting, the supernatant was diluted 10-, 20-, 40-, 80-fold in PBST, and then was detected by sandwich ELISA. Table 3 listed the detailed results of recoveries with different matrices in different dilution ratios. Due to the presence of interference factors, the recoveries carried out by sandwich ELISA averaged from 79.1 to 100.5 %; however, most of them averaged from 90 to 100 %. High recovery rates is one

Table 1 The sandwich ELISA parameters with different matrix from non-transgenic maize Z31

Parameters	Leaf	Seed	Pollen	Stem	Tassel	Root	Husk leaf	Silk
LOD (ng/mL)	0.37	0.40	0.51	0.28	0.30	0.43	0.27	0.32
LOQ (ng/mL)	0.57	0.49	0.78	0.42	0.38	0.49	0.29	0.36
Quantification range (ng/mL)	0.59–12.43	0.61–13.04	0.76–15.24	0.79–13.23	0.57–13.05	0.63–15.71	0.45–14.40	0.63–13.65

Table 2 Average recoveries of Cry1Ie protein spiked in maize samples by the sandwich ELISA

Spiked concentration (μg/g)	Recovery (%) ± SD							
	Leaf	Seed	Pollen	Stem	Tassel	Root	Husk leaf	Silk
16.0	95.6 ± 4.1	93.3 ± 4.3	96.8 ± 4.6	95.3 ± 4.1	93.5 ± 4.6	98.6 ± 6.7	95.6 ± 4.9	97.3 ± 6.5
8.0	93.5 ± 6.6	95.4 ± 8.4	97.8 ± 5.2	97.8 ± 6.3	80.6 ± 7.2	99.2 ± 5.8	86.4 ± 1.4	95.8 ± 3.1
4.0	93.4 ± 2.9	78.4 ± 3.7	79.8 ± 1.1	86.7 ± 6.5	83.8 ± 5.6	78.1 ± 0.1	93.6 ± 2.2	92.3 ± 6.4
2.0	92.3 ± 3.7	77.2 ± 3.5	84.8 ± 3.1	95.6 ± 7.9	83.5 ± 3.6	97.0 ± 14.3	87.2 ± 3.7	94.1 ± 8.9
1.0	87.8 ± 6.2	81.3 ± 5.1	87.0 ± 1.9	96.8 ± 4.5	81.5 ± 4.8	86.7 ± 9.5	90.4 ± 10.8	97.7 ± 6.3
0.5	84.7 ± 2.2	87.3 ± 4.1	80.9 ± 5.5	88.2 ± 3.4	80.7 ± 5.5	83.7 ± 6.1	85.3 ± 3.4	99.5 ± 4.5
0.25	86.3 ± 9.9	79.7 ± 10.6	76.8 ± 6.2	88.9 ± 4.5	80.4 ± 6.8	75.1 ± 9.5	87.2 ± 5.3	90.0 ± 6.2
0	ND	ND	ND	ND	ND	ND	ND	ND

Different samples from non-transgenic maize were fortified with Cry1Ie protein at varying concentrations for recovery studies. Data are means ± standard deviations (SD) of three independent replicates

ND not detectable

important parameter for the reliability of the ELISA measurement. Guertler et al. [18] have developed sensitive and specific ELISA for Cry1Ab protein from feed into bovine milk with 97 % recovery rate. All the results of recovery tests and dilution agreement tests indicated that the sandwich ELISA would be a reliable and sensitive method to detect Cry1Ie protein in maize samples.

Sandwich ELISA analysis of the real-world samples

To verify the specificity of sandwich ELISA, leaves and seeds from 36 maize non-GM cultivars (Z31, LuoDan No.12, YunDuan No.2, BeiYu No.20, DaTian No.1, HongDan No.6, LuDan No.10, ShengYu No.5, LuDan No.3, BiDan No.18, HuiDan No.4, ZhiJin No.3, DeDan No.5, YaYu 27, AoYu 3628, MingZeng 127, XiShan 70, ZhengDa 615, JinKa 688) were analyzed. No Cry1Ie toxin had been detected in any cultivars, except in the leaves (6.95 ± 0.11 μg Cry1Ie toxin/g fresh weight) and seeds (0.43 ± 0.07 μg Cry1Ie toxin/g dry weight) of the transgenic maize producing Cry1Ie protein. The results clearly indicated that the Sandwich ELISA could specifically recognize the Cry1Ie toxin.

Besides, we analyzed the temporal and spatial expression of Cry1Ie protein in transgenic maize IE034 which has a single copy of *Cry1Ie* gene [12]. For the temporal expression analysis, the results showed that Cry1Ie toxin contents decreased with plant development (Fig. 5a). And for the spatial expression analysis, the results showed the highest accumulation of the Cry1Ie toxin in the pollen; relatively high expression levels in the root, tassel, leaf, stem, and husk leaf; and lower accumulation in the silk and seed (Fig. 5b). Different spatial and temporal expression patterns were observed on transgenic maize MON810 which *Cry1Ab* was driven by 35S promoter [35]. These indicate that constitutive promoters, i.e., 35S, Ubiquitin promoter, could also lead to the specific expression of the interested genes, and plant tissue and plant development are also the main parameters affecting the Bt protein contents in transgenic crops. The developed sandwich ELISA for Cry1Ie protein should be very useful for the large-scale monitoring of Cry1Ie expression in the field if transgenic maize expressing *Cry1Ie* goes into commercialization.

Table 3 Average recoveries of Cry1Ie protein spiked in maize samples for dilution agreement tests

Dilution ratios	Recovery (%) ± SD							
	Leaf	Seed	Pollen	Stem	Tassel	Root	Husk leaf	Silk
10-fold	93.5 ± 6.6	95.4 ± 8.4	97.8 ± 5.2	97.8 ± 6.3	92.4 ± 6.5	99.2 ± 5.8	86.4 ± 1.4	95.8 ± 3.1
20-fold	94.5 ± 6.3	89.8 ± 3.7	79.1 ± 2.9	91.5 ± 2.5	91.6 ± 1.3	86.4 ± 8.5	92.4 ± 5.4	83.1 ± 7.8
40-fold	95.1 ± 4.5	97.8 ± 6.8	101.8 ± 4.7	94.9 ± 5.7	97.1 ± 3.5	100.5 ± 5.8	91.8 ± 3.7	90.6 ± 6.5
80-fold	96.4 ± 3.5	86.6 ± 5.1	91.2 ± 6.8	92.1 ± 1.1	97.5 ± 5.5	94.8 ± 3.4	93.7 ± 4.1	92.0 ± 2.7

Different samples from non-transgenic maize were spiked with Cry1Ie protein at a concentration of 8.0 μg/g for dilution agreement tests. After extracting, the supernatant was diluted for 10-, 20-, 40-, 80-folds in PBST, respectively, and then detected by sandwich ELISA. Data are means ± standard deviations (SD) of three independent replicates

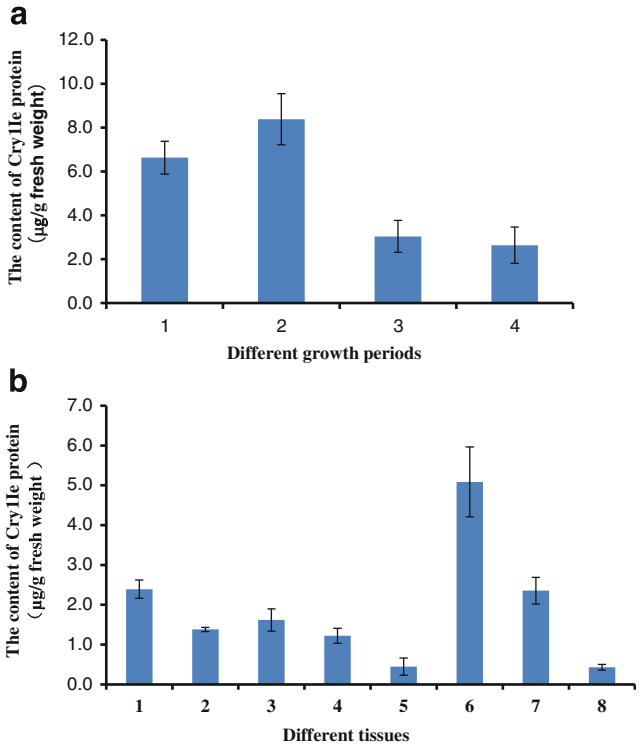

Fig. 5 The temporal and spatial expression of Cry1Ie protein in transgenic maize. **a** The temporal expression of Cry1Ie protein in leaves of transgenic maize IE034. *1*, V3 (third leave stage); *2*, V8 (eighth leave stage); *3*, V12 (12th leave stage); *4*, VT (tasseling stage). **b** The spatial expression of Cry1Ie protein in transgenic maize IE034. *1*, root; *2*, stem; *3*, leaf; *4*, husk leaf; *5*, silk; *6*, pollen; *7*, tassel; *8*, seed

Conclusion

To the best of our knowledge, this is the first report describing a procedure for expression of the full-length Cry1Ie protein in the soluble supernatant instead of in the inclusion bodies. The purified Cry1Ie protein was validated by sequencing and bioassay and then was used as immunogen for the development of mAb and pAb. The titers of the mAb $1G_42D_6$ and pAb were 3.2×10^4 and 2.5×10^5, respectively. The mAb $1G_42D_6$ has no cross-reactivity with other Bt toxins. A sensitive sandwich ELISA method with resistance to the matrix effect was developed to determine the Cry1Ie protein with the LOD of 0.56 ng/mL, whose working range was between 0.83 and 11.74 ng/mL. This method was performed well in recovery tests and dilution agreement tests, and was successfully used to quantify the Cry1Ie protein in real-world samples. Overall, the results displayed that the immunoassay could be used for the quick and convenient determination of Cry1Ie protein in maize samples.

Acknowledgments We thank Prof. Baomin Wang in China Agricultural University for the help on the antibody production. This work was funded by the Agricultural Science and Technology Innovation Program (ASTIP) of CAAS.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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