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Establishment of a sandwich enzyme-linked immunosorbent assay for specific detection of *Bacillus thuringiensis* (Bt) Cry1Ab toxin utilizing a monoclonal antibody produced with a novel hapten designed with molecular model

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Abstract Cry1Ab toxin is commonly expressed in genetically modified crops in order to control chewing pests. At present, the detection method with enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibody cannot specifically detect Cry1Ab toxin for Cry1Ab's amino acid sequence and spatial structure are highly similar to Cry1Ac toxin. In this study, based on molecular design, a novel hapten polypeptide was synthesized and conjugated to keyhole limpet hemocyanin (KLH). Then, through animal immunization with this antigen, a monoclonal antibody named 2C12, showing high affinity to Cry1Ab and having no cross reaction with Cry1Ac, was produced. The equilibrium dissociation constant (K_D) value of Cry1Ab toxin with MAb 2C12 was 1.947×10^{-8} M. Based on this specific monoclonal antibody, a sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was developed for the specific determination of Cry1Ab toxin and the LOD and LOQ values were determined as 0.47 ± 0.11 and 2.43 ± 0.19 ng mL^{-1}, respectively. The average recoveries of Cry1Ab from spiked rice leaf and rice flour samples ranged from 75 to 115%, with coefficient of variation (CV) less than 8.6% within the quantitation range (2.5–100 ng mL^{-1}), showing good accuracy for the quantitative detection of Cry1Ab toxin in agricultural samples. In conclusion, this study provides a new approach for the production of high specific antibody and the newly developed DAS-ELISA is a useful method for Cry1Ab monitoring in agriculture products.

Keywords Monoclonal antibody · Cry1Ab toxin · Cry1Ac toxin · DAS-ELISA · Cross-reactivity

Introduction

The Cry1Ab and Cry1Ac toxins, produced by the bacterium *Bacillus thuringiensis* (Bt) during sporulation stage, are insecticidal proteins which are lethal to Lepidoptera and Coleopteran insect larvae [1–4]. They will be cleaved into 65 kD active proteins under the action of proteases in the alkaline midgut environment after ingestion by sensitive larvae, then bound to specific receptors existing in the intestinal cells, resulting in the formation of pore and midgut epithelial cell lysis, finally inducing larval death [5–8]. On the other hand, Cry1Ab and Cry1Ac toxins are regarded as nontoxic to human and farm animals for the lack of corresponding receptors in mammals [9–13].

At present, Cry1Ab and Cry1Ac genes have been widely introduced into genetically modified (GM) crops like cotton, corn, and soybean, whose planting areas have increased every year globally, beneficial to reducing pesticide use and increasing the crop yield [14–19]. However, the increasing areas of GM crops also bring safety concerns surrounding the potential risks of GM crops to human health and nature, such as accumulation of transgenic proteins in the human food chain, interfusion with the genes of other related species, and the emergence of resistant pests to GM crops [20–26]. It had been reported Cry1Ab toxin was detected in maternal, fetal, and non-pregnant women blood samples [27]. GM crops have become a controversial issue and focus of concern, and several

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countries have accordingly enforced labeling thresholds for GM crops and relevant products [28]. Implementation of these labeling policies requires the establishment of reliable detection methods for genetically modified organisms (GMOs).

The common reliable detection methods for GMOs in plants and products are mainly focused on nucleic acid and protein analyses. The DNA-based PCR methods are highly sensitive in Bt gene detection, but need to be operated by experienced person using specific equipment, which are time-consuming and expensive [29]. The protein-based methods, such as enzyme-linked immunosorbent assay (ELISA) and lateral flow strip that using specific antibodies, have been used widely for saving time and reducing costs [30]. In addition, electrochemical immuno-assay based on specific nanobody had been reported for the detection of Cry1Ab toxin [31]. Electrochemical immunoassay was more sensitive than ELISA, but the expression of nanobody was unstable and deactivation occurred sometimes in the process of expression and storage. What is more, electrochemical immunoassay was not suitable for field on-site detection.

Sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is the preferable immunoassay used for the detection of Cry1Ab and Cry1Ac toxins, where the analytes are sandwiched in the middle of the capture antibody and the detection antibody. However, the monoclonal antibody produced for Cry1Ab toxin has high cross reaction with the Cry1Ac toxin for Cry1Ab and Cry1Ac have high homologous sequence (higher than 80%) and high similar three-dimensional (3D) structure, corresponding to the report that about 90% of the monoclonal antibody (MAb) against antigens with similar structure have some cross reaction [32]. At present, there has been a report on the specific determination of Cry1Ac toxin which has no cross reaction with Cry1Ab toxin based on specific MAb obtained through immunization using Cry1Ac protein [33]. However, no report or commercialized immunoassay kits at present can specifically detect Cry1Ab toxin in GMOs while avoiding the cross reaction with Cry1Ac toxin [34–38].

The objective of the present study was to develop a specific MAb against Cry1Ab toxin without cross reaction to Cry1Ac toxin through immunization using polypeptide which was obtained by molecular design and establish a sensitive DAS-ELISA for the specific detection of Cry1Ab toxin in GM crops and their products.

Materials and methods

Chemicals and instruments

The standards of Cry1 toxins (purity ≥95%) used in this study were from *B. thuringiensis*, provided by You Long Bio. Co. Ltd (Shanghai, China). Dimethylformamide (DMF), keyhole limpet hemocyanin (KLH), 3,3′5,5′-tetramethyl benzidine (TMB), Freund's complete and incomplete adjuvants (FCA and FIA),

hypoxanthine thymidine (HT), hypoxanthine aminopterin thymidine (HAT), polyethylene glycol (PEG, 50%, *w/v*), dimethyl sulfoxide (DMSO), and mouse monoclonal antibody isotyping kit were purchased from Sigma (St. Louis, MO, USA). HRP-goat-anti-mouse/rabbit antibody (1 mg mL^{-1}) was purchased from KPL Inc. (Gaithersburg, MA, USA). Purified anti-Cry1Ab polyclonal antibody (PAb) was produced in our laboratory according to the method described by Zhu et al. [39]. RPMI-1640 medium and fetal calf serums (FCS) were purchased from Gibco (USA). M-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) was purchased from Thermo (USA). BIA maintenance kit, amine coupling kit [1.0 M ethanolamine-HCl pH 8.5, N-Hydroxysuccinimide (NHS), 1-ethyl-3-(3-diethylaminopropyl)-carbodiimide hydrochloride (EDC)], HBS buffer, pH 7.4 [10×, 1.5 M NaCl, 0.1 M HEPES, 30 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% *v/v* surfactant P20], and sensor chip CM5 were all purchased from GE Healthcare (Sweden). Unless otherwise stated, all reagents used were analytical grade or better.

Cell culture plates (6, 24, and 96 wells) and culture flasks were purchased from Corning (Beijing, China); 96-well automated microplate washer and automatic microplate reader from Thermo (USA); magnetic stirring apparatus from SCILOGEX (USA); HD-4 nucleic acidprotein detector from Ke Ge Chuang Bio. Co. Ltd (Nanjing, China); NanoVue Plus, HiTrap Protein G column and Sephadex G-25 resin from GE Healthcare (China); dialysis bag from Sigma (USA); and Biacore X100 from GE Healthcare (Sweden); SP2/0 myeloma cells were provided by College of Veterinary Medicine of Jiangsu Academy of Agricultural Sciences; female Balb/c mice of SPF grade were purchased from Comparative Medicine Center of Yangzhou University. The animal experiments were performed according to the Regulation Guideline for Experimental Animals (Ministry of Science and Technology of China).

Carbonate buffer solution (CBS) was 50 mmol L^{-1}, pH 9.6; phosphate buffer solution (PBS) was 50 mmol L^{-1}, pH 7.4; block buffer (MPBS) was 3% milk (*m/v*) in PBS; washing buffer (PBST) was PBS with 0.5% (*v/v*) Tween-20; stock solutions (1 mg mL^{-1}) of the Cry1 toxins were prepared by dissolving 1 mg standards in 1 mL CBS and stored at −20 °C; working solutions of each toxin were prepared by diluting the stock solutions in CBS and stored at 4 °C; substrate solution system was composed by 10 mL pH 5.0 phosphate-citrate buffer, 0.1 mL TMB (10 mg mL^{-1}, dissolved in DMSO), and 25 μL hydrogen peroxide (0.65%, *v/v*); complete 1640 medium was RPMI 1640 medium containing 20% (*v/v*) FCS. HAT/HT medium was complete 1640 medium containing HAT/HT.

Preparation of immunogen

Theoretical models and synthesis of hapten polypeptide

The amino acid sequences of Cry1Ab and Cry1Ac were searched from GenBank database and analyzed by

DNAMAN software. Then, DNAStar software was used to find out the strong antigenicity and hydrophilicity parts of the two toxins to predict the antigen epitope [40–42]. Based on the 3D structure of Cry1Ac which had been obtained by X-ray crystallography, the 3D structure model of Cry1Ab toxin was constructed by homology modeling using the SWISS-MODEL website. Three commonly used online websites, Ramachadran plot, ERRAT, and Verify3D, were used to evaluate the 3D model of Cry1Ab. And then, the non-overlapping areas of the two Cry1 toxins were found out after analyzing the models with SWISS-Pdb Viewer 4.1.0 software. Comprehensively compared the above analysis results, a same sequence of both toxins completely exposed in different space conformation with strong antigenicity and hydrophilicity was screened out as the target polypeptide. The polypeptide was named T4 and synthesized by Nanjing Genscript Biotechnology Co., LTD. A cysteine residue was added at N terminal of the polypeptide chain in order to conjugate with the carrier protein KLH.

Preparation of conjugates

T4 was conjugated to KLH to prepare the immunogen for its molecular weight was too low to stimulate the immune response in body. T4 and KLH were conjugated by MBS via -SH group on the N terminal of T4. All reagents were equilibrated to room temperature before experiment. The solution of KLH dissolved with PBS at the concentration of 10 mg mL^{-1} was transferred to dialysis bag and dialyzed against PBS at 4 °C overnight. MBS was dissolved with DMF at the concentration of 10 mg mL^{-1} in a vial. KLH solution and MBS solution were mixed at the ratio of 10:1 (W/W) in the bottle and incubated to generate activated KLH at room temperature for 30 min. The bottle was shaken several times during incubation. Nucleic protein detector was connected with a column vial silicone tube and PBS was added onto the column loaded with Sephadex G-25 resin to wash resin until the absorbance at A280 is stable. The prepared solution containing the activated KLH was added onto column to remove excess MBS and reaction byproducts. The effluence containing the activated KLH was collected in a tube with the assistance of nucleic acid protein detector. A bottle with T4 dissolved in the bottom was added with the activated KLH by the ratio of 1:1 (W/W) and incubated to generate T4-KLH conjugates at room temperature for 3 h. The solution containing T4-KLH conjugates was transferred to dialysis bag and dialyzed against PBS buffer at 4 °C overnight, and then stored at −20 °C until use.

Production of monoclonal antibodies

Five hundred micrograms T4-KLH conjugate was dissolved in 250 μL PBS and emulsified with 250 μL FCA, equally intraperitoneal injected into four 8–10 weeks old female Balb/c mice for the first immunization. Subsequently,

injections were administered every 2 weeks at the same dose with FIA. One week after the third immunization, the antiserum was collected from the tail vein of each mouse and tested the anti-Cry1Ab antibody titer by indirect ELISA as described below (antibody titer was determined as the maximum antiserum dilution that gave an absorbance of 1.0 in the indirect ELISA). Finally, 100 μg T4-KLH conjugate dissolved in PBS without adjuvant was injected into the mouse which has the highest antibody titer 3 days before cell fusion.

Cell fusion was performed according to Nowinski et al. [43]. The spleen cells collected from the mouse were fused with SP2/0 myeloma cells at a ratio of 5:1–10:1 using PEG 2000. The fused cells were suspended in 80 mL HAT medium and equally added into eight 96-well plates which containing feeder cells and cultured at 37 °C with 5% CO$_2$ [44]. Half HAT medium was changed to HT medium 10 days after cell fusion and replaced entirely by fresh HT medium 2 weeks after fusion. When cell density reached approximately 1/10-1/5 of the well bottom, the culture supernatants were tested by indirect ELISA to detect anti-Cry1Ab antibodies, and the positive hybridoma cells were sub-cloned several times using the limiting dilution method [45] until the stable antibody-secreting clones were screened out. Finally, the monoclonal strains were proliferated in 25 mL culture flasks.

The expanded monoclonal cells were collected and intraperitoneally injected into five multiparous Balb/c mice which were given 0.5 mL sterile atoleine previously [46]. Ascitic fluids were obtained after abdomen enlargement of the mice. The MAbs were separated and purified from ascitic fluids using affinity chromatography on protein G columns and the concentrations were determined by NanoVue Plus [47]. Subclass of the selected antibody was measured by an antibody isotyping kit. All the antibodies were stored at −20 °C until use.

Indirect ELISA

In the whole process of screening and titer determination, Cry1Ab was used as coating antigen. Firstly, 96-well microtitre plates were coated with 2 μg mL^{-1} of Cry1Ab (100 μL well^{-1}) and incubated overnight at 4 °C. The plates were then blocked by 3% MPBS (250 μL well^{-1}) at 37 °C for 2 h after washed three times with PBST. Subsequently, another round of washing was carried out and analytes (antisera, supernatants of hybridoma cells, or purified MAbs, 100 μL well^{-1}) in PBS were added and incubated at 37 °C for 1 h. The unbound antibodies were removed by washing the plates for three times. Then, the plates were filled with 100 μL well^{-1} goat-anti-mouse IgG-HRP (1:5000 in MPBS) and incubated at 37 °C for 1 h. After four times washing with PBST, the plates were filled with enzyme substrate solution (100 μL well^{-1}) and incubated in dark at 37 °C for 15 min. Finally, 50 μL well^{-1} of 2 M H$_2$SO$_4$ was added to the plates to

stop the enzyme catalyzed reaction and the absorption values at 450 nm were read by a microplate reader.

Affinity determination

The affinities of Cry1Ab and Cry1Ac toxins with the produced MAb were determined by surface plasmon resonance (SPR) experiment as described before [48]. Five concentrations (7.5, 15, 30, 60, and 120 $\mu g \cdot mL^{-1}$) of toxins and a blank were included. The equilibrium dissociation constant (K_D) was calculated based on association rate constant (k_a) and dissociation rate constant (k_d). Biacore X100 software (version 2.0.1) was used for kinetic curve-fitting and data processing.

DAS-ELISA optimization and establishment

The optimal concentrations of MAbs and PAbs were determined according to the method described by Roda et al. [32] with some modification. MAbs were coated to 96-well microtiter plates as capture antibody at different dilutions ranged from 0.1 to 1 $\mu g\ mL^{-1}$ while the PAbs were fixed at 0.5 $\mu g\ mL^{-1}$ as detection antibody. The standard curve for DAS-ELISA assay was constructed using Cry1Ab protein with the concentrations of 0, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1000, 2000, and 4000 ng mL^{-1}. A similar procedure was employed to optimize the concentration of anti-Cry1Ab PAbs within 0.05–0.5 $\mu g\ mL^{-1}$ as the concentration of MAbs fixed at the optimal concentration determined above. Different tracer antibody (HRP-goat-anti-rabbit, 1 mg mL^{-1}) concentrations (1:5000–1:10,000 in MPBS) and color developing time (5–15 min) were also preliminary evaluated. Finally, based on the optimized conditions, the new DAS-ELISA method for detecting Cry1Ab toxin was established using the method described below.

Anti-Cry1Ab MAbs (capture antibody) in PBS at the optimal concentration was dispensed into 96-well plates (100 $\mu L\ well^{-1}$) and incubated overnight at 4 °C. After the microtitre plates were washed three times using a 96-well microplate washer, 3% MPBS (250 $\mu L\ well^{-1}$) were added and incubated at 37 °C for 2 h to occupy the excess binding sites. Subsequently, 100 μL samples (standard solutions of Cry1 toxins in CBS or spiked extracts) were added after the wells were washed three times. A blank control (CBS) was also added to the plates. The plates were incubated for 1 h at 37 °C and washed three times with PBST. Then, 100 μL of optimized concentration of anti-Cry1Ab PAbs was filled to the plates and incubated at 37 °C for 1 h. After three successive rinses with PBST, the goat-anti-rabbit antibody labeled with HRP was added into each well (100 $\mu L\ well^{-1}$ in MPBS) and incubated at 37 °C for 1 h. Then, each well was added 100 μL freshly prepared enzyme substrate solution after washed four times with PBST. After incubated optimal time at 37 °C in dark, the reaction was terminated with the addition of 50 μL 2 M H_2SO_4 per well. Absorption values at 450 nm were read

using a microplate reader. All the data was managed by GraphPad Prism 5 software (version 5.01).

Validation of the DAS-ELISA

Sensitivity

The sensitivity of the DAS-ELISA could be reflected by the limit of detection (LOD) and limit of quantification (LOQ), and they are two important parameters for detection method. In this study, the standard Cry1Ab toxin of different concentrations (1, 2.5, 5, 10, 25, 50, 75, 100, and 200 ng mL^{-1}) was detected by the established DAS-ELISA. And the LOD for the assay was calculated as the blank control value plus five times standard deviation. The LOQ of the method was determined with the same criteria, but considered as the blank signal plus ten times its standard deviation [49].

Quantitative range

The quantitative range was defined as the lower and upper limits of Cry1Ab concentrations that could be detected accurately. In this study, the quantitative range was concluded from the data obtained in the above section.

Specificity

The method specificity was evaluated by cross-reactivity and matrix effect tests. In this study, the cross-reactivities of the established DAS-ELISA with Cry1Ac, Cry1B, Cry1C, and Cry1F toxins were verified by establishing dose–response curves using the standard toxins between 0 and 4000 ng mL^{-1} in CBS treatment along with Cry1Ab toxin. Matrix interference is a common problem existed in the process of immunoassays, which may cause false positive or negative results in real sample analysis [50]. Usually, the impact of the matrix can be reduced by a simple dilution with assay buffer before analysis. In this study, the matrix effect was evaluated by comparing the absorbance values of the rice leaf and rice flour extracts and their 5-fold, 10-fold dilutions with CBS buffer at 450 nm.

Accuracy

Accuracy was determined by recovery test in spiked rice leaf and flour samples. In brief, 5 g non-transgenic rice leaf (finely chopped) and rice flour samples was spiked with Cry1Ab toxin at seven concentrations (10, 25, 50, 100, 250, 500, 1000 ng g^{-1}). The samples were vortex shaken at room temperature for 30 min and kept overnight at 4 °C. On the second day, 5 mL CBS containing 0.05% Tween-20 was added to the samples as extraction solution and gently shaken for 2 h at room temperature. Then, the mixture was centrifuged at

3000 rpm for 10 min and the supernatant was diluted with CBS for ten times (actual concentrations of Cry1Ab were 1–100 ng mL^{-1}) and then used for DAS-ELISA assay without any further purification procedure. A blank (free toxin sample) was also treated as above. Each test was in triplicate and repeated three times.

Results and discussion

Preparation of immunogen

The 3D structure models of Cry1Ab and Cry1Ac toxins, as well as the 3D structure evaluation results of Cry1Ab toxin have been reported before [48]. Comprehensively considered the amino acid sequence, antigenicity and hydrophilicity, as well as the 3D structures of the two toxins, T4 was determined with the amino acid sequence of SGTVDSLDEIPPQNNNVPPR. Its 3D structure was shown in Fig. 1. T4 had relatively high antigenicity and hydrophilicity. Although T4 was a common sequence existed in the two toxins, its 3D structure in Cry1Ac toxin owned a α-Helice, which was different from Cry1Ab in this position (Fig. 1).

The selection of the right hapten is the key step for the whole process of producing specific MAb [51, 52]. It has been reported that the cross-reactivity of the antibodies is positively related to the overlapping degree of the haptens, so different structure parts of the target analytes should be selected as haptens for the production of high specific antibody [53–58]. Molecular modeling is an efficient way to find out the difference in 3D structures. In addition, another important factor in the hapten polypeptide selection and design is the prediction of antigenic determinant in protein molecules. According to previous reports, strong antigenicity, high hydrophilicity, and stable conformation of epitope have great influences on the immunogenicity of antigen. In this study,
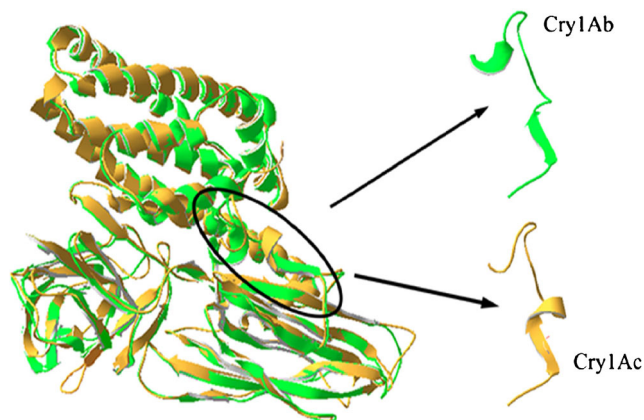
Fig. 1 Three-dimensional structures of the selected antigen polypeptide T4 in Cry1Ab and Cry1Ac toxins

T4 meet the criteria and was completely exposed to the surface. And antigen epitope analysis found that the strongest antigenicity part was just in the middle of T4 which composed the α-Helice in Cry1Ac protein.

Due to T4's weak immunogenicity, it will be coupled with KLH, the carrier protein, to elicit immune response [59]. In this research, the polypeptide T4 and KLH were coupled via -SH group on T4 to make sure the peptide was completely exposed and presented to the immune system to produce the needed antibody. It is reported that the hapten-carrier antigen would induce production of various antibodies against the hapten and the carrier [60]. However, there was no need to worry about the interference of nonspecific antibodies, for they were ruled out by coating Cry1Ab toxin in the whole process of screening.

Production of monoclonal antibodies

After three times immunization, all the four Balb/c mice produced antibodies against Cry1Ab toxin under the induction of the T4-KLH conjugate. Among them, mouse 3# giving the highest antibody titer (12.8×10^5) was chosen for the final immunization and subsequent fusion experiment.

Five clones secreting MAbs against Cry1Ab were obtained after sub-cloning and one stable hybridoma clone named 2C12 specifically recognizing Cry1Ab and had no cross reaction with Cry1Ac was expanded for ascites production. Ten days after the monoclonal cells intraperitoneally injected into the Balb/c mice, ascitic fluids were collected. The ascites were purified and the concentration was 1 mg mL^{-1}. The titer of the MAb against Cry1Ab toxin was higher than 5.1×10^5. The MAb belongs to IgG1 class, having kappa light chain.

The specific recognition of MAb 2C12 to Cry1Ab toxin may be mainly for that the antigenic determinant recognized by 2C12 was exposed in the natural form of Cry1Ab protein molecule, but just buried internally in Cry1Ac after folded into α-Helice structure. This assumption need to be confirmed with more research in the future. In addition, there are multiple factors that affect the final production of MAb, such as hapten selection, immune process, cell fusion and screening, etc. One cell fusion process can obtain multiply hybridoma cell strains, and the secreted antibodies can response to the same immunogen through screening. But antibodies may be different if more than one antigenic determinant existed on the immunogen. Even with the same antigenic determinant, different affinity may be observed for different types and sub-types of antibodies. The affinity between antigen and antibody depended on the compatibility of antigenic determinant and antibody. High affinity antibody would exhibit high sensitivity in the immunoassay.

At present, the production of anti-Cry1Ab antibody was mainly based on immunization with Cry1Ab toxin itself. This traditional method was convenient, simple, and easy to succeed. However, because of the particularity of Cry1Ab and Cry1Ac toxins, the monoclonal antibody produced for

Cry1Ab toxin has high cross reaction with Cry1Ac toxin. Unlike the traditional method, in this study the immunogen was synthesized with the hapten which was selected from the different part in Cry1Ab and Cry1Ac based on the analysis of physical properties and 3D structures of the two toxins. This method for antigen selection was more targeted and purposeful, and could provide more probabilities to produce high specific antibodies. It is a new way for hapten selection and antibody production.

Affinity determination

The affinities of MAb 2C12 and Cry1Ab/Cry1Ac toxins were determined by kinetic analysis. The sensogram of Cry1Ab toxin against MAb 2C12 was shown in Fig. 2a. From the figure we can see that all the data for Cry1Ab toxin could be entered into kinetic analysis, which indicated the high accuracy and repeatability of these results. The K_D value of Cry1Ab toxin with MAb 2C12 was 1.947×10^{-8} M (Fig. 2b). On the contrary, MAb 2C12 cannot combined with Cry1Ac which confirmed its specificity to Cry1Ab toxin. This ensured the applied value of MAb 2C12 in the specific detection of Cry1Ab toxin.

DAS-ELISA optimization and establishment

Although ELISA assay is comparatively simple, it can be influenced by a number of factors [61], including reagent quality, storing temperature, adding method, washing buffer, washing time and number, reaction time and temperature, antibody concentrations, and so on. In this study, the concentrations of capture, detection and tracer antibodies, as well as the developing time were examined in order to improve the performance of immunoassay.

The absorbance values at 450 nm had significantly positive correlations with the capture and detection antibody concentrations. In the established DAS-ELISA, considering the quantified dynamic range and linearity coefficient of determination (R^2) of the curve, as well as low consumption of antibodies, the optimal concentrations of the anti-Cry1Ab mouse MAb and anti-Cry1Ab rabbit PAb were determined as 0.5 and 0.2 µg mL^{-1}, respectively (Fig. 3a, b). In addition, 1:7500 dilution of the tracer antibody (HRP-goat-anti-rabbit, 1 mg mL^{-1}) and 5 min color developing time were observed to be the best criteria for the linearity of the curve and limit of detection. Higher tracer antibody concentration and longer developing time will improve the background values. The optimized antibody concentrations and measurement conditions for determination of Cry1Ab toxin were used in subsequent assays.
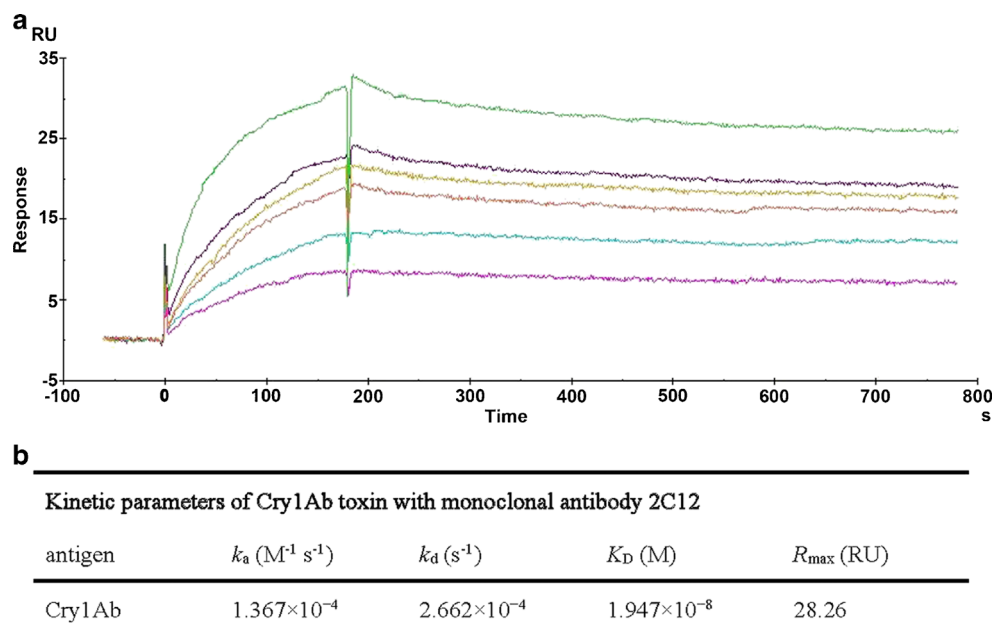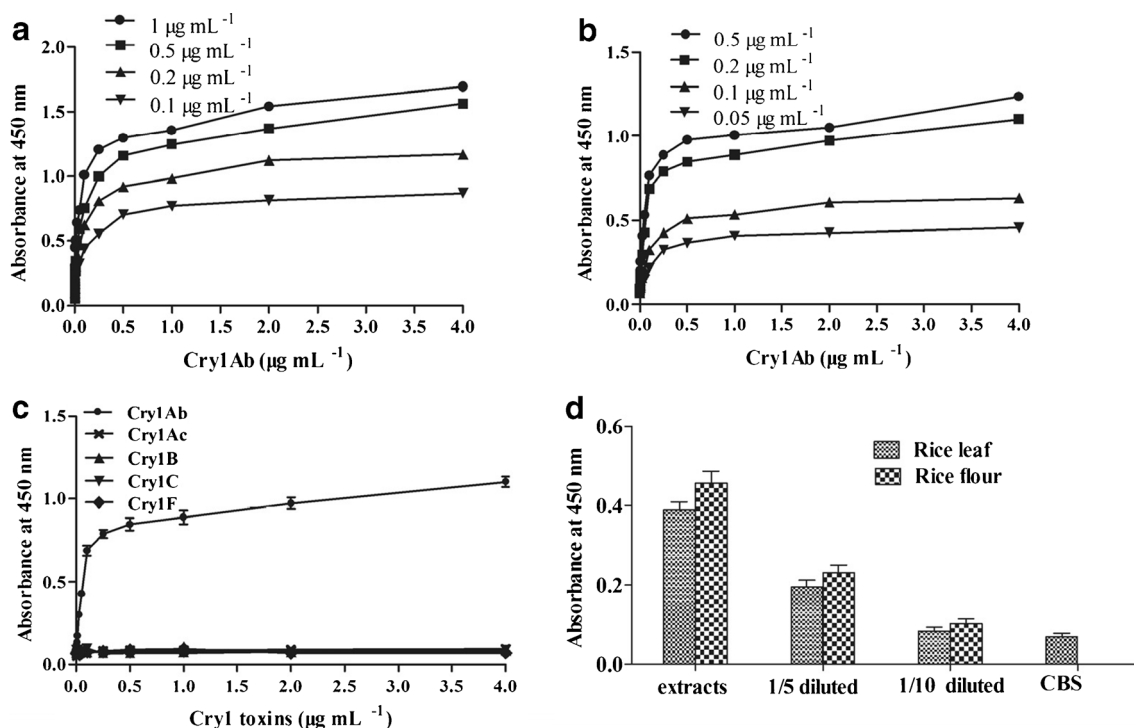
Validation of the DAS-ELISA

Sensitivity

To determine the LOD and LOQ, series concentrations of Cry1Ab standard toxin between 1 and 200 ng mL^{-1} were used. There were good correlations between the absorbance values and the concentrations of Cry1Ab toxin between 1 and 100 ng mL^{-1} which could be described by a cubic polynomial equation:

$$y = A + Bx + Cx^2 + Dx^3$$

In the equation, y is the absorbance value (OD) and x is the Cry1Ab concentration (ng mL^{-1}). A, B, C, and D are coefficients which were 0.08580, 0.00938, -0.00004, and -1×10^{-6}, respectively (Fig. 4). And, the LOD and LOQ were determined as 0.47 ± 0.11 and 2.43 ± 0.19 ng mL^{-1},

Fig. 2 a Sensogram and **b** kinetic parameters of binding between Cry1Ab toxin and MAb 2C12

Kinetic parameters of Cry1Ab toxin with monoclonal antibody 2C12

antigen	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D (M)	R_{max} (RU)
Cry1Ab	1.367×10^{-4}	2.662×10^{-4}	1.947×10^{-8}	28.26

Fig. 3 Optimization of the **a** anti-Cry1Ab MAb (capture antibody) and **b** anti-Cry1Ab PAb (detection antibody) concentrations and **c** cross-reactivities of the optimized DAS-ELISA with Cry1Ac, Cry1B, Cry1C, and Cry1F toxins, as well as **d** absorbance at 450 nm of different dilution gradients of the rice leaf and flour extracts. Fixed antibody concentrations were as follows: **a**, detection antibody 0.5 μg mL^{-1}; **b**, capture antibody 0.5 μg mL^{-1}; **c**, capture antibody 0.5 μg mL^{-1} and detection antibody 0.2 μg mL^{-1}. Each value is mean ± standard deviation of three replicates

respectively. The detection limit of the established DAS-ELISA method in this study was the same level as the commercial ELISA kits of Cry1Ab/Cry1Ac [32, 62] and the established DAS-ELISA can be used for specific quantitative determination of Cry1Ab toxin.

Quantitative range

Figure 4 shows that the absorbance values have good correlations with the concentrations of Cry1Ab toxin between 1 and 100 ng mL^{-1}. Meanwhile, considering the LOQ, the quantitative range was determined as 2.5–100 ng mL^{-1}, in which range the Cry1Ab toxin can be accurately quantified by the standard curve and equation. However, higher concentration of Cry1Ab (>100 ng mL^{-1}) needs a further dilution for accurate quantification.

Specificity

The cross-reactivity of the optimized DAS-ELISA was determined against Cry1Ac, Cry1B, Cry1C, and Cry1F toxins and the results were showed in Fig. 3c. There were no correlation between the absorbance values and the concentrations (up to 4 μg mL^{-1}) of those Cry1 toxins. The results showed that the established DAS-ELISA was highly specific to Cry1Ab toxin and had no cross reaction with Cry1Ac, Cry1B, Cry1C, and Cry1F.

The Cry1Ab, Cry1Ac, Cry1B, Cry1C, and Cry1F toxins are all members of *B. thuringiensis* family and have been widely introduced into GM crops. Among them, Cry1Ab and Cry1Ac toxins have highly similar sequences and 3D structures [48, 63]. As reported by many researchers and manufacturers, even if the MAbs and PAbs were raised against the Cry1Ab toxin, they can also strongly bind to the Cry1Ac

$$y = 0.08580 + 0.00938\,x - 0.00004\,x^2 - 1 \times 10^{-6}x^3$$

$$r^2 = 0.99957$$
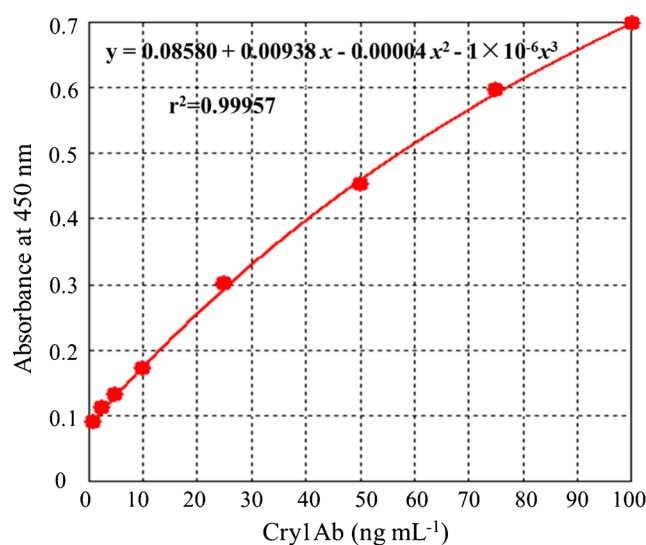
Fig. 4 Polynomial regression line calculated using the standard Cry1Ab toxin within the range of 1–100 ng mL^{-1}. Each value is mean of three replicates

Table 1 Recoveries of Cry1Ab toxin from spiked rice leaf and flour samples by the developed DAS-ELISA

Matrix	Spiked Cry1Ab (ng g^{-1})	Amount determined (ng g^{-1})[a]	Mean recovery ± SD (%)	CV (%)
Rice leaf	10	12.4	124 ± 8.4	6.8
	25	26.5	106 ± 4.2	4.0
	50	48.1	96 ± 5.5	5.8
	100	93.2	93 ± 5.5	5.9
	250	229.8	92 ± 4.7	5.1
	500	435.2	87 ± 6.3	7.2
	1000	805	81 ± 3.4	4.2
Rice flour	10	13.4	134 ± 10.9	8.1
	25	28.7	115 ± 7.9	6.8
	50	54.2	108 ± 9.4	8.6
	100	99.2	99 ± 6.6	6.7
	250	220.1	88 ± 7.5	8.6
	500	412.2	82 ± 5.8	7.1
	1000	754.5	75 ± 4.1	5.4

[a] The values are the mean of three replicates

protein (indeed, Cry1Ab immunoassay kits are usually commercialized as Cry1Ab/Cry1Ac assays). In this study, the greatest advantage is the established DAS-ELISA could specifically recognize and detect Cry1Ab toxin without cross reaction to other Cry1 toxins, especially Cry1Ac toxin.

The matrix interferences of rice leaf and flour were analyzed by testing 5-fold and 10-fold dilutions. The results were shown in Fig. 3d, no significant difference was observed in the absorbance between the 10-fold dilution of sample extracts and the CBS, indicating that a 10-fold dilution with CBS could basically reduce matrix effect for the detection of Cry1Ab toxin without any further cleanup steps. The simple sample preparation process makes the approach saving in both time and cost, and convenient for users to operate without access to expensive and complex equipment, which meet the requirement of rapid determination.

Accuracy

Accuracy is evaluated by measuring the mean recoveries of Cry1Ab from fortified samples at several concentration levels in the quantitative range. Usually, the recoveries between 70 and 120% with coefficient of variations (CV) less than 20% for each fortification level are considered to be practicable.

In this study, the accuracy of the developed DAS-ELISA for Cry1Ab detection was assessed by measuring spiked rice leaf and flour samples based on the established cubic polynomial equation and the results were listed in Table 1. As shown in Table 1, the average recoveries of

25–1000 ng g^{-1} Cry1Ab fortified rice leaf and flour samples ranged between 75 and 115% with CV less than 8.6% (actual concentrations of spiked extracts used for assay were 2.5–100 ng mL^{-1}) which met the criteria of accuracy. When the fortified concentration of Cry1Ab was 10 ng g^{-1} in rice leaf and flour, i.e., 1 ng mL^{-1} for spiked extracts, the recoveries were 124 and 134%, respectively. These recoveries were out of the accurate range, which indicated Cry1Ab could not be accurately measured but still could be detected at this concentration.

These results suggested that within quantitative range, the established DAS-ELISA was accurate for the quantitative determination of Cry1Ab toxin. The results showed the developed DAS-ELISA, the sample extraction method, and dilution agreement were satisfactory and accurate for the detection of Cry1Ab toxin in agricultural samples.

Conclusions

At present, Cry1Ab toxin cannot be specifically detected by ELISA method based on monoclonal antibody for its amino acid sequence and 3D structure were highly similar with Cry1Ac toxin. Under such background, an attempt utilizing the difference existed in the 3D structures of the two toxins to design a novel polypeptide as immunogen and produce specific MAb against Cry1Ab toxin was presented. Then, a DAS-ELISA was established based on the specific MAb. The LOD and LOQ values were determined as 0.47 ± 0.11 and 2.43 ± 0.19 ng mL^{-1}, respectively. Recoveries of Cry1Ab from spiked rice leaf and flour samples ranged from 75 to 115% with CV less than 8.6% during quantitation range (2.5–100 ng mL^{-1}), demonstrating that the DAS-ELISA method based on MAb 2C12 is sensitive and accurate for the specific detection of Cry1Ab toxin in samples. Compared with previous methods for antibody preparation, this method based on molecular design can be more targeted, and it provided a new approach to produce high specific antibody with low cross reaction to their analogues which can be used to distinguish the high similar antigens.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

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