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A novel sandwich ELISA system using rabbit monoclonal and polyclonal antibodies for rapid detection of Bt-Cry1Ac protein

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ABSTRACT. Crystal proteins (Cry) from *Bacillus thuringiensis* (Bt) are well-known pesticidal proteins in genetically modified (GM) crops and often used as the target proteins in detection of GM food. Instead of the classic mouse hybridoma, new rabbit monoclonal antibodies (McAb) were first produced in detection of GM food. Rabbit McAb and polyclonal antibodies (PcAb) were prepared using Bt-Cry1Ac protein as the immune antigen (Ag). Sandwich ELISA with either PcAb as the coating antibody and peroxidase horseradish (HRP) labeled McAb as the detecting antibody (PcAb-Ag-McAb), or McAb as the coating antibody and HRP labeled PcAb as the detecting antibody (McAb-Ag-PcAb), was developed for detecting Bt-Cry1Ac protein. The analytical sensitivity of McAb-Ag-PcAb was higher with the linear detection range of 0.97-62.50 ng mL^{-1} and the limit of detection (LOD) of 0.24 ng mL^{-1}. There was no cross-reaction with Cry1Ab and Cry1c proteins and the mean recovery was 103.35% in corn leaves. The LOD and the linear detection range of the proposed ELISA are more satisfactory than other

ELISA systems using an anti-Cry1Ac PcAb or two Cry1Ac-specific McAb for detection of the target protein and the research provides an effective instrument for detecting Cry1Ac protein in GM crops and their derivatives.

Keywords*: Cry1Ac; Sandwich ELISA; Rabbit monoclonal antibody; Genetically modified food; Detection*

1. Introduction

Since the first genetically modified (GM) food emerged in 1996 in USA, research and production of GM food have aroused more and more attention all over the world. In 2008, the GM plants were committed to grow in twenty-five countries and GM food was permitted to import in thirty countries [18]. GM technology has accelerated the development of biology and brought much convenience to human life [15]. However, it also brings many potential concerns about ecological safety and human health[9]. For these reasons, many countries have set their upper limit content of GM components, e.g., 0.9% in European Union, 3% in South Korea, and 5% in Japan [3]. With the purpose of safeguarding the security of animals and human and protecting our ecology, since labeling GM food and seed is regulated, it is very important to develop a fast and sensitive method to detect and determine the GM components' contents [9].

Bacillus thuringiensis (Bt) crystal proteins (Cry) are common pesticidal proteins in GM plant food. Cry1Ac protein is a typical and widely studied Bt-Cry protein that exists in many anti-pest GM plants, such as maize, potato, cotton and corn, with the expansion of planting area annually in many countries [8,20]. Enzyme-linked immunosorbent assay (ELISA) is one of the most common detection methods for GM food [4,14], based on strong specificity and affinity of antibodies and high efficient catalytic ability of enzymes [2,21]. Previous research claimed that ELISA based on Cry1Ac-specific antibodies was more specific than DNA-based PCR, which was another common method for GM foods determination [1]. ELISA method can detect target protein both qualitatively and quantitatively, expected to provide a fast, sensitive and precise result [4,5].

In ELISA, polyclonal antibodies (PcAb) are a mixture of immune globulins against different epitope specificities of immune antigen (Ag) and have the main

merits of low cost and short time in its production, while monoclonal antibodies (McAb) are specific to a single epitope of Ag and have more sensitivity and specificity to their specific antigen. Recently, mouse McAb [19,25,29] and rabbit PcAb [7,10,17,23,24,26] have been used to detect several Bt proteins. However, there is no related ELISA system using rabbit McAb or both rabbit McAb and rabbit PcAb for detection of GM food. In our previous research, some high-affinity rabbit McAb have been already developed for detection of veterinary drug residues in milk [11,12,13]. Compared to mouse McAb, rabbit McAb have simpler structure, wider repertoire, higher binding affinity, robuster reproduction and easier humanizing, which has increasingly gained attention for clinical application [6,13]. Besides, there is limited availability of specific methods to detect Cry1Ac protein in GM plants and the used antibodies are either rabbit anti-Cry1Ac PcAb [26] or two mouse Cry1Ac-specific McAb [19]. ELISA with both PcAb and McAb has higher sensitivity than that only using PcAb, and lower cost than that using two specific McAb.

Thus, the main purpose of this paper was to develop a new sandwich ELISA system for detecting Cry1Ac protein specifically using both rabbit McAb and rabbit PcAb. Based on high-quality rabbit antibodies, it is probable to lower the limit of detection (LOD), improve the precision and accuracy of analytical results, and widen the scope of the determination techniques of GM food. The new ELISA will provide an effective instrument for detecting and identifying Cry1Ac protein in GM crops.

2. Materials and methods

2.1. Materials

The purified recombinant Cry1Ac, Cry1Ab, Cry1c toxin proteins (different Bt proteins), transgenic corn samples with *Cry*1Ac gene and non-transgenic corn samples (*Oryza sativa* Minghui 63) in this study were supplied by China National Rice Research Institute (CNRRI). Tetramethylbenzidine substrate (TMB) was from National Biochemicals Corp. Molecular weight markers were from TaKaRa Biotech. HRP was from solon industry. The rest of the chemicals were of analytical grade. SPECTRAmax Plus348 microplate reader was from molecular device corporation

(USA).

2.2. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Twelve percent separation gel, 5% stacking gel, SDS running buffer (0.348 mol L^{-1} glycine, 0.05 mol L^{-1} Tris, 0.1% SDS), sample buffer (2% SDS, 5% β-mercaptoethanol, 0.02% bromophenol blue in 0.01 mol L^{-1} Tris-HCL buffer) were used in this experiment.

2.3. Preparation of antibodies

This part was finished with cooperation of Epitomics Biotech (Hangzhou) Co., Ltd. In this study, five rabbits were immunized in order to obtain PcAb and McAb that against Cry1Ac protein. PcAb were prepared by immunizing two rabbits with 500 μg of Cry1Ac protein. Thereafter every rabbit was injected 200 μg of Cry1Ac protein every two weeks at 4 sites. The anti-Cry1Ac serum of the rabbits was obtained and the serum with higher titer was purified by Sepharose Protein-A. Other three rabbits were immunized as the method of preparing PcAb. Splenic cells of the rabbit with highest serum titer were obtained and the B cells were fused with myeloma cells. The positive fusion cells were screened by indirect ELISA. After cultured for two weeks, three optimal fusion cells were chosen for obtaining recombinant clones. And then one recombinant clone with highest affinity to Cry1Ac protein was used for McAb production [25,27]. The McAb were purified by Sepharose Protein-A immunosorbent column.

2.4. Conjugating antibody with peroxidase horseradish (HRP)

Forty-five microliter $NaIO_4$ solution (20 mg mL^{-1}) was added to 400 μL HRP solution (5 mg mL^{-1}), kept at room temperature for 20 min, and the oxidation process was stopped by 40 μL glycol. The oxidated HRP and antibodies of 2 mg were put together at room temperature and pH 9.0-10.0 for 2 h. The conjugated solution was transferred into a brown container, and then 45 μL $NaBH_4$ was added at 4°C for 2 h. The HRP labeled antibodies were purified by 50% saturated $(NH_4)_2SO_4$. At last, the conjugates were resolved by phosphate buffered saline (PBS,

10 mmol L^{-1}, pH 7.2) and dialyzed against PBS at 4°C for 12 h, and then the same volume of glycerol was added. The product was stored at -20°C.

2.5. Sandwich ELISA

In this study, two different sandwich ELISA methods either McAb as the coating antibody or PcAb as the coating antibody were developed to choose a sandwich ELISA with higher assay sensitivity to analyze Cry1Ac protein. Micro-well plates were coated with 100 μL antibodies of either McAb (5 μg mL^{-1}) or PcAb (8 μg mL^{-1}) per well at 4°C over night. After washing the plates two times with PBST (PBS with 0.05% Tween 20), nonfat milk (3%, m/v) of 200 μL was added to block the unbounded sites at 37°C for 1 h. Then the plates were washed three times with PBST. For the immunoassay, 100 μL protein solution (diluted in PBS) was added to each well, and incubated at 37°C for 1 h with shaking. After washing the plates four times, 100 μL conjugate HRP of either HRP-PcAb (1:1000 dilution) or HRP-McAb (1:200 dilution) was added to each well. After being incubated at 37°C for 1 h with shaking, the plates were washed five times with PBST. TMB substrate solution (5 mg mL^{-1} TMB, 0.04% H_2O_2) of 100 μL was added to each well, after 15 min the reaction was stopped by 2 mol L^{-1} H_2SO_4 and the absorbance was read at 450 nm.

2.6. Method validation

In the optimal ELISA, assay specificity, recovery and precision were evaluated.

Extraction of whole corn leaves protein: All the corn leaves were collected at the seedling stage and the protein extract buffer was PBST with 2.0 mg mL^{-1} vitamin C. Clean and dry leaf samples of 23.0 ± 2.0 g were mashed and soaked in 500 μL extract buffer for 60 min on ice. The extracts were centrifuged at 6000 g for 5 min and the supernatant was stored at 4°C until use [17].

Specificity: Another two Bt proteins (Cry1Ab and Cry1c) were used to validate the specificity of the optimal ELISA reaction with the positive control Cry1Ac in this assay. The concentrations of these proteins (Cry1Ac, Cry1Ab, Cry1c) were 2000, 1000, 500, 250, 125, 62.50, 31.25, 15.62, 7.81, 3.90 ng mL^{-1}.

Recovery: To determine the accuracy of the optimal sandwich ELISA method,

recovery experiments were taken. Cry1Ac protein was fortified into non-transgenic corn leaves' tissue at different concentration levels (31.25, 15.62, 7.81, 3.90 ng mL^{-1}), and then it was extracted together with whole tissue proteins in the non-transgenic corn leaves and measured by the optimal sandwich ELISA. Each concentration level of fortified extract was run four replicates.

Precision: Extracted tissue proteins of transgenic corn leaves were analyzed to assess the precision of this optimal ELISA. The protein extract was prepared and diluted 1:3 with PBST for assay.

3. Results and discussion

3.1. Analysis of Cry1Ac purity

To obtain McAb towards Cry1Ac toxin protein successfully, the purity grade of Cry1Ac protein should be higher than 90%. Before immunization, the antigen's purity was analyzed by SDS-PAGE (Fig. 1). There was only one clear visible band of Cry1Ac protein in SDS-PAGE, which indicated that the purity of Cry1Ac protein was at an extremely high level (> 90%) and it could be used as an antigen for antibody production. In Fig. 1, it was showed that the molecular weight of Cry1Ac protein was between 64 kD and 67 kD, large enough to induce immune response solely.

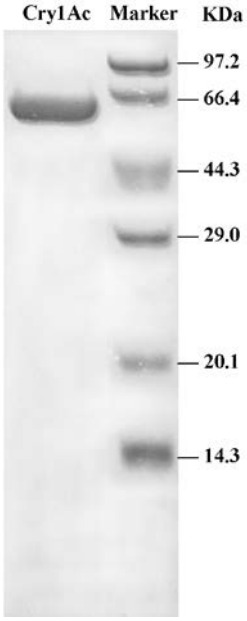

Fig. 1 Purity of Cry1Ac protein by SDS-PAGE.

3.2. Sandwich ELISA

PcAb detect a multiplicity of epitopes and could recognize the antigen from different orientations while McAb detect a single epitope and have proved effective reagents in terms of specificity for clinical diagnostic tests [16]. Both McAb and PcAb used in the experiment are rabbit antibodies. Rabbit antibody usually contains a high affinity and can recognize more types of epitopes than murine antibody. Compared to the rat and the mouse, the rabbit has a larger spleen, so more fusion experiments can be performed using rabbit antibody and the high throughput screening of fused cells becomes possible. Rabbit McAb is able to recognize the antigen of small molecules more precisely with the technological support of the cooperative Epitomics Company, which has many related patents. This is the first report about using the rabbit McAb to detect transgenic proteins.

In a sandwich ELISA bases on McAb and PcAb, there is no common sense of whether McAb could be the capture antibody and PcAb is the detection antibody or

PcAb could be the capture antibody and McAb is the detection antibody. Two different sandwich ELISA methods were developed and evaluated by their sensitivity and linear detection range to choose an optimal sandwich ELISA for further study. In Fig. 2, the result of McAb as the coating antibody and HRP labeled PcAb as the detection antibody (McAb-Ag-PcAb) showed that the LOD capability of this assay method was 0.24 ng mL^{-1}, which was defined as the concentration corresponding to three standard deviation of the signal of the blank control [22,26]. This LOD reached the detection level of lower than 1 ng mL^{-1}. The linear detection range was 0.97-62.50 ng mL^{-1}, which means the range that could be detected quantitatively. The correlative equation was $y=0.0318x+0.0418$ with $R^2=0.9994$. This detection level was better than that reported before by studying Cry1Ac protein, which was 0.5 ng mL^{-1}[19]. In the reports of other Bt proteins [7,25,28,29], only Zhu's research studying Bt-Cry1Ab protein showed a better LOD, which was lower than 10 pg mL^{-1} by dot-based fluorescence-linked immunosorbent assay (QD-FLISA), but a bad precision left for improvement.

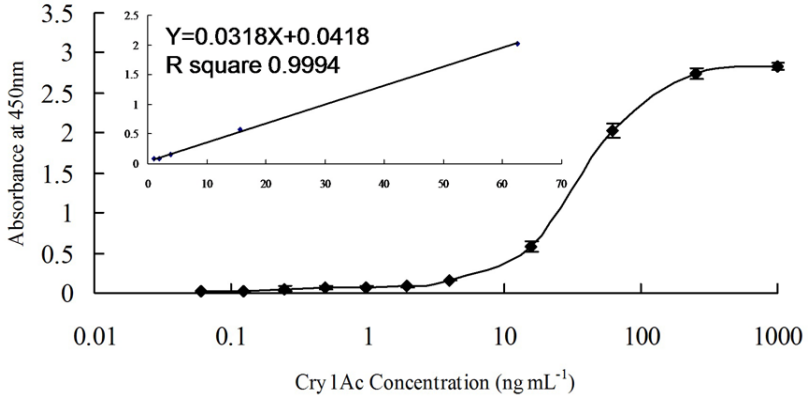

Fig. 2 Determination of Cry1Ac toxin protein by a sandwich ELISA, in which the coating antibodies were McAb and the detection antibodies were HRP labeled PcAb. The concentrations of Cry1Ac protein were 1000, 250, 62.50, 15.60, 3.90, 1.95, 0.97, 0.49, 0.24, 0.12, 0.06 ng mL^{-1}.

The result of PcAb as the coating antibody while HRP labeled McAb as the detection antibody (PcAb-Ag-McAb) (Fig. 3) showed that the LOD was about 1.95 ng mL^{-1}, which was lower than 10 ng mL^{-1}. The linear detection range of the second

ELISA was 3.90-62.50 ng mL^{-1} and the correlative equation was y=0.0287x-0.0204 with R^2=0.9967. The data of PcAb-Ag-McAb seems not better than McAb-Ag-PcAb. In previous related reports, the ELISA method to detect Cry1Ac protein was set up with easily got two McAb or only PcAb instead of both PcAb and McAb and sometimes the detection limit could be given in the unit μg per g dry/fresh weight of leaves or other related samples [7,10,24]. The results demonstrated that the sandwich ELISA with both PcAb and McAb had better LOD than previous ELISA methods for the detection of Cry1Ac protein.

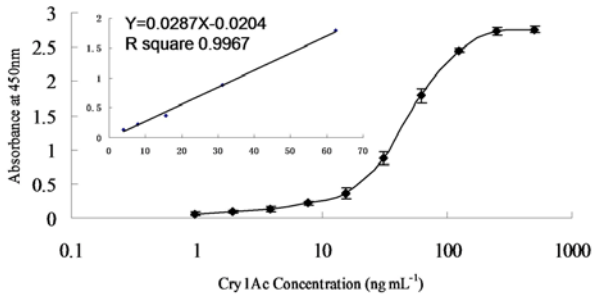

Fig. 3 Determination of Cry1Ac toxin protein by a sandwich ELISA, in which the coating antibodies were PcAb and the detection antibodies were HRP labeled McAb. The concentrations of Cry1Ac protein were 500, 250, 125, 62.50, 31.25, 15.60, 7.80, 3.90, 1.95, 0.97 ng mL^{-1}.

The results of the two assay methods indicated the sensitivity of McAb-Ag-PcAb was higher and the linear detection range of McAb-Ag-PcAb was wider than that of PcAb-Ag-McAb. Therefore, McAb-Ag-PcAb assay method was chosen for further study.

3.3. ELISA method validation

3.3.1. Specificity

Two other Bt-Cry proteins (Cry1Ab and Cry1c) were taken to study the cross-reaction. The result (Fig. 4) indicated that these anti-Cry1Ac antibodies had no obvious cross-reaction with other Bt-Cry proteins Cry1Ab and Cry1c. These McAb

and HRP labeled PcAb could be highly specific for Cry1Ac protein without significant cross-reaction with other Bt-Cry proteins assessed here, which is in accordance with previous study using mouse McAb or rabbit PcAb [19,26]. Among many reported Bt proteins, three used Bt proteins (Cry1Ac, Cry1Ab, Cry1c) are typical proteins in GM crops [25], and in most cases sandwich ELISA developed with McAb could detect target antigen availably without obvious interference from other proteins. Hence, this assay could provide a significant specific detection method against Cry1Ac protein in GM crops.

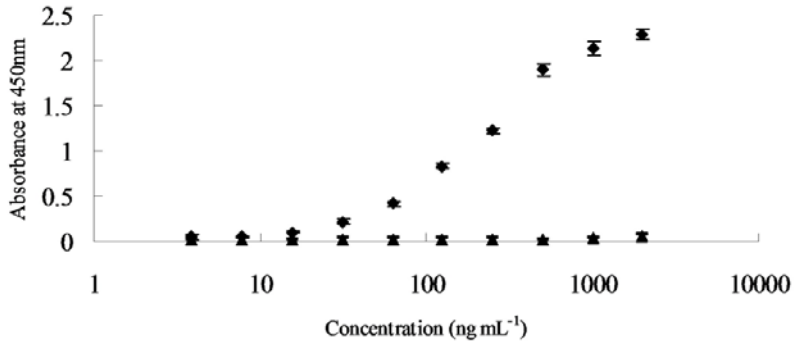

Fig. 4 Cross-reaction results between antibodies and other Bt proteins by the newly developed ELISA (McAb-Ag-PcAb). ◆Cry1Ac —Cry1Ab ▲Cry1c

3.3.2. Recovery

The analytical performance of this optimal ELISA was assessed by fortifying Cry1Ac protein into non-transgenic corn leaves. Cry1Ac protein was extracted with other proteins in corn leaves together. The immunoassay performed well when Cry1Ac protein was fortified in negative control samples and the recoveries ranged from 89.20% to 121.92% with the mean value of 103.35% (Table 1). The result indicated that the immunoassay method had good assay accuracy and could efficiently detect Cry1Ac protein by extra-assay.

Table 1 Accuracy results achieved by fortifying Cry1Ac protein in negative corn leaves

Cry1Ac protein		Recovery rate (%)
Amount added (ng mL^{-1})	Amount measured (ng mL^{-1})[a]	
31.25	34.53 ± 2.55	110.49
15.62	13.94 ± 0.89	89.20
7.81	7.17 ± 0.26	91.77
3.90	4.76 ± 0.23	121.92
Mean recovery		103.35[b]

[a] Mean value ± standard deviation (four replicates)

[b] Mean recovery (%)

3.3.3. Precision

Tissue protein extracting from corn leaves was collected and detected by the newly developed sandwich ELISA. Positive-1 and Positive-2 were from different GM corn leaves which were grown in different fields. Absorbance at 450 nm (OD_{450}) of positive samples was obviously higher than that of control and negative samples (Fig. 5), which indicated that this sandwich ELISA could be used to detect some crops with Cry1Ac protein. Furthermore, the Cry1Ac protein in the positive samples was 1.465 μg g^{-1} dry leaf (Positive-1) and 1.673 μg g^{-1} dry leaf (Positive-2) separately. This demonstrated that the novel immunoassay method could be used to detect Cry1Ac protein in some crops quantitatively. In fact, loss of tissue protein can not be avoided during the extracting process, so reducing the loss of protein will improve the precision of the measurement. Previous ELISA systems for Cry1Ac detection in GM plant includes an ELISA based on Cry1Ac-specific PcAb, which could detect the target protein from GM cotton leaves with the similar efficiency to PCR method [26] and an ELISA based on Cry1Ac-specific McAb, which could be used in detection of cotton tissues - boll, leaf, pollen and seed [19]. Compared to them, the new sandwich ELISA system using McAb-Ag-PcAb has better precision and efficiency in detection of Cry1Ac GM plants.

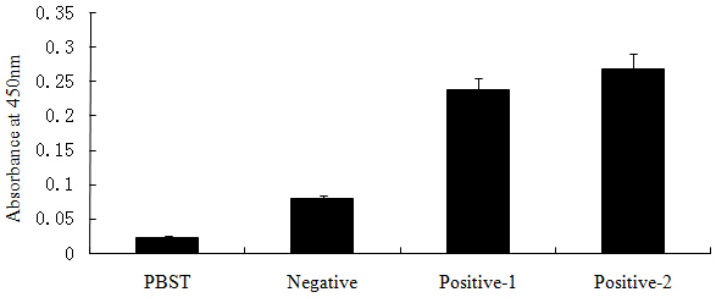

Fig. 5 Detection of positive samples by the optimal ELISA. PBST was tested as control. Positive-1 and Positive-2 represent sample test of transgenic corn leaves, and Negative represents sample test of non-transgenic corn leaves.

4. Conclusions

In this study, rabbit McAb were used for the first time in detection of transgenic proteins. Both McAb and PcAb against different epitope specificities of Cry1Ac protein were obtained. It is proved that sandwich ELISA with McAb as coating antibody and HRP labeled PcAb as detection antibody was the optimal choice for detection of Cry1Ac protein. The detection limit and the linear detection range of the new sandwich ELISA were better than reported ELISA methods using an anti-Cry1Ac PcAb or two Cry1Ac-specific McAb. Meanwhile, the antibody in this immunoassay showed highly specific for Cry1Ac without significant cross-reaction with Cry1Ab and Cry1c proteins. This developed immunoassay method performed well with fortifying Cry1Ac protein in negative samples and the recoveries ranged from 89.20% to 121.92% with the mean value of 103.35%. Tissue protein extracts of the positive corn leaves were used to evaluate the precision of this developed ELISA and it showed that this immunoassay method could detect Cry1Ac in some positive crops qualitatively and quantitatively with accuracy.

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