Preparation of Artificial Antigen and Egg Yolk-derived Immunoglobulin (IgY) of Citrinin for Enzyme-Linked Immunosorbent Assay[1]

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Objective To prepare artificial antigens and anti-citrinin egg yolk-derived immunoglobulin (IgY) to build an enzyme-linked immunosorbent assay (ELISA) for citrinin (CTN). **Methods** CTN was conjugated with bovine serum albumin (BSA), ovalbumin (OVA) with formaldehyde condensation method to prepare artificial antigens and identified by ultraviolet (UV) spectrometry and Infrared (IR) spectrometry. Artificial antigens for CTN and anti-CTN IgY were purified with polyethylene glycol two-step precipitation method and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). ELISA with IgY was established. Cross-reactivity of IgY with various structural similarities to CTN and possible co-occurrence with CTN in agricultural commodities were studied. **Results** UV and IR absorption spectra suggested that CTN was correlated with the carrier protein of BSA or OVA. SDS-PAGE patterns showed that the anti-CTN IgY was almost pure with a molecular weight of approximate 100 KD. The indirect competitive ELISA showed that the detection limit of CTN was 10 ng mL^{-1}, with a good linearity ranging 20-640 ng mL^{-1}. **Conclusion** Artificial antigens of CTN can be successfully synthesized. The established ELISA can be used to determine CTN- contaminated samples.

Key words: Citrinin; Artificial antigen; IgY; ELISA

INTRODUCTION

Citrinin (CTN) [(3R, 4S)-4,6-dihydro-8-hydroxy-3,4,5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylc acid], a toxic secondary metabolite, was first isolated from filamentous fungus *Penicillium citrimun*[1]. It is also produced with other species of *Penicillium*[2], *Aspergillus*[3] and *Monascus*[4]. CTN is known to be nephrotoxic and carcinogenic[5-6], teratogenic[7] and mutagenic[8] to humans and to a wide variety of animals, and is often found together with another mycotoxin, ochratoxin A[9] which is a severe dangerous food and feed contaminant. CTN is present in wheat[10], oats[11], barley[12], corn[13], rye[14], wheat flour[15], feed rains[16], and red rice[17] and can be assayed using thin-layer chromatography techniques (TLC)[18], high-performance liquid chromatography (HPLC)[19], with UV or fluorescence detection(FD)[20], and ELISA[21]. Recently, LC-MS, and GC-MS[22] techniques have become available for qualitative and quantitative determination of CTN. Cloning and sequence analysis of full-length cDNA of a novel yp05 gene associated with CTN production in *Monascus aurantiacus* has been reported[23]. The development of immunochemical assay techniques allowed Vrabcheva *et al.*[24] to perform the first large-scale examination of contamination on cropped grain. Since Willianms and his associates found the egg yolk immunoglobulin, IgY has been identified mainly in the yolk of bird eggs. With the development of IgY technique, an increasing number of reports on IgY for ELISA are available as a result

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of in-depth IgY studies[25-28]. However, no reports on anti-CTN IgY for ELISA are available.

This paper discusses the method for the preparation of artificial immune antigens, the development of plate coating antigens for CTN, and the possession of purified anti-CTN IgY. A rapid test of anti-CTN IgY was established by indirect competitive enzyme-linked immunosorbent assay (ELISA) as previously described[29]. The anti-CTN IgY can be used to detect if patients are poisoned with citrinin by analyzing blood serum and emiction, which is beneficial to people's health and can lay a foundation for the development of immunological methods to detect other biotoxins.

MATERIAL S AND METHODS

Materials and Instruments

CTN (C1017, Sigma, USA), formaldehyde (Kemeng, China), bovine serum albumin (BSA), ovalbumin (OVA) (Bio,China), anti-chicken IgY HRP conjugate (G135A, Promega, USA), were used in this study.

UV spectra were recorded with a TU-1901 instrument (Pgeneral, China). IR spectra were recorded with a Nicolet 380 instrument (Thermo, the Unites States). The value of optical density (OD) was measured using a Bio-Rad microplate reader (No:680, Bio-Rad, USA).

Preparation and Identification of Immunogen and Plate Coating Antigen for CTN

Immunogen and plate coating antigen for CTN were prepared in accordance with the description by D.Abramson (1995) and following optimizing modifications. BSA (10.0 mg) was dissolved in 2 mL of sodium acetate solution (0.1 mol L^{-1}, pH 4.2), 0.2 mg of CTN was dissolved in 80 μL of methanol, and then 100 μL of 37% formaldehyde mixture solution was then added. After incubated at 37 ℃ for 24 h, the mixture was dialyzed at 4 ℃ for 2 d against four changes of phosphate buffer saline (PBS) containing 0.01 mol L^{-1} phosphate and 0.1 mol L^{-1} NaCl at pH 7.4, followed by four changes of distilled water. CTN-BSA conjugation was obtained and lyophilized at -60 ℃ for 12 h. The final products were preserved at -20 ℃. OVA was used to substitute BSA for preparing the plate coating antigen CTN-OVA.

UV spectra of CTN, BSA, OVA, CTN-BSA and CTN-OVA (aqueous solutions) were tested at a wavelength of 240-400 nm. IR spectrogra of BSA, OVA, CTN-BSA, and CTN-OVA were scanned respectively by FTIR with a pressing potassium bromide troche.

Preparation and Purification of Anti-CTN IgY

Immunized white Leghorn laying hens (Shavers SX 288) at the age of approximately 20 weeks were used to produce eggs following the standard animal care regulation. One mg of prepared CTN-BSA conjugate was dissolved in 1 mL of 0.1 mol L^{-1} PBS and emulsified with an equal volume of complete Freund's adjuvant as the immunogen for injection. Intramuscular (pectoral muscles) and lap injection of 0.5 mL emulsion was given to each hen. Four booster injections using the same preparation method substituting complete Freund's adjuvant for incomplete Freund's adjuvant were given subsequently on days 14, 24, 34, and 44. Eggs were collected every day one month after the final boost with purification and stored at 4 ℃.

Anti-CTN IgY was purified as previously described[30] with the following yield-optimizing modifications.

In general, 1 volume of yolk was diluted with 3 volumes of a 4% polyethylene glycol 6000 solution containing 40 g of polyethylene glycol 6000 dissolved in 2910 mL (pH 7.0) and 0.01 mol L^{-1} phosphate buffered saline (PBS)) and 0.3 volume of chloroform. The mixture was shaken up, followed by centrifugation at 9690 r/min for 10 min at 10 ℃. The top aqueous layer was obtained and filtrated with tissues, the rest mixture was then precipitated twice with a 40% polyethylene glycol 6000 solution containing 640 g polyethylene glycol 6000 and 960 mL PBS. The precipitate of IgY was washed 4 times with PBS. Twelve hours after lyophilization at -60 ℃, IgY was stored at -20 ℃. The IgY preparations for routine use were kept at 4 ℃ after diluted to 3 mg mL^{-1}.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify the anti-CTN IgY. Proteins in the SDS-PAGE buffer system were denatured by heating in a buffer containing sodium dodecyl sulfate(SDS) and thiol reducing agents such as 2-mercaptoethanol (ME) as previously described[30]. This method is particularly useful for the calculation of molecular weights and separation of substances in the antibody on account of separation of proteins in correspondence to their own molecular weight. In this study, the resolving gel was 15% and the sracking gel was 5%. The electrophoresis time was 1 h at 200 volts with a 10 μL sample.

Indirect Competitive ELISA for Quantitative Determination of CTN and Specificity of IgY

The prepared coating antigen (CTN-OVA) dissolved in a 0.05 mol L^{-1} carbonate buffer (pH 9.6)

was used to coat ELISA plates (1.0 μg of 100 μL per well) at 37 ℃ and deposited overnight at 4 ℃. The plates were washed twice with PBS containing 0.5% tween-20 and blocked for 1 h at 37 ℃ with PBS containing 3% skim milk powder (200 μL per well). Emptied plates were washed three times with PBS containing 0.5% tween-20, dried and stored at 4 ℃. Multiple diluted anti-CTN antibodies (1 mg mL^{-1}) in PBS containing 0.5% skim milk powder (100 μL per well) were added into the ELISA plates, incubated for 1 h at 37 ℃, washed three times with PBS containing 0.5% tween-20, and dried. One hundred μL of horseradish peroxidase labeled rabbit anti-chicken IgY diluted at 1:5000 in PBS with 0.5% skim milk powder was added and incubated for 1 h at 37 ℃. The plates were washed three times with PBS containing 0.5% tween-20 and dried thoroughly. One hundred μL of an OPD substrate solution containing 0.4 mg mL^{-1} OPD (pH 5.0), 0.1 mmol L^{-1} citrate-phosphate buffer, and 1 μL mL^{-1} H_2O_2 was added to each well and incubated for 15 min at 37 ℃. Then 2 mol L^{-1} H_2SO_4 (50 μL per well) was added to ELISA plates for termination. Colour changes were observed, and the plates were read directly on a Bio-rad microplate reader at 492 nm.

The ELISA plates coated with coating antigen were prepared as above. Ten-well polystyrene microtitration plates with six lines were used as follows: well 1:100 μL_PBS with 0.5% skim milk powder was added; wells 2-9: 100 μL, 10 ng·mL^{-1}, 20 ng mL^{-1}, 40 ng mL^{-1}, 80 ng mL^{-1}, 160 ng mL^{-1}, 320 ng mL^{-1}, 640 ng mL^{-1}, 1280 ng mL^{-1} of CTN were added in PBS containing 0.5% skim milk powder; well 10: 200 μL PBS with 0.5% skim milk powder was added.

Then 100 μL 0.125 μg/μL anti-CTN IgY purified with a chloroform polyethylene glycol procedure was added to wells 1-9 of six-lined plates. The six-lined plates were incubated for 1 h at 37 ℃, 100 μL was added to each well of six-lined plates pre-coated and blocked with 10 μg/mL CTN-OVA, The plates were incubated for 1 h at 37 ℃, washed three times with PBST and dried. One hundred μL of horseradish peroxidase labeled rabbit anti-chicken IgY diluted at 1:5000 in PBS with 0.5% skim milk powder was added to each well of the plates. The plates were incubated for 1 h at 37 ℃, washed three times with PBS containing 0.5% tween-20 and dried thoroughly. One hundred μL of an OPD substrate solution containing 0.4 mg mL^{-1} OPD (pH5.0), 0.1 mmol L^{-1} citrate-phosphate buffer and 1 μL mL^{-1} H_2O_2 was added to each well of the plates and incubated for 15 min at 37 ℃ followed by addition of 2 mol L^{-1} H_2SO_4 (50 μL per well) to ELISA plates for termination. Color changes were observed, and data were obtained directly on the Bio-rad microplate reader at 492 nm.

The specificity of IgY was studied with different mycotoxins which are similar in structure to CTN and possiblely co-occur with CTN in agricultural crops. Patulin (PAT), Ochratoxin A (OA) and aflatoxin B_1 (AFB_1) produced by *Penicillium* and *Aspergillus*, share similar structural features with CTN and occur simultaneously with CTN in cereals. Deoxynivalenol (DON) produced by *Fusarium*, also known as Vomitoxin contaminates cereals such as wheat, barley and maize, while *Zearalenone* (ZON) has comparatively low acute toxicities. These five substances were used in investigation of the specificity of IgY.

RESULTS

Preparation and Identification of Immunogen and Plate Coating Antigen for CTN

The samples were identified by ultraviolet spectrometry. Their characteristic absorptions are demonstrated in Fig. 1. The carrier of protein reached its peak at 278 nm and was also found in the absorption curve for conjugated CTN-BSA and CTN-OVA. Meanwhile, CTN reached its peak at 333 nm, CTN-BSA reached its at 329 nm and CTN-OVA reached its peach at 316 nm, respectively after coupled with protein.

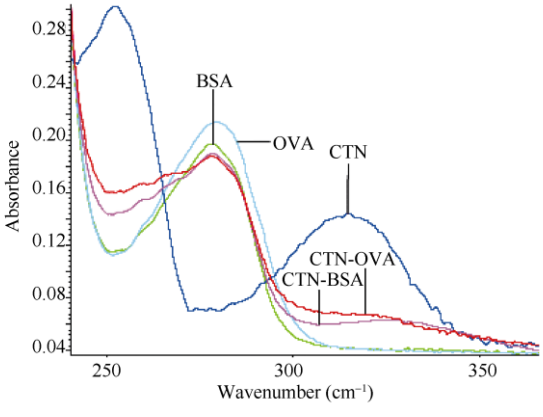

FIG.1. UV absorption spectra of BSA (1), OVA (2), CTN (3), CTN-BSA (4) and CTN-OVA (5).

Infrared spectrogra of BSA and OVA showed the characteristic absorption peak of proteins, such as 3306 cm^{-1} amidocyanogen (N-H) band, the 1653 cm^{-1} amide Ⅰ and 1536 cm^{-1} amide Ⅱ bands, *etc.*[31]. Compared with the IR spectra of BSA, the IR spectra of artificial antigen BSA-CTN, which is regarded as

immunogen, represented a hydroxy characteristic absorbance band at 3400 cm^{-1}. Moreover, the shrunk vibration bands at 1000-1300 cm^{-1} showed a C-N bond, suggesting that CTN is correlated with BSA. The IR spectra of OVA-CTN, which is regarded as a plate coating antigen, demonstrated successfully conjugated CTN and OVA (Fig. 2).

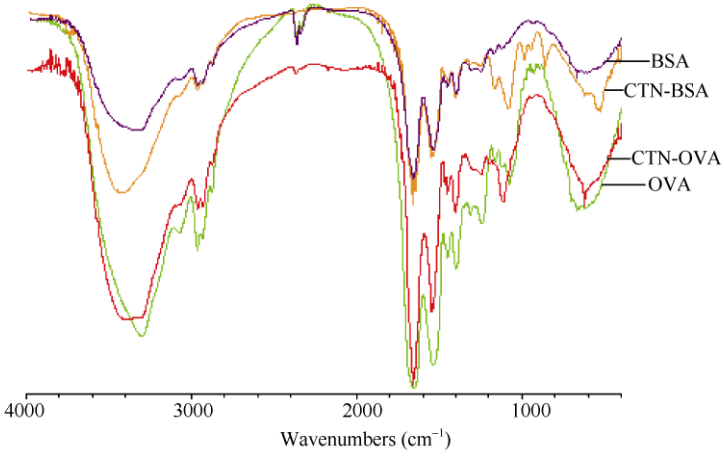

FIG. 2. IR absorption spectra of BSA (1), OVA (2), CTN (3), CTN-BSA (4), and CTN-OVA (5).

UV and IR spectrometry findings suggested that the artificial antigen BSA-CTN and plate coating antigen OVA-CTN were successfully synthesized with the formaldehyde condensation method.

Preparation and Purification of Anti-CTN IgY

The SDS-PAGE of anti-CTN IgY is demonstrated in Fig. 3 (lanes 1-2 reveal the IgY antibody purified with a chloroform polyethylene glycol procedure), displaying two bands in SDS-PAGE. The molecular weight of one band, a heavy chain of IgY, was approximately 100 KD, while the molecular weight of the other band, a light chain of IgY, was approximately 55 KD. The middle band, representing, the protein with a little impurity, had a good purity of the final anti-CTN IgY preparation.

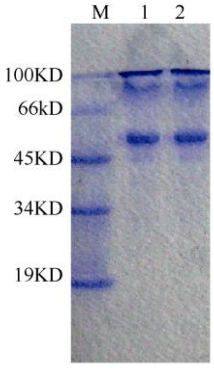

FIG. 3. SDS-PAGE analysis of anti-CTN IgY. Lanes 1 and 2: IgY purified with a chloroform polyethylene glycol procedure; lane M: protein markers.

Indirect Competitive ELISA for Quantitation of CTN in IgY

Antibody titer was used to measure the quantity of recognised antibody corresponding to particular epitope, which is commonly expressed as the maximum dilution ratio (or its reciprocal). ELISA performed to determine the antibody titer. The results of anti-CTN IgY are shown in Table 1.

The optimum dilution for plate coating antigen CTN-OVA and anti-CTN IgY was determined with checkerboard titration method. The optimal dilution of CTN-OVA was finally determined to be 10 $\mu g\ mL^{-1}$. the maximum assay sensitivities of anti-CTN IgY dilution were confirmed to be 1:8 by choosing the titer with its relevant OD_{492} value of approximately 1.0. According to the antibody titer judging method to determine the ratio of positive hole value and negative control ($P/N \geqslant 2.1$), the titer of anti-CTN IgY is 1:256.

The standard curve for CTN detection by competitive ELISA with anti-CTN IgY is shown in Fig. 4. The Y axis represents the inhibition rate and the X axis represents the logarithm of 2 for the base number. The concentration of CTN added to polystyrene microtitration plates (C) was divided by 10 ng/mL (C0) for antilogarithm, revealing that the concentration of CTN at the limit of detection is 10 $ng\ mL^{-1}$, with a good linearity ranging 20-640 $ng\ mL^{-1}$ and the squared correlation coefficient is expressed as R^2=0.9635.

TABLE 1

Titer of Anti-CTN IgY

Titer	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	Negative Control
OD_{492} (n=6)	2.554 ±0.032	1.628 ±0.036	1.050 ±0.022	0.594 ±0.024	0.314 ±0.020	0.183 ±0.009	0.113 ±0.005	0.068 ±0.003	0.045 ±0.001	0.028 ±0.001

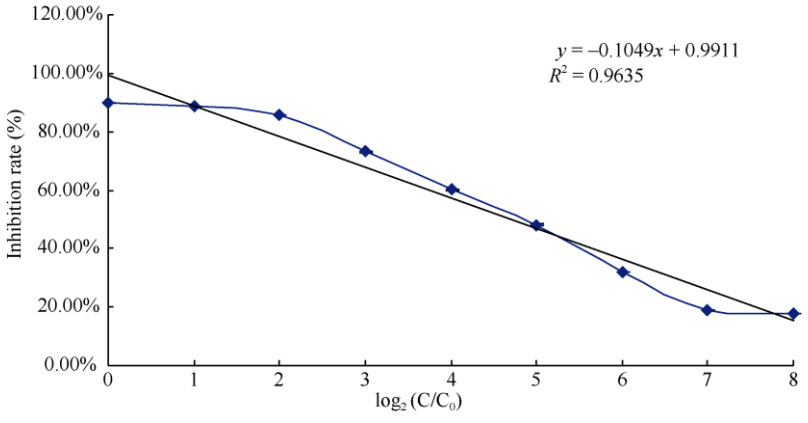

FIG. 4. Standard curve detection of CTN by competitive ELISA with anti-CTN IgY.

Specificity of IgY

For testing the specificity of IgY, ELISA were carried out with CTN substituted OA, AFB_1, PAT, DON, and ZON. The cross-reactivity is shown in Table 2. None of the toxins inhibited anti-CTN IgY of solid phase antigen at a concentration of 10 000 ng mL^{-1}, demonstrating a good specificity of anti-CTN IgY.

TABLE 2

Cross-reactivity of Chicken Anti-CTN IgY with Various Structural Similarities to Citrinin

Structure-related Molecules	Cross-reactivity (%)
Ochratoxin A (OA)	0.08
Deoxynivalenol (DON)	0.02
Aflatoxin B_1 (AFB_1)	0.05
Zearalenone (ZON)	0.28
Patulin (PAT)	1.56

DISCUSSION

In this paper, the artificial immune antigen BSA-CTN and plate coating antigen OVA-CTN were successfully synthesized with the formaldehyde condensation method. The identification with UV and IR spectrometry was carried out for the two products and compared with BSA, OVA, and CTN. Instrumental analysis showed that CTN was coupled with the carrier of protein. The anti-CTN IgY was obtained by immunization of hens. Polyethylene glycol two-step precipitation method was used to purify the anti-CTN IgY and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was set up for identification in this process. All the above preparations aimed to establish ELISA for detecting CTN. The results of ELISA for quantitative determination of CTN showed that the maximum assay sensitivity of anti-CTN IgY dilution was 1:8, and the titer of anti-CTN IgY was 1:256. The determination range was 20-640 ng mL^{-1}, and LOD was 10 ng mL^{-1}. The anti-CTN IgY possesses a good specificity and can be used in determination of the CTN-contaminated samples. Comparatively, Abramson *et al.*[21] in 1995 conjugated CTN with keyhole limpet hemocyanin (KLH) to obtain the immune antigen CTN-KLH. They established an indirect competitive enzyme immunoassay using rabbit antisera that could detect CTN in buffer

solutions at 1-13 ng/mL. The recovery rate of CTN added to wheat flour at 200-2000 ng/g was 89%-104%, with a coefficient of 6.9%-13%. Hence, direct and indirect competitive ELISA was established to detect CTN in barley, with a linear working range of 0.4-0.8 ng/mL. The recovery rate of CTN added to wheat flour at 100-2000 ng/g was 105%-112%, with a coefficient of f 4.5%-12%[32]. Vrabcheva *et al.* [33] in 2000 analyzed CTN by ELISA with detection of 5 ng/g. However, in 2001, Heber[34] established an indirect competitive ELISA to detect CTN in red yeast rice, but the detection was only 15 μg/g. Kononenko reported a rapid method to determinate CTN in feed by indirect competitive ELISA with an analytical concentration range of 2-500 μg/kg for CTN[35]. The above findings suggest that the recovery and LOD are significantly different when different antibody and different detection methods are used.

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