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Analytical Methods

A monoclonal antibody-based enzyme-linked immunosorbent assay for detection of ustiloxin A in rice false smut balls and rice samples

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ABSTRACT

Ustiloxin A, a cyclopeptide mycotoxin, was isolated from the pathogenic fungus *Villosiclava virens* that causes rice false smut, a worldwide devastating rice disease. A monoclonal antibody (mAb) 2D3G5 was generated with ustiloxin A–bovine serum albumin conjugate. A highly sensitive and specific indirect competitive enzyme-linked immunosorbent assay (icELISA) was then developed. It possessed a median inhibition concentration (IC_{50}) of 13.8 ng/mL and a working range of 2.8–72 ng/mL. The mAb 2D3G5 recognized ustiloxin B with the cross-reactivity as 4%. The average recoveries of ustiloxin A from rice false smut balls and peeled rice samples ranged from 92% to 117% and from 92% to 107%, respectively. Comparison of the concentrations of ustiloxin A in rice false smut balls detected by both icELISA and high performance liquid chromatography–photodiode array detection indicated that the developed icELISA was suitable for detection of ustiloxin A in rice food and feed samples.

1. Introduction

Rice false smut is an emerging and economically important disease infected by *Villosiclava virens* (Nakata) Tanaka & Tanaka (anamorph: *Ustilaginoidea virens* Takahashi) (Tanaka, Ashizawa, Sonoda, & Tanaka, 2008) in most rice (*Oryza sativa* L.) producing countries such as China, India, Myanmar, and Japan (Ashizawa, Takahashi, Moriwaki, & Hirayae, 2010; Wang et al., 2008, 2014). *V. virens* infects the rice filament and grows intercellularly. The rice false smut balls are then formed in the infected rice spikelets (Hu, Luo, Wang, Liu, & Li, 2014; Tang et al., 2013). The recent widespread cultivation of hybrid rice and heavy application of nitrogenous fertilizer have been considered as being responsible for the increased rice false smut disease (Guo et al., 2012; Zhang et al., 2014). This disease results in yield loss, polluted rice grains, and even more important, generating toxins poisoning to humans and domestic animals (Koiso et al., 1994; Zhou et al., 2012). Two kinds of mycotoxins, namely ustiloxins and ustilaginoidins, have been isolated and identified from rice false smut pathogen (Lu et al., 2014; Zhou et al., 2012). Ustiloxins belong to the cyclopeptides containing a 13-membered cyclic core structure with an ether linkage. Five ustiloxins have been identified and named as ustiloxins A, B, C, D and F. Among them, ustiloxin A is the most toxic and represents about 80% of the total ustiloxin content (Koiso et al., 1992, 1994, 1998; Shan et al., 2012). When domestic animals were fed with the rice grains and feedstuff contaminated by the rice false smut pathogen, they showed a variety of symptoms such as diarrhea, hemorrhage, poor growth, ovarian atrophy, abortion, liver, heart and kidney damage (Lu et al., 2014; Zhou et al., 2012). Ustiloxin A and the crude water extract of rice false smut balls were reported to cause necrosis of the liver and kidneys in mice quite similar to that observed in lupinosis caused by phomopsin A (Nakamura et al., 1994). Meanwhile, ustiloxins functioned as the phytotoxins by inhibiting the radicle and plumule growth during seed germination of rice, wheat and maize, even inducing an abnormal swelling of the seeding roots (Koiso et al., 1992, 1994). Furthermore, ustiloxins had antimitotic activity by inhibiting microtubule assembly and cell skeleton formation of plant and animal cells (Li, Koiso, Kobayashi, Hashimoto, & Iwasaki, 1995; Luduena et al., 1994; Morisaki et al., 1998). Both false smut balls and false smut pathogen-infected rice food and forage have created the concerns for food and feed safety (Zhou et al., 2012). In order to monitor contents of ustiloxins in rice, rice products, and feedstuff contaminated by rice false smut pathogen,

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a sensitive, rapid and accurate detection for ustiloxins is urgently needed.

To analyze ustiloxins, some methods have been developed, which includes high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC–MS) (Ji, Cao, Xu, Yin, & Shi, 2012; Miyazaki, Matsumoto, Uchihara, & Morimoto, 2009; Shan et al., 2012). Conventional instrumental methods are accurate and reliable, but usually require expensive instruments and highly skilled professionals. Enzyme-linked immunosorbent assay (ELISA) has been regarded as a rapid and sensitive method based on the immune reaction between antigen and antibody, which needs a very small amount of samples and easy pretreatments. These features convert ELISAs into very powerful tools for mycotoxin analysis (Watanabe, Miyake, & Yogo, 2013; Yan, Li, Yan, & Su, 2014; Zheng, Richard, & Binder, 2006).

To our knowledge, there was no published report about ELISA for the analysis of ustiloxins. In the present study, we developed a rapid, sensitive, and specific indirect competitive ELISA (icELISA). This new assay was based on the monoclonal antibodies against ustiloxin A and it was evaluated for the analysis of ustiloxin A in the rice samples including rice false smut balls, peeled rice, and unpeeled rice grains.

2. Experimental

2.1. Chemicals and immunochemicals

The reagents and chemicals including cell freezing medium-dimethyl sulfoxide (DMSO) (serum-free), hypoxanthine, aminopterin, and thymidine (HAT), hypoxanthine and thymidine (HT) medium supplements, L-glutamine, penicillin, streptomycin, goat anti-mouse IgG conjugated with horseradish peroxidase (IgG–HRP), bovine serum albumin (BSA), ovalbumin (OVA), complete and incomplete Freund's adjuvant, and *o*-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO, USA). Polyethylene glycol (PEG)-2000 was from Fluka (Buchs, Switzerland). Cell culture media (Dulbecco's modified Eagle's medium, DMEM) and fetal bovine serum (FBS) were obtained from Gibco BRL (PaisLey, Scotland). Methanol and trifluoroacetic acid (TFA) in HPLC grade were purchased from Tianjin Tianhao Chemical Industry Co. Ltd. (Tianjin, China). All other reagents and solvents were of analytical grade.

2.2. Buffers and solutions

The buffers and solutions used in the present work were the same as those previously used (Zhao et al., 2006). They included coating buffer (0.05 M carbonate buffer, pH 9.6), phosphate-buffered saline (PBS) (0.1 M phosphate buffer containing 0.9% NaCl, pH 7.5), PBS with 0.1% (v/v) Tween-20 (PBST), PBST containing 0.5% (w/v) gelatin (PBSTG), citrate–phosphate buffer (0.01 M citric acid and 0.03 M Na_2HPO_4, pH 5.5), substrate solution (4 μL of 30% H_2O_2 at w/w added to 10 mL citrate–phosphate buffer containing 2 mg/mL OPD), and a stop solution (2 M H_2SO_4).

2.3. Preparation of ustiloxins A and B

Ustiloxins A and B (Fig. 1) were isolated and purified as described previously (Shan et al., 2012). Briefly, 800 g of dry rice false smut balls were ground and extracted with deionized water for three times (2 L for each time) at room temperature. The water solution was filtered and then concentrated under vacuum at 60 °C by a rotary evaporator to afford a water extract. The water extract was subjected to repeated column chromatography on macroporous adsorption resin HP-20, ODS-AQ, Sephadex LH-20, and Sephadex G15 to obtain 90 mg of ustiloxin A and 12 mg of ustiloxin B, respectively. Both ustiloxins A and B were structurally identified according to our previous report (Shan et al., 2012).

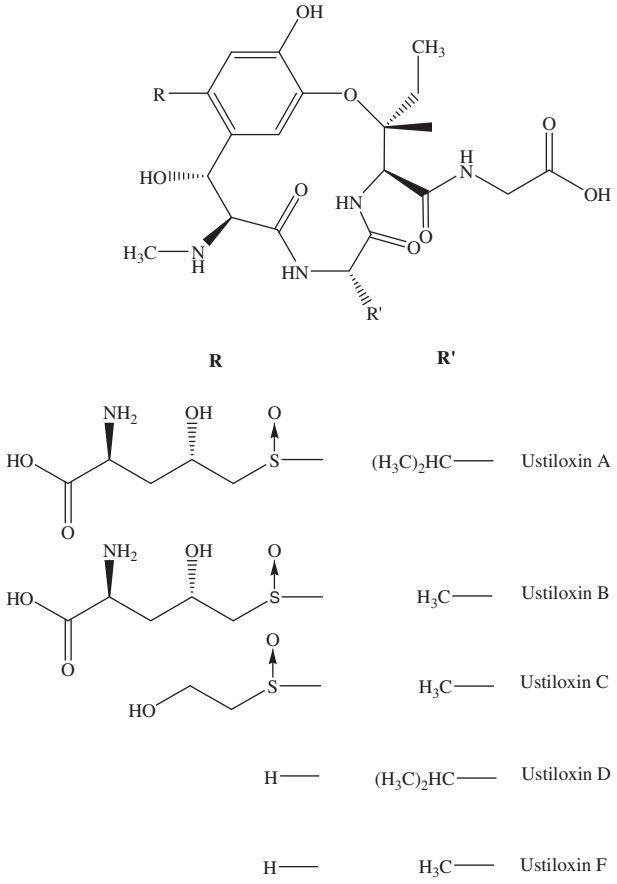

Fig. 1. Chemical structures of the ustiloxins.

2.4. Preparation of immunogen and coating antigen

Ustiloxin A was conjugated with BSA and OVA to prepare the immunogen (UA–BSA) and coating antigen (UA–OVA), respectively, using the glutaraldehyde method (Cliquet, Cox, Van Dorpe, Schacht, & Goddeeris, 2001). Ustiloxin A (2.3 mg) was dissolved in 1 mL of N,N-dimethylformamide (DMF) and split into two equal volumes. 500 μL of ustiloxin A solution was added, while stirring, into 1 mL of PBS containing BSA (11.35 mg) or OVA (7.54 mg), followed by addition of 6.8 μL of 5% glutaraldehyde solution into the mixture. The reaction mixture was stirred overnight at 4 °C. Conjugates were dialyzed against 2 L of PBS for 3 days, with two changes per day, and stored at −20 °C. The conjugation of haptens with proteins (UA–BSA and UA–OVA) was confirmed by UV–VIS spectra, according to the reported method (Zhang et al., 2007).

2.5. Antibody preparation

The monoclonal antibodies (mAb) were produced and purified according to the procedure previously described (Zhao et al., 2006). Briefly, six female Balb/c mice (6–8 weeks of age) were immunized with UA–BSA conjugates (*ca.* 100 μg per mouse, 50 μg was injected intraperitoneally, 50 μg was injected subcutaneously) at 2-week intervals. The spleen cells were collected from the mouse which had the highest titer and best specificity. They were fused with the SP2/0 (purchased from China Institute of

Veterinary Drug Control, Beijing, China) cell line using PEG-2000 at a ratio of 10:1 of spleen to myeloma cells. The hybridoma cells were selectively cultured in complete medium (DMEM supplemented with 20% FBS (v/v), 0.2 M L-glutamine, 50,000 U/L penicillin, 50 mg/L streptomycin) with 1% (v/v) HAT for approximately two weeks at 37 °C in a CO_2 incubator (5% CO_2 in air) (Thermo, Vantaa, Finland). The supernatants were screened by icELISA. Positive hybridoma cells were cloned by limiting dilution and clones were further selected by icELISA. The clone with a high antibody titer and good sensitivity in the culture supernatant was expanded in mice for ascites production. The mAbs were purified from ascite fluids by ammonium sulfate precipitation.

2.6. icELISA

2.6.1. Establishment of conventional icELISA

The coating antigen, purified mAb and goat anti-mouse IgG–peroxidase conjugate were dissolved in a mixed solution of PBS and glycerol (50:50) and stored in −20 °C. Ustiloxin A was dissolved in sterile water and stored in 4 °C. The coating buffer, PBSTG and substrate solution were freshly prepared or stored in 4 °C for less than one week.

Suitable dilutions of the coating antigen UA–OVA (0.03–1.00 μg/mL) and mAb 2D3G5 (0.06–1.00 μg/mL) were screened by checkerboard titration.

The protocol for icELISA was the same as described previously (Zhao et al., 2006). A 96-well microplate (Corning, New York, USA) was coated with the coating antigen (UA–OVA) solution (1 μg/mL, 100 μL per well) at 37 °C for 3 h. The plate was washed with PBST for four times on an automatic plate washer (Wellwash 4 MK2, Thermo, Vantaa, Finland). 50 μL of various concentrations of the standard or analytes in PBSTG was pipetted into each well, followed by addition of 50 μL of sera, supernatant, or purified mAb solution diluted in PBSTG. The plate was incubated at 37 °C for 30 min and then washed again with PBST for four times to remove the unbound antibodies. An aliquot of 100 μL per well goat anti-mouse IgG–peroxidase conjugate diluted in PBSTG (1 μg/mL) was added. After being incubated at 37 °C for 30 min, the plate was washed with PBST again. 100 μL of substrate solution was added into each well. The reaction was terminated by adding 50 μL of 2 M H_2SO_4 per well. Absorbance was read at 492 nm on a Multiskan MK3 microplate reader (Thermo, Vantaa, Finland).

2.6.2. Antibody specificity

The mAb specificity was evaluated by cross-reactivity (CR) with a set of structural analogs via icELISA. CR of ustiloxin B was calculated according to the formula: CR (%) = [IC_{50} (ustiloxin A)/IC_{50} (ustiloxin B)] × 100.

2.6.3. Sample extraction and recovery tests

Rice false smut balls collected from Qionglai (Sichuan, China) were used for ustiloxin A recovery study. The samples were extracted according to the procedure previously described (Shan et al., 2012, 2013) with minor modifications. Briefly, 50 mg powdered sample of rice false smut balls spiked with ustiloxin A at concentrations of 0.0, 0.2, 0.5, 1.0, 2.0, 4.0, 8.0, and 14.0 mg/g was extracted with deionized water for three times (3 × 1.5 mL, 30 min for each time) in an ultrasonic bath at room temperature. The extracts were combined and adjusted to 5.0 mL. The combined extracts were then diluted 2000-fold with PBSTG, followed by icELISA analysis in triplicate. The spiked concentrations of ustiloxin A in the diluted extracts were at 0, 1, 2.5, 5, 10, 20, 40 and 70 ng/mL.

The peeled rice samples purchased from the local market were also used for recovery study. The extraction procedure of peeled rice samples was similar to that described above (Section 2.6.3) with minor modifications. Briefly, 1 g of the samples spiked with ustiloxin A at concentrations of 0, 20, 50, 100, 200, 250, 400 and 700 ng/g was extracted with deionized water for two times (2 × 9 mL, 30 min for each time) in an ultrasonic bath at room temperature. The extracts were combined and then concentrated to dryness by vacuum freeze dryer. The residue was dissolved in 1 mL of PBSTG, diluted 10-fold and analyzed by icELISA in triplicate. The spiked concentrations of ustiloxin A in the diluted solutions were at 0, 2, 5, 10, 20, 25, 40 and 70 ng/mL.

2.7. icELISA and HPLC analysis of ustiloxin A in rice false smut ball samples

The rice false smut balls collected from different areas of China were extracted with deionized water according to the procedure described above (Section 2.6.3) with minor modifications. Briefly, 200 mg powdered sample of rice false smut balls was extracted with deionized water for three times (3 × 6 mL, 30 min for each time) in an ultrasonic bath at room temperature. The water extract was concentrated by a rotary evaporator to dryness under vacuum at 60 °C, which the residue was dissolved in 1 mL of methanol–water solution (15:85, v/v) in a test tube. After 8000-fold dilution with PBSTG, each extract was analyzed by icELISA in triplicate.

For HPLC analysis, the concentrated solution was then filtered through a filter (pore size, 0.22 μm) before analysis. Each solution was analyzed on a Shimadzu LC-20A high-performance liquid chromatograph system (Kyoto, Japan) that consisted of two LC-20AT solvent delivery units, an SIL-20A autosampler, an SPD-M20A photodiode array detector, a CBM-20Alite system controller, and a Synergi reversed-phase Hydro-C_{18} column (250 mm × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA). The injection volume was 20 μL. The mobile phase, composed of methanol–water (15:85, v/v) containing 0.02% TFA (v/v), was set at a flow rate of 1.0 mL/min, in isocratic elution mode at a temperature of 30 °C. The detection wavelength was at 220 nm. A total analysis time was 20 min (Shan et al., 2012).

2.8. icELISA analysis of ustiloxin A in rice samples

Both peeled and unpeeled rice samples were used for icELISA analysis. The peeled rice samples were purchased from the local supermarket in 2013. The unpeeled rice samples were collected from the false smut free field in Beijing as well as the rice false smut infected field in Hunan, China in October 2013. The rice samples were extracted with deionized water according to the same procedure described above with minor modifications. Briefly, 5 g of the samples was extracted with deionized water for two times (2 × 45 mL, 30 min for each time) in an ultrasonic bath at room temperature. The combined extracts were concentrated by a rotary evaporator to dryness under vacuum at 60 °C. The residue was dissolved in 2 mL of distilled water, diluted in PBSTG in appropriate dilutions and analyzed by icELISA in triplicate.

3. Results and discussion

3.1. Preparation of hapten–protein conjugates

The hapten load on carrier proteins were estimated via UV–VIS spectral measurement at 200–400 nm (Cui et al., 2014). The results showed that the haptens were successfully coupled with the carrier proteins. The molar ratios of ustiloxin A to proteins were estimated to be 6:1 and 8:1 for UA–BSA and UA–OVA, respectively.

3.2. Production and characterization of mAbs

Three days after the fourth immunization, blood samples were obtained from mice immunized with the immunogen (UA–BSA) to determine titer and inhibition of the antisera against ustiloxin A. The resulting antisera derived from UA–BSA showed high affinity to ustiloxin A. When the concentration of the coating antigen (UA–OVA) was 0.5 μg/mL and the antisera were diluted with PBSTG at 1:1000, the best inhibition by ustiloxin A standard solution at 2000 ng/mL was 82% for antisera from the six mice immunized with UA–BSA. The mouse that produced the best inhibition antisera was then used to collect its spleen cells for *in vitro* hybridoma cell production. After hybridoma cell lines were cloned using limiting dilution, four clones secreting mAbs against ustiloxin A were obtained. One clone, named as 2D3G5, with the best inhibition by ustiloxin A was expanded for ascites production. The titer (the antiserum dilution that gave an absorbance of 1.0 in the noncompetitive assay conditions) of the ascites was 3.2×10^4. The mAb was confirmed as an IgG1 isotype.

3.3. Development of icELISA

3.3.1. Optimization of icELISA conditions

Suitable dilutions of the coating antigen UA–OVA, mAb 2D3G5 and goat anti-mouse IgG-HRP were screened by checkerboard titration. Optimum concentrations of the coating antigen, purified mAb, and the IgG–HRP conjugate for icELISA were at 1.0, 0.25, and 1.0 μg/mL, respectively. An icELISA under the optimized conditions was then developed.

3.3.2. Assay sensitivity

Under the optimal conditions, icELISA measurements were conducted with a series of concentrations of ustiloxin A dissolved in PBSGT. A representative inhibition curve (Fig. 2) for ustiloxin A generated by icELISA was established. The IC_{50} value of the icELISA was 13.8 ng/mL; the detection limit was 1.1 ng/mL (10% inhibition); and the calibration range, based on 20–80% of inhibition of binding of mAb 2D3G5 to the immobilized hapten–OVA, was from 2.8 to 72 ng/mL.

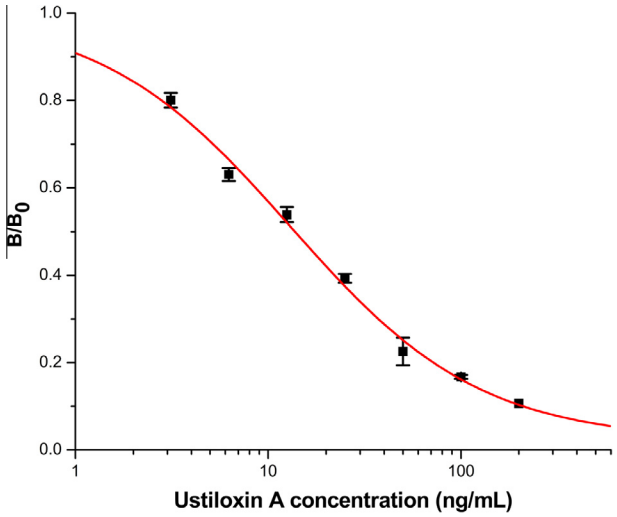

Fig. 2. Inhibition curve of ustiloxin A in icELISA format (each value represents the mean of triplicate ± standard deviation. B_0 and B are the absorbance values at 492 nm in the absence and presence of ustiloxin A, respectively).

3.3.3. Assay specificity

Ustiloxin A is the most toxic and predominant (about 80%) among all characterized ustiloxins, followed by ustiloxin B (Shan et al., 2012). The specificity of mAb 2D3G5 against ustiloxin A was estimated with ustiloxin B. The cross-reactivity of ustiloxin B was 4.1% (Table 1). Ustiloxin A was conjugated with carrier proteins via $-NH_2$ at C-5′ position via the glutaraldehyde method. We assume that the site involved in the reaction with the antibody might be the cyclopeptide far from the conjugation site. The structure of ustiloxin B is the most similar to ustiloxin A among the five known ustiloxins. There is a minor difference with two methyl groups at C-24 position (Fig. 1) between ustiloxins A and B. Ustiloxin C and F with $-CH_3$ at C-24 position, being structurally similar to ustiloxin B, might also have very small cross-reactivity. Ustiloxin D with $-CH(CH_3)_2$ might interfere the analysis. It was, however, very different with ustiloxin A at C-12 position. Furthermore, the content of ustiloxin A determined in rice false smut ball sample, after 8000-, 16,000- and 32,000-fold dilutions, were very close to each other (1.03, 1.06 and 0.96 mg/g, respectively) and agreed well with that by HPLC (1.08 mg/g). Therefore, the other ustiloxins might not interfere the icELISA. The low cross-reactivity of mAb 2D3G5 with ustiloxin B makes the assay specific and practically useful for ustiloxin A analysis.

3.3.4. Recoveries of ustiloxin A from the spiked samples

To test the assay reliability, ustiloxin A fortified in rice false smut balls and peeled rice samples was determined by icELISA. The average recoveries of ustiloxin A from the spiked rice false smut balls and peeled rice samples ranged from 92% to 117% and 92% to 107%, respectively (Tables 2 and 3). The results demonstrated the developed icELISA can be used to quickly and reliably monitor ustiloxin A in rice food and feed samples.

3.4. Comparison of icELISA and HPLC for analysis of ustiloxin A in rice false smut ball samples

The content of ustiloxin A in six rice false smut balls collected from different areas of China was determined by icELISA and HPLC. The HPLC calibration curves of ustiloxin A showed good linearity, $Y = 35548.29919X - 6346.467662$, $R^2 = 0.999942247$, where Y is the peak area of analyte, and X is the concentration (μg/mL) of analyte.

The ustiloxin A content varied in rice false smut ball samples from different areas and ranged from 0.34 to 0.95 mg/g (Table 4). Ustiloxin A content of the rice false balls from Shandong Province was the highest, followed by the samples from Fujian and Hunan Provinces, while ustiloxin A content of the samples from Sichuan and Liaoning Provinces was the lowest.

The HPLC results were quite similar to those of icELISA (Table 4). A good correlation between the results of HPLC (Y) and icELISA (X) was obtained with the linear regression equation of $Y = 1.2081X - 0.0591$ ($R^2 = 0.997$), which suggested that the developed icELISA could be used as an effective and accurate method for ustiloxin A analysis.

Table 1
Cross-reactivity of ustiloxin A and ustiloxin B.

Ustiloxin	IC_{50} (ng/mL)	Cross-reactivity (%)
Ustiloxin A	12.1 ± 0.4	100 ± 3.3
Ustiloxin B	292 ± 8.5	4.1 ± 0.1

Data represent means of triplicate ± standard deviations.

Table 2
Average recoveries of ustiloxin A spiked in rice false smut ball samples.

Spiked content (mg/g)	Detected content (mg/g)	Mean recovery (%)
0.0	0.36[a] ± 0.12	–
0.2	0.59 ± 0.09	117[b] ± 15
0.5	0.82 ± 0.02	92 ± 17
1.0	1.39 ± 0.09	103 ± 7
2.0	2.39 ± 0.06	101 ± 3
4.0	4.44 ± 0.19	102 ± 2
8.0	8.28 ± 0.36	99 ± 3
14.0	13.87 ± 1.04	97 ± 7

[a] Data represent means of triplicate ± standard deviations.
[b] Recoveries were determined after subtraction of the background content of ustiloxin A. The spiked concentration of ustiloxin A in diluted solutions (ng/mL): 0, 1, 2.5, 5, 10, 20, 40, and 70. The detected concentration of ustiloxin A in diluted solutions by icELISA (ng/mL): 1.80, 2.97, 4.10, 6.94, 11.93, 22.22, 41.40 and 69.36.

Table 3
Average recoveries of ustiloxin A spiked in peeled rice samples.

Spiked content (ng/g)	Detected content (ng/g)	Mean recovery (%)
0	–	–
20	21.46[a] ± 0.71	107[b] ± 4
50	52.66 ± 5.49	105 ± 11
100	102.41 ± 10.22	102 ± 10
200	184.06 ± 3.43	92 ± 2
250	233.15 ± 14.14	93 ± 6
400	371.38 ± 46.25	93 ± 12
700	669.67 ± 70.46	96 ± 10

[a] Data represent means of triplicate ± standard deviations.
[b] Recoveries were determined after subtraction of the background content of ustiloxin A. The spiked concentration of ustiloxin A in diluted solutions (ng/mL): 0, 2, 5, 10, 20, 25, 40 and 70. The detected concentration of ustiloxin A in diluted solutions by icELISA (ng/mL): –, 2.15, 5.27, 10.24, 18.41, 23.32, 37.14 and 66.97.

Table 4
Comparison of ELISA and HPLC analysis of ustiloxin A in rice false smut ball samples collected from different areas of China.

Collected area of rice false smut balls	Content of ustiloxin A by ELISA analysis (mg/g)	Content of ustiloxin A by HPLC analysis (mg/g)
Linyi, Shandong	0.95 ± 0.03	1.08 ± 0.07
Hanshou, Hunan	0.64 ± 0.03	0.73 ± 0.11
Qionglai, Sichuan	0.36 ± 0.09	0.37 ± 0.02
Donggang, Liaoning	0.34 ± 0.09	0.34 ± 0.03
Jianou, Fujian	0.71 ± 0.01	0.78 ± 0.02
Jianyang, Fujian	0.68 ± 0.05	0.78 ± 0.01

Data represent means of triplicate ± standard deviations.

3.5. Analysis of ustiloxin A in rice samples with icELISA

Table 5 shows the ustiloxin A content in six rice samples collected from the local supermarkets and different areas of China detected by the developed icELISA. The ustiloxin A content ranged from 0.015 to 2.24 μg/g. The content of ustiloxin A in peeled rice samples purchased from local supermarket were all below 0.06 μg/g, while it varied among unpeeled rice samples collected from different areas. The unpeeled rice samples collected from Hanshou of Hunan, which was a false smut seriously-infected area, had the highest content (2.24 μg/g) of ustiloxin A.

With the increasing of rice false smut disease, there are more rice false smut balls mixed in rice and rice grain. The analytical results for ustiloxin A in rice false smut ball samples showed that the content were quite high (0.34–1.08 mg/g). In many areas, especially in most rural areas, people would consume the rice grains mixed with rice false smut balls or use them to feed livestock, which would cause health concerns. Monitoring content of ustiloxins in rice grains, rice products, and feedstuff contaminated by rice false smut pathogen is very important. The sensitive, rapid, and accurate icELISA reported here is needed and can be useful for ustiloxin A analysis.

Table 5
ELISA analysis of ustiloxin A content in rice samples.

Sample (collected area in China)	Ustiloxin A content (μg/g)
Peeled rice (Shuangyashan, Heilongjiang)	0.047 ± 0.002
Peeled rice (Suihua, Heilongjiang)	0.056 ± 0.003
Peeled rice (Jiansanjiang, Heilongjiang)	0.015 ± 0.001
Unpeeled rice (Beijing)	0.17 ± 0.020
Unpeeled rice (Beijing)	0.064 ± 0.001
Unpeeled rice (Hanshou, Hunan)	2.24 ± 0.07

Data represent means of triplicate ± standard deviations.

4. Conclusions

Ustiloxin A was used to produce monoclonal antibodies to develop a useful ELISA for the analysis of ustiloxin A. Ustiloxin A was conjugated with BSA and OVA via glutaraldehyde method to prepare an immunogen and coating antigen, respectively. The established icELISA based on mAb 2D3G5 possesses an IC_{50} value of 13.8 ng/mL and a working range of 2.8–72 ng/mL. The mAb 2D3G5 recognized ustiloxin B with the cross-reactivity as 4%. Moreover, spiked rice false smut balls and peeled rice samples analyzed by icELISA had average recoveries of 92–117% and 92–107%, respectively. The content of ustiloxin A in rice false smut balls determined with the icELISA agreed well with that by HPLC ($R^2 > 0.99$). The rice samples including rice false smut balls as well as peeled and unpeeled rice samples collected from different areas of China were analyzed by the developed icELISA. The results indicate that the developed icELISA is suitable for monitoring content of ustiloxin A in rice food and feed samples.

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References

Ashizawa, T., Takahashi, M., Moriwaki, J., & Hirayae, K. (2010). Quantification of the rice false smut pathogen *Ustilaginoidea virens* from soil in Japan using real-time PCR. *European Journal of Plant Pathology, 128*, 221–232.
Cliquet, P., Cox, E., Van Dorpe, C., Schacht, E., & Goddeeris, B. M. (2001). Generation of class-selective monoclonal antibodies against the penicillin group. *Journal of Agricultural and Food Chemistry, 49*, 3349–3355.
Cui, Y., Liu, K., Xu, C., Liu, F., Li, Q. X., Liu, S., et al. (2014). Development of a sensitive monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay for analysing chlorantraniliprole residues. *Food Chemistry, 143*, 293–299.
Guo, X., Li, Y., Fan, J., Li, L., Huang, F., & Wang, W. (2012). Progress in the study of false smut disease in rice. *Journal of Agricultural Science and Technology A, 2*, 1211–1217.
Hu, M., Luo, L., Wang, S., Liu, Y., & Li, J. (2014). Infection process of *Ustilaginoidea virens* during artificial inoculation of rice panicles. *European Journal of Plant Pathology, 139*, 67–77.
Ji, F., Cao, H., Xu, J., Yin, X., & Shi, J. (2012). Simultaneous quantitative determination of ustiloxin A and ustiloxin D in rice grains by high performance liquid chromatography–tandem mass spectrometry. *Chinese Journal of Rice Science, 26*, 246–250.
Koiso, Y., Li, Y., Iwasaki, S., Hanaoka, K., Kobayashi, T., Sonoda, R., et al. (1994). Ustiloxins, antimitotic cyclic peptides from false smut balls on rice panicles caused by *Ustilaginoidea virens*. *The Journal of Antibiotics, 47*, 765–773.
Koiso, Y., Morisaki, N., Yamashita, Y., Mitsui, Y., Shirai, R., Hashimoto, Y., et al. (1998). Isolation and structure of an antimitotic cyclic peptide, ustiloxin F: Chemical interrelation with a homologous peptide, ustiloxin B. *The Journal of Antibiotics, 51*, 418–422.

Koiso, Y., Natori, M., Iwasaki, S., Sato, S., Sonoda, R., Fujita, Y., et al. (1992). Ustiloxin: A phytotoxin and a mycotoxin from false smuth balls on rice panicles. *Tetrahedron Letters, 33*, 4157–4160.

Li, Y., Koiso, Y., Kobayashi, H., Hashimoto, Y., & Iwasaki, S. (1995). Ustiloxins, new antimitotic cyclic peptides: Interaction with porcine brain tubulin. *Biochemical Pharmacology, 49*, 1367–1372.

Lu, S., Tian, J., Sun, W., Meng, J., Wang, X., Fu, X., et al. (2014). Bis-naphtho-γ-pyrones from fungi and their bioactivities. *Molecules, 19*, 7169–7188.

Luduena, R. F., Roach, M. C., Prasad, V., Banerjee, M., Koiso, Y., Li, Y., et al. (1994). Interaction of ustiloxin A with bovine brain tubulin. *Biochemical Pharmacology, 47*, 1593–1599.

Miyazaki, S., Matsumoto, Y., Uchihara, T., & Morimoto, K. (2009). High-performance liquid chromatographic determination of ustiloxin A in forage rice silage. *The Journal of Veterinary Medical Science, 71*, 239–241.

Morisaki, N., Mitsui, Y., Yamashita, Y., Koiso, Y., Shirai, R., Hashimoto, Y., et al. (1998). Synthesis and anti-tubulin activity of ustiloxin D derivatives. *The Journal of Antibiotics, 51*, 423–427.

Nakamura, K., Izumiyama, N., Ohtsubo, K., Koiso, Y., Iwasaki, S., Sonoda, R., et al. (1994). "Lupinosis"-like lesions in mice caused by ustiloxin produced by *Ustilaginoidea virens*: A morphological study. *Natural Toxins, 2*, 22–28.

Shan, T., Sun, W., Liu, H., Gao, S., Lu, S., Wang, M., et al. (2012). Determination and analysis of ustiloxins A and B by LC-ESI–MS and HPLC in false smut balls of rice. *International Journal of Molecular Sciences, 13*, 11275–11287.

Shan, T., Sun, W., Wang, X., Fu, X., Sun, W., & Zhou, L. (2013). Purification of ustiloxins A and B from rice false smut balls by macroporous resins. *Molecules, 18*, 8181–8199.

Tanaka, E., Ashizawa, T., Sonoda, R., & Tanaka, C. (2008). *Villosiclava virens* gen. nov., comb. nov., the teleomorph of *Ustilaginoidea virens*, the causal agent of rice false smut. *Mycotaxon, 106*, 491–501.

Tang, Y.-X., Jin, J., Hu, D.-W., Yong, M.-L., Xu, Y., & He, L.-P. (2013). Elucidation of the infection process of *Ustilaginoidea virens* (teleomorph: *Villosiclava virens*) in rice spikelets. *Plant Pathology, 62*, 1–8.

Wang, S., Li, M., Dong, H., Liu, X. Z., Bai, Y. J., & Yang, H. (2008). Sporulation, inoculation methods and pathogenicity of *Ustilaginoidea albicans*, the cause of white rice false smut in China. *Journal of Phytopathology, 156*, 755–757.

Wang, F., Zhang, S., Liu, M.-G., Lin, X.-S., Liu, H.-J., Peng, Y.-L., et al. (2014). Genetic diversity analysis reveals that geographical environment plays a more important role than rice rice cultivar in *Villosiclava virens* population selection. *Applied and Environment Microbiology, 80*, 2811–2820.

Watanabe, E., Miyake, S., & Yogo, Y. (2013). Review of enzyme-linked immunosorbent assays (ELISAs) for analyses of neonicotinoid insecticides in gro-environments. *Journal of Agricultural and Food Chemistry, 61*, 12459–12472.

Yan, X., Li, H., Yan, Y., & Su, X. (2014). Developments of pesticides analysis by multi-analyte immunoassay: A review. *Analytical Methods, 6*, 3543–3554.

Zhang, Q., Wang, L., Ahn, K. C., Sun, Q., Hu, B., Wang, J., et al. (2007). Hapten heterology for a specific and sensitive indirect enzyme-linked immunosorbent assay for organophosphorus insecticide fenthion. *Analytica Chimica Acta, 596*, 303–311.

Zhang, Y., Zhang, K., Fang, A., Han, Y., Yang, J., Xue, M., et al. (2014). Specific adaptation of *Ustilaginoidea virens* in occupying host florets revealed by comparative and functional genomics. *Nature Communications, 5*, 3849.

Zhao, J., Li, G., Wang, B., Liu, W., Nan, T., Zhai, Z., et al. (2006). Development of a monoclonal antibody-based enzyme-linked immunosorbent assay for the analysis of glycyrrhizic acid. *Analytical and Bioanalytical Chemistry, 386*, 1735–1740.

Zheng, M. Z., Richard, J. L., & Binder, J. (2006). A review of rapid methods for the analysis of mycotoxins. *Mycopathologia, 161*, 261–273.

Zhou, L., Lu, S., Shan, T., Wang, P., Sun, W., Chen, Z., et al. (2012). Chemistry and biology of mycotoxins from rice false smut pathogen. In B. J. Melborn & J. C. Greene (Eds.), *Mycotoxins: Properties, applications and hazards* (pp. 109–129). New York: Nova Science Publishers.