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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).

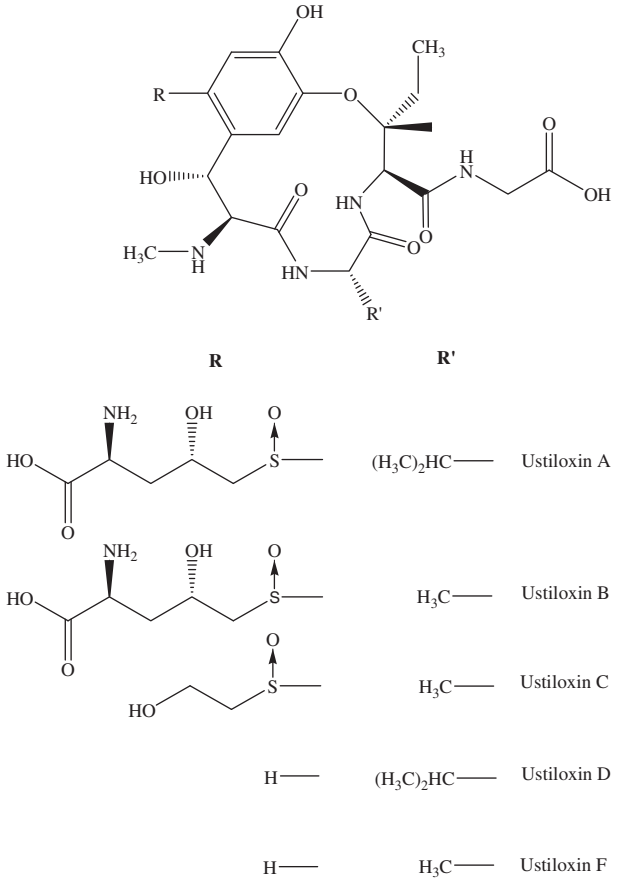

Fig. 1. Chemical structures of the ustiloxins.

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