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Gold nanoparticle-enhanced multiplexed imaging surface plasmon resonance (iSPR) detection of *Fusarium* mycotoxins in wheat

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ABSTRACT

A rapid, sensitive and multiplexed imaging surface plasmon resonance (iSPR) biosensor assay was developed and validated for three *Fusarium* toxins, deoxynivalenol (DON), zearalenone (ZEA) and T-2 toxin. The iSPR assay was based on a competitive inhibition format with secondary antibodies (Ab_2) conjugated to gold nanoparticles (AuNPs) used as a signal amplification tag. Signal was amplified nearly 25-fold for DON, 90-fold for ZEA and 12-fold for T-2 toxin assay using Ab_2-AuNPs. Analyses, including steps to regenerate the sensor, took 17.5 min. The antigen coated sensor chip was used for more than 46 cycles without affecting signal intensity (< 12%). Matrix matched calibration curves were used to determine *Fusarium* toxins in wheat. The mean recoveries ranged from 87% to 103% with relative standard deviations of repeatability of less than 5%. The limits of detection were 15 μg/kg for DON, 24 μg/kg for ZEA and 12 μg/kg for T-2 toxin. This provided sufficient sensitivity to monitor contamination of these mycotoxins in wheat in accordance with European Commission (EC) limits. Cut off levels for all three *Fusarium* toxins were validated using blank wheat and wheat spiked either at the EC regulated levels (100 μg/kg for ZEA and T-2 toxin) or at one third of the EC level (for DON: 400 μg/kg). The assay was successfully applied and further validated with naturally contaminated wheat samples. This is the first reported AuNP enhanced iSPR assay to detect and classify three agriculturally important *Fusarium* toxins in wheat.

1. Introduction

Mycotoxins are naturally occurring secondary metabolites produced by three major types of fungal genera, *Aspergillus*, *Penicillium* and *Fusarium*. Among these, *Fusarium* is the most widespread pathogen in cereals and is a heterogenous mycotoxin producer (Nathanail et al., 2015). Their widespread geographical distribution depends on several factors, including environmental conditions, crop production, storage, and processing and transportation methods (McNamee et al., 2017). The major toxins produced by *Fusarium* are the trichothecenes (deoxynivalenol, T-2 toxin and HT-2 toxin), fumonisins, and zearalenone (ZEA). After the aflatoxins, *Fusarium* toxins are the most reported mycotoxins in raw agricultural commodities (CAST, 2003). These toxins can enter into the food/feed chain and exert a wide range of adverse effects in humans and animals.

Among the trichothecenes, the most prevalent mycotoxin is deoxynivalenol (DON), produced by *F. graminearum* and *F. culmorum*. DON is frequently observed in U.S., Canadian and European wheat after invasion of the fungi, particularly during cool, wet growing and harvest seasons (CAST, 2003). In 1990s, DON was a severe problem in grain in the United States (U.S.). DON causes vomiting in animals and humans (D'Mello et al., 1999; Bhat et al., 1989). It is also affects the intestinal, hematopoietic, immune, endocrine, and nervous systems (Lin and Guo, 2016). The maximum level of DON permitted by the European Commission (EC, 2006) in unprocessed cereals, other than durum wheat, oats and maize is 1250 μg/kg.

Natural contamination with T-2 toxin (T-2) is less common in the U. S., but is observed more frequently in European grains, in particular oat and oat-based products (CAST, 2003; EFSA, 2011). T-2 is highly toxic, on the order of ten-fold more toxic than DON in mammals (Ueno, 1983). T-2 can penetrate skin and can cause non-specific symptoms such as weight loss, feed refusal, dermatitis, vomiting, diarrhea, hemorrhages and necrosis of the epithelium of stomach and intestinal, bone marrow, spleen, testis and ovary (EC, 2001). The EC has recommended a guidance level of 100 μg/kg in wheat for T-2 and HT-2 toxin (EC, 2013).

ZEA is an estrogenic mycotoxin, mainly produced by *F. graminearum*. In the U. S. and Canada, co-contamination of grain with ZEA

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and DON is frequently observed. The production of ZEA is favored by high humidity and low temperature conditions that are common in the Mid-western U.S. during autumn harvest (CAST, 2003). The commodities most commonly contaminated with ZEA are corn (maize), moldy hay, and pelleted commericial feed. ZEA and related congeners have estrogenic effects. These include edema of the vulva, prolapse of the vagina, enlargement of the uterus, atrophy of testicles, atrophy of ovaries, enlargement of mammary glands, and abortion (CAST, 2003). The maximum allowable levels for ZEA are 100 μg/kg in unprocessed cereals and 200 μg/kg in maize (EC, 2006).

Due to the public health concern and large economic losses caused by these toxins, a rapid, sensitive and economical analytical method is needed. Analytical methods for mycotoxins are generally divided into those that are confirmatory (quantitative analysis) or those that are used for rapid screening. Confirmatory methods such as high performance liquid chromatography, gas chromatography and mass spectrometry, are usually expensive, consume large volumes of solvent, and require skilled personnel. On the other hand, immunoassays such as enzyme linked immunosorbent assay (ELISA) and lateral flow devices ("dipsticks") are most commonly used to provide rapid, easy and sensitive detection. Most such assays offer only single toxin analysis. Therefore, rapid and sensitive multi-toxin screening assay is an attractive alternative.

Imaging surface plasmon resonance (iSPR) is an emerging semi-quantitative technique for the reliable, label free and sensitive detection of mycotoxins (Joshi et al., 2016). iSPR measures the changes in the refractive index due to biomolecular interactions at the sensor surface, the interface between a metal or dielectric media. Typically, SPR biosensors are suitable for medium and large compounds as the signal is proportional to the mass of the target molecules (Karczmarczyk et al., 2016). As mycotoxins are low molecular weight compounds, their binding with the antibodies is generally not adequate to produce sufficient SPR signal for practical use (Hu et al., 2014). A good alternative format uses the competition between free toxin and immobilized toxin (antigen) for limited amounts of primary (anti-toxin) antibodies added to the sample. The SPR signal can be further amplified by biofunctionalized nanomaterials such as gold nanoparticles (AuNPs), magnetic nanoparticles (MNPs), fluorophores or quantam dots (QDs) (Sendroiu et al., 2009; Yuan et al., 2011, Shabani and Tabrizian et al., 2013). AuNPs offer several advantages, including higher sensitivity, stability and selectivity, relatively easy and inexpensive synthesis, stability, unique optoelectronic properties, and high surface to volume ratio with excellent biocompatibility (Daniel and Astruc, 2004).

Although AuNPs have been successfully applied as amplification tags in the SPR-based detection of DNA, proteins and drug molecules, only a few mycotoxin studies have incorporated AuNPs. These include assays for ochratoxin A (OTA) (Karczmarczyk et al., 2016), and a combination of aflatoxins, OTA and ZEA (Hu et al., 2014). Karczmarczyk et al. (2016) reported a gold enhanced SPR assay for OTA in red wine using an indirect inhibition immunoassay format. Because multiplexing allows multiple toxins to be detected in a single run and because it reduces analysis time and the cost for reagents, multiplexing is becoming increasingly popular. Hut et al. (2014) described a multiplexed iSPR method for three mycotoxins in peanut samples. In both of these reports, however, the analysis times were long (~ 40 min or more), which is not suitable for practical purposes. Herein a rapid, reproducible, and sensitive iSPR assay is reported for three major *Fusarium* toxins in wheat. The method combines the multiplexing capability of iSPR with the strategy of using colloidal gold for amplification. Furthermore, the method was rapid and took only 17.5 min including the regeneration steps. To the best of our knowledge, no multiplex iSPR assay has been reported for the detection of DON, ZEA and T-2 in wheat that uses AuNPs for signal amplification.

2. Materials and methods

2.1. Chemicals and reagents

Gold (III) chloride trihydrate (HAuCl4. $3H_2O$) and mycotoxins (DON, ZEA and T-2) were purchased from Sigma Aldrich (MO, USA). PEG_3-OH was purchased from SensoPath Technologies (MT, USA) and PEG_6-COOH was purchased from Dojindo Molecular Technologies (MD, USA). 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Sulfo-N-hydroxysucccinimide (Sulfo-NHS) were purchased from Thermo Scientific (USA). Goat anti-mouse IgG (secondary antibody: Ab_2) was purchased from Fisher Scientific (USA). The run buffer consisted of 0.1% w/v bovine serum albumin (BSA) in 10 mM sodium phosphate and 150 mM sodium chloride in water at pH 7.2. The water used in all experiments was purified by a nanopure water purification system (Fisher Scientific). Naturally contaminated wheat samples were purchased from Trilogy Analytical Laboratory (Missouri, USA). HPLC grade methanol was purchased from EMD Milipore (MA, USA). All other chemicals and reagents were purchased from Fisher Scientific.

2.2. Monoclonal antibodies

Mouse monoclonal antibodies (primary antibody: Ab_1) cross-reactive with DON, ZEA and T-2 toxin were produced at Envigo (Indianapolis, USA) using cell lines developed previously at the USDA-National Center for Agricultural Utilization Research (NCAUR, Peoria, IL). The monoclonal antibodies (Mab) used were Mab#1 for DON, Mab#22 for ZEA, and Mab#2–13 for T-2 toxin. The cross reactivities of the DON, ZEA and T-2 Mabs in several immunoassay formats were reported previously (Maragos et al., 2012, 2013; Maragos and Kim, 2006). For this manuscript the cross-reactivities of the antibodies were determined in the iSPR format. Calibration curves for the target mycotoxins and mycotoxin analogs were determined in blank wheat matrix, and the concentrations causing 50% inhibition (IC_{50}s) were calculated. The cross-reactivities were calculated as: (IC_{50} of the target mycotoxins/IC_{50} of the analog) × 100%. The analogs tested included 15-acetyl-deoxynivalenol (15-ADON), 3-acetyl-deoxynivalenol (3-ADON), nivalenol (NIV), α-zearalenol, β-zearalenol and HT-2 toxin (HT-2).

2.3. Preparation of mycotoxin-protein conjugates (antigens)

DON-β-lactoglobulin (DON-BLG) was synthesized using the carbodiimide technique described by Xiao et al. (1995) and Maragos and McCormick (2000). The protein concentration of the conjugate was determined with a commercial BCA assay (Pierce, Rockford, IL, USA). The conjugate was diluted to 1 mg/mL with 100 mM phosphate buffer saline (PBS), then distributed as 0.2 mL portions and lyophilized. To make the ZEA-BSA conjugate, the 6′-carboxymethyloxime of ZEA (ZEA-CMO) was first prepared according to the method of Thouvenot and Morfin (1983). The ZEA-CMO was then reacted with BSA as described by Maragos and Kim (2006). The T2-BSA protein conjugate was prepared according to the method of by Xiao et al. (1995) using 1, 1′-carbonyldiimidazole to link the 3-hydroxyl group of T-2 toxin to BSA. The lyophilized conjugates were reconstituted with water to achieve solutions of 1 mg/mL, which were then diluted as necessary with water during the immobilization procedure.

2.4. iSPR biosensor

An iSPR biosensor (iSPRimager®II) system was purchased from GWC technologies (Wisconsin, USA). The system used chips (slides) with 17 gold spots affixed in a grid pattern, with each spot surrounded by a hydrophobic polymer layer (SpotReadyTM chips, GWC Technologies). The chips were affixed to the optics of the instrument with a refractive index-matching fluid. The optical system (CCD

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