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Phage-borne peptidomimetics as immunochemical reagent in dot-immunoassay for mycotoxin zearalenone

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ABSTRACT

The advantageous characteristics of phage probes and facility of immunoassays were combined to develop a rapid dot-immunoassay for the mycotoxin zearalenone (ZEN). A peptide library of random 12-mers displayed on phage was panned against anti-ZEN antibody. Selected phage-borne peptidomimetics were used as substitute for coating antigen and applied in dot-immunoassay for rapid detecting of ZEN. The binding specificities and reaction kinetics between selected phages and antibody were analyzed by phage ELISA and surface plasmon resonance, respectively. The equilibrium dissociation constant (KD) measured for selected phage (Z5): antibody was 39.8 nM. The cut-off level for this phage-based dot-immunoassay method of detecting ZEN in cereal samples, assessed visually, was 50 μg kg^{-1} and the final results can be obtained within 10 min. The validation of the method was performed by analyzing the spiked samples with ZEN at five levels (15, 30, 45, 60, and 75 μg kg^{-1}) and naturally contaminated cereal samples, the results were in good agreement with the obtained by the commercial ZEN ELISA kit. These results suggest that phage can act as a useful immunochemical reagent in dot-immunoassay for toxic small molecules.

1. Introduction

Mycotoxins are secondary metabolites produced by fungi and contaminate various agricultural commodities. Several recent studies have shown that mycotoxins have a range of detrimental effects on human's health such as immune suppression, micronuclei, chromosome aberrations, DNA fragmentation, etc. (Abbes et al., 2007; Daly et al., 2000; Wagacha & Muthomi, 2008). Because of the toxic effects the presence of mycotoxins should be monitored in human foods and feedstuff. Currently, many methods such as high-performance liquid chromatograph (HPLC), liquid chromatography-mass spectrometry and gas chromatograph (GC) have been developed for the identification of mycotoxins (Basova et al., 2010; Krska & Molinelli, 2007; Sulyok, Krska, & Schuhmacher, 2010). On the other hand, immunoassays such as enzyme-linked immunosorbent assay (ELISA), fluorescence polarization immunoassay, automatic flow-though immunosensor assay, fluoroimmunoassay, dipstick immunoassay and lateral-flow immunoassay, which are simple, robust, and low-cost techniques, have been developed and found widespread application as rapid screening techniques for mycotoxins (Gachok et al., 2008; He et al., 2012; Knopp, 2006; Quinton et al., 2010; Shim, Kim, & Chung, 2009; Thongrussamee et al., 2008; Urraca, Benito-Pen, Perez-Conde, Moreno-Bondi, & Pestka, 2005; Yuan, Deng, Lauren, Aguilar, & Wu, 2009).

Unfortunately, since the development of immunoassay for mycotoxins involves using the mycotoxin itself, both in free and conjugated forms, it might be toxic and expensive to manufactures and users. Also, the efficiency of chemical conjugation of mycotoxin to a carrier protein or an enzyme is low because such conjugation involves extensive modification and blocking stages and causes substantial bridge group interference and unwanted cross-reaction (Xiao, Clarker, Marquardt, & Frohlich, 1995).

For the above mentioned reasons, it is vital to develop substitute as immunochemical reagent which replaces the toxic compound mycotoxins and its conjugates forms in an immunoassay. One approach for doing this is via generation of anti-idiotype antibodies, whose structures mimic the surface structures of small molecules. However, this technology is time-consuming and costly (Yuan et al., 1999). Recently, filamentous phage-displayed peptide has been shown to be an alternative to molecular recognition

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element for various biological targets (Mount et al., 2004; Samoylova et al., 2003). A phage displayed peptide mimotope or epitope mimic is a peptide that will mimic the antibody binding site on the antigen and compete with the native antigen for binding (Casey, Coley, Parisi, & Foley, 2009). By well-know means, phages, which bind to the desired target, can be selected, isolated, and rapidly reproduced in great numbers (Gonzalez-Techera et al., 2007). Due to these advantages, labeled or immobilized phage probes have been used in ELISA, dipstick assays, quartz crystal microbalances, and the surface plasmon resonance (SPR) to detect bacterial, protein, and mammalian targets. However, most of these technologies are complex, restricted to macromolecules, require expensive instrumentation or are used as substitute for competing antigen in the aqueous phase (Kim, McCoy, Gee, Gonzalez-Sapienza, & Hammock, 2011; Lai, Fung, Xu, Liu, & Xiong, 2009; Liu, Liu, Ding, Zhu, & Yu, 2009).

In this study, phage-borne peptidomimetics were used as substitute for coating antigen and applied in dot-immunoassay for rapid detecting of mycotoxin. The major advantage of the phage-based assay is that it avoids laborious synthesis of a large panel of toxic coating antigen, which is required for the development of a standard indirect competitive immunoassay. For this we used zearalenone (ZEN), a potent estrogenic metabolite mycotoxin produced by the genus *Fusarium*, as a representative mycotoxins. To the best of our knowledge, the phage probes-based dot-immunoassay assay for mycotoxin zearalenone has not yet been reported.

2. Material and methods

2.1. Chemicals and reagents

The Ph.D.™ Phage Display Peptide Library Kit was purchased from New England Biolabs, Inc. (Beverly, Mass). ZEN, bovine serum albumin (BSA), horseradish peroxidase-conjugated goat anti-mouse IgG (secondary antibodies), and the 3′,3′,5,5′-tetramethylbenzidine (TMB), N-hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). ZEN-BSA conjugates, anti-ZEN monoclonal antibodies (IgG_1, cross-reactivity with ZEN, α-zearalanonl, β-zearalanol was 100%, 5%, and 3%, respectively), and P4 (7-mer random peptides displayed on M13 phage with specific binding to antibody, amino acid sequence: HHCHWWH) were prepared as described previously (He et al., 2011). Polyvinylidene fluoride (PVDF) membranes (0.45 μm, 26.5 × 3.75 cm) were purchased from Millipore Co. (Bedford, MA). Mycotoxin ZEN ELISA kits were purchased from the R-Biopharm group (Darmstadt, Germany). All inorganic chemicals and organic solvents were of analytical reagent grade.

2.2. Phage selection and identification

A peptide library of random 12-mers displayed on phage was panned against anti-ZEN antibody. Biopanning experiments were carried out as described in the NEB phage display manual. Briefly, ELISA wells were coated with 100 μg ml^{-1} purified anti-ZEN antibody and phages were selected from the library by performing three rounds of panning. The procedure of selection and separation of phages by panning-elution was described in the previous study (Liu, Yu, He, & Xu, 2007). In all cases, the number of input phages remained the same (2.0×10^{11} pfu). The concentration antibody in the coating was reduced from 75 to 50 μg ml^{-1}, and the concentration of Tween-20 was raised from 0.25% to 0.5% (v/v) in the second and third round of selection. After three rounds of panning-elution selection, individual plaques from third round panned phages were picked from LB/IPTG/Xgal plates and used to infect *Escherichia coli* ER2738 cells for phages amplication and identification as described in the NEB manual. After phage selection by panning-elution, phage ELISA was set up as described in the previous study to screen phages capable of specific binding to antibody. In the specificity test, the cross-reactivity of phage ELISA with ZEN analogs (zearalanone, α-zearalanonl, β-zearalanol, α-zearalenol, and β-zearalenonl) and other mycotoxins (aflatoxin B_1, fumonisin B_1, deoxynivalenol, and ochratoxin A) was evaluated as described previously by Wang et al. (2013).

2.3. Surface plasmon resonance analysis

A BIAcore 3000 instrument (GE Lifesciences) was used to determine the surface-binding affinities of the selected phages : antibody. A sensor chip (research grade C1, BIAcore) was activated by injecting activation solution (200 mM EDC and 50 mM NHS at a volume ratio of 1:1) at a flow rate of 5 μl min^{-1} for 12 min. The selected phage (2.5×10^{10} pfu ml^{-1} diluted with sodium acetate buffer, pH 4.0) was injected into the sensing channel at a flow rate of 5 μl min^{-1} for 12 min. The remaining activated sites on the chip were blocked by injecting 1 M ethanolamine at a flow rate of 5 μl min^{-1} for 12 min. Antibody was injected in PBS (pH 7.4), at concentrations ranging from 25 nM to 400 nM, at a flow rate of 30 μl min^{-1} for 3 min. The surface was then regenerated by injection of 50 mM NaOH at a flow rate of 30 μl min^{-1} for 1 min. The K_D (equilibrium dissociation constant) between selected phages and antibody were calculated using BIA evaluation software Version 4.1 (BIAcore). To compare the phage performance as a recognition reagent in the biosensor, ZEN-BSA conjugates were also immobilized on sensor chip (research grade CM5, BIAcore) and analyzed by SPR as described previously by Kadota et al. (2010).

2.4. Preparation of phage probes-based test device

An adhesive rubber fence (90 × 45 × 2.0 mm), with 6 rectangular holes (10 × 30 mm) was fixed to a PVDF membrane; the membrane was divided into 6 independent rectangular membrane reaction zones (He et al., 2012). After being soaked into methanol for 10 s with gentle shaking, membrane was then semidried by gently shaking in the air. A piece of wetted filter paper (400 × 400 mm) was placed under the membrane. A 3-μl volume of selected phage, diluted to an appropriate concentration with PBS (pH 7.4), was applied to the membrane reaction zones. The membrane was then incubated at 37 °C for 2 h at 70% relative humidity. In order to block free protein binding sites, membrane was immersed in PBS (pH 7.4) containing 3% non-fat dry milk for 1 h with gentle shaking at room temperature. Finally, the membrane was dried at 37 °C and a piece of scotch tape was applied to the back (Fig. 1).

2.5. Extraction of samples

Corn samples were milled in a grinder and 4-g portion of each sample were soaked in 10 ml of methanol-PBS (60:40, v/v) and extracted for 5 min by gently shaking. The suspension was filtered and the filtrate diluted 4-fold in PBS (pH 7.4) and used directly in assay.

2.6. Phage-based dot-immunoassay

ZEN standards in solution with methanol-PBS (15:85, 100 μl) were mixed with the anti-ZEN antibody (100 μl, diluted in PBS, pH = 7.4) in a plastic tube. The negative control solutions without ZEN and sample extraction solution were prepared accordingly. The positive control, negative control, and sample solutions were