Detection of deoxynivalenol based on a single-chain fragment variable of the antideoxynivalenol antibody

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Abstract

Immunological detection of low molecular weight toxins, such as deoxynivalenol using single-chain antibody fragment (scFv), is a potentially novel and safe method of diagnosing fungal infection and food contamination. To develop a deoxynivalenol detection procedure based on scFv, deoxynivalenol was first conjugated to horseradish peroxidase (HRP) for immunoassay. Deoxynivalenol was initially activated using N-[[p-maleimidophenyl] isocyanate and subsequently conjugated to S-acetyl thiglycolic acid N-hydroxysuccinimide-activated HRP. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and enzyme-linked immunosorbent assay (ELISA) analysis confirmed covalent attachment of deoxynivalenol to HRP successfully. Competitive direct-ELISA based on antideoxynivalenol scFv was used to detect deoxynivalenol in spiked and natural contaminated wheat samples, and the results showed that scFv is applicable in deoxynivalenol detection of contaminated wheat samples. This work confirms that sensitive and specific scFv against fungal toxins can be applicable in diagnosis of fungal infection.

Introduction

Deoxynivalenol (DON, vomitoxin), a trichothecene mycotoxin, is mainly produced by the fungus Gibberella zeae (Schwein) Petch (anamorph, Fusarium graminearum Schwabe), which is the causing agent of wheat scab, corn ear rot diseases (Yuan et al., 1999; Fox et al., 2004). Biochemically, deoxynivalenol is an inhibitor of protein synthesis and has hemolytic activity, causing apoptosis (cytotoxicity) and immunotoxicity to eukaryotic organisms. In animals, deoxynivalenol can cause anorexia and vomiting (Rottet et al., 1996; Naef et al., 2006), and it can also cause human skin irritation, hemorrhaging, hematological changes, lymphocyte blastogenesis impairment, radioimmunometric effects and apoptosis (Rottet et al., 1996; Goyarts & Danicke, 2006). In plants, deoxynivalenol is a virulence factor in the pathogenesis of G. zeae on wheat (Naef et al., 2006). A major way to eliminate deoxynivalenol from human and animal foods is to detect contaminated raw materials and divert them from feed and contaminated foods.

Because of the known toxicity of deoxynivalenol combined with its prevalence, several methods for deoxynivalenol detection, including PCR, GC, GC-MS, HPLC and thin-layer chromatography (Scott, 1982; Fazekas et al., 2000; Li et al., 2005) are available. But these methods are time-consuming and require technical expertise. Enzyme-linked immunosorbent assays (ELISA) employing polyclonal (Ram et al., 1986; Danicke et al., 2004; Yoshizawa et al., 2004) or monoclonal (Dixon et al., 1987) antibodies have been developed to detect mycotoxins. It is known that ELISA is a rapid, sensitive and relatively simple method, requiring little sample preparation. In addition, enzyme immunoassays lack the health hazards associated with radioimmunoassays.

The monoclonal antibody, most commonly used in immunology reactions, can be generated by a modified hybridoma technology, but the entire procedure is extremely complicated, highly expensive and depends on expertise. Single-chain antibody fragment (scFv), a small engineered antibody, in which the variable heavy chain (VH) and light chain (VL) of the antibody molecule are connected by a short, flexible polypeptide linker (Wang et al., 2005, 2006) is developed to replace the monoclonal antibody to some extent. The obvious advantages of scFv are that it retains the original antigen-binding site, allowing it to maintain its specific affinity for the antigen, and it can be produced in
large scale in Escherichia coli at low cost (Dai et al., 2003; Wang et al., 2006).

Since the pioneer work of Smith (1985) 20 years ago, the phage antibody technique has become one of the most remarkable achievements in antibody technology. With the technique, several scFv antibodies (Wang et al., 2005, 2006) were obtained. In this paper, a procedure of deoxynivalenol detection based on antideoxynivalenol scFv by competitive direct (CD)-ELISA is described. Results of this study suggested that the scFv-based CD-ELISA could be a possible replacement of the monoclonal antibody in the detection of deoxynivalenol in the real cereal samples.

Materials and methods

Reagents

Crude ovalbumin, bovine serum albumin (BSA), horse-radish peroxidase (HRP) and deoxynivalenol were purchased from Sigma Chemical Co. (St Louis, MO). S-acetyl thioglycolic acid N-hydroxysuccinimide (SATA) and N-[p-maleimidophenyl] isocyanate (PMPI) were purchased from Pierce Biotechnology Inc. (Rockford, IL). Ampicillin, isoproyl- b-D-thiogalactopyranoside (IPTG), HRP-labeled goat antimouse IgG was purchased from Sino-American Biotechnology Company, China. All other reagents used were of analytical-reagent grade. All the plasmids and host strains were stored in the authors’ laboratory.

Expression and isolation of antideoxynivalenol scFv–E-tag

Single clone deoxynivalenol1.3G2/HB2151 containing antideoxynivalenol scFv (GenBank accession numbers: AAN75452 and AAN75453) and E-tag fusion gene was grown overnight at 37 °C in liquid Luria–Bertani (LB) medium containing 100 μg mL⁻¹ ampicillin, diluted 100-fold in the same medium and grown until an OD₆₀₀ nm of 0.3 was observed. IPTG was then added (1 mM final concentration) and the bacteria were grown for an additional 4 h at 30 °C. Bacteria were pelleted and then resuspended in 1/20 of the original culture volume of 50 mM Tris/HCl, pH 8.0 buffer, containing 30% sucrose and 1 mM EDTA. After incubation for 30 min at 0 °C, the bacteria were pelleted and subjected to osmotic shock by the addition of 1/20 of the original culture volume of 5 mmol L⁻¹ MgSO₄ and incubated for 30 min at 0 °C. The bacterial debris was pelleted and the soluble periplasmic extract contained scFv–E-tag fusion proteins.

For Western blotting, scFv–E-tag fusion protein was electrophoresised on SDS gel and then transferred to nitrocellulose membrane and blocked with nonfat milk. The membrane was incubated for 1 h at 37 °C with a preparation containing the anti–E-tag antibody. The membrane was washed three times with PBST, and the secondary antibody HRP/anti-IgG conjugate was added for 1 h at 37 °C. The rest was according to standard procedure. Control gel was stained with Coomassie blue to detect proteins.

Preparation of deoxynivalenol–HRP conjugates

Preparation of deoxynivalenol–HRP conjugates was conducted according to Fox et al. (2004) with slight modifications. S-acetyl thioglycolic acid N-hydroxysuccinimide [SATA; 1 mg/50 μL in dimethylformamide (DMF)] was added to 10 mL of HRP [1 mg mL⁻¹ in 50 mM potassium phosphate, 150 mM NaCl, 1 mM EDTA (buffer A), pH 7.8]. The solution was mixed gently, allowed to incubate for 1 h and then dialysed extensively against buffer A at pH 6.8. Deoxynivalenol (2 mg mL⁻¹ in DMSO) was activated through available hydroxyl groups by adding 100 μL N-[p-maleimidophenyl] isocyanate (PMPI; 30 mg mL⁻¹ in DMSO) and brought to a final volume of 310 μL with DMSO, which represented a fivefold molar excess of PMPI over deoxynivalenol. After reacting for 1 h at room temperature (RT), PMPI-activated deoxynivalenol (260 μL) was added to 2 mL SATA–HRP (0.5 mg mL⁻¹ in buffer A, pH 6.8). This combination of reactants represented a 20- and twofold molar excess of activated deoxynivalenol to HRP. After 2 h incubation, deoxynivalenol–HRP conjugates were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA.

Deoxynivalenol–HRP conjugate analysis

Antideoxynivalenol scFv–E-tag was diluted in 200 mM sodium carbonate (1 μg mL⁻¹), pH 9.6, and used to coat 96-well microtiter plates (100 μL well⁻¹) at 37 °C for 2 h. After coating, microtiter plates were washed three times with phosphate-buffered saline-0.05% (v/v) Tween-20 (PBST) followed by the addition of blocking solution [PBSM, 4% (w/v) nonfat milk in PBS, 200 μL well⁻¹]. After washing, deoxynivalenol–HRP conjugates were added to the wells. Following incubation for 1 h, the plate was washed four times and tetramethylbenzidine substrate was added for 10 min (Yeo & Wong, 2002; Fox et al., 2004). Free HRP and modified deoxynivalenol–HRP conjugates were also evaluated by SDS-PAGE following the standard procedure.

CD-ELISA procedures

Anti-E-tag antibody was diluted in 200 mM sodium carbonate, pH 9.6. Flat-bottomed microtiter plates were subsequently coated at 37 °C for 1 h with saturating amounts of anti-E-tag (15 μg mL⁻¹; 100 μL well⁻¹). After coating, microtiter plates were washed three times with phosphate-buffered saline-0.05% (v/v) Tween-20 (PBST) followed by...
addition of blocking solution [1%(w/v) BSA in coating buffer; 200 μL well\(^{-1}\)]. Antideoxynivalenol scFv–E-tag was diluted to 1/1000 in PBST and added to a final volume of 100 μL well\(^{-1}\). Various concentrations of deoxynivalenol were mixed with deoxynivalenol–HRP conjugates, and added to the wells. Following 1 h incubation, the plate was washed four times and tetramethylbenzidine substrate was added for 10 min. The reaction was stopped by adding 1 M H\(_2\)SO\(_4\) and the absorbance was read at 450 nm (Yuan et al., 1997).

**Deoxynivalenol detection in spiked wheat samples**

Different amounts of deoxynivalenol were spiked into 5 g clean wheat powder (nondeoxynivalenol detected with Neo-gen Varatox kit) to create a series of deoxynivalenol concentrations expected at 0.05, 0.1, 0.2 and 0.4 p.p.m. The samples were shaken at 250 r min\(^{-1}\) in a rotary shaker for 5 min, and filtered. The same volume of extract and deoxynivalenol-HRP conjugates were mixed. Then 100 μL of the solution mixture was transferred into wells coated previously with 100 μL of 15 μg mL\(^{-1}\) anti-E-tag antibody and blocked with PBSM. The plate was incubated at RT for 60 min and washed three times with distilled PBST, then added with 100 μL of substrate. It remained at RT for 10 min to develop colors and was then stopped by the stop solution, and absorbance was read at 450 nm. In total, three batches of spiking were conducted, and every batch had three replications. A Neogen veratox 5/5 kit was used to compare batches.

**Detection of wheat samples**

Fifty-five natural contaminated wheat samples were tested side by side using scFv and monoclonal antibody-based CD-ELISA (Neogen veratox 5/5 kit). The procedure to prepare samples was the same as the detection of the spiked wheat samples except no deoxynivalenol was added. Each sample was tested three times.

**Results**

**Expression, isolation and Western blotting of antideoxynivalenol scFv–E-tag**

Strain deoxynivalenol1.3G2/HB2151 containing the gene encoding antideoxynivalenol scFv fused with E-tag was used to produce recombinant protein scFv–E-tag in E. coli. The single clone deoxynivalenol1.3G2/HB2151 was grown in LB medium until an OD\(_{600\text{nm}}\) of 0.6 was reached, then IPTG was added to induce the recombinant protein over expression. The bacteria were grown for an additional 4 h and analyzed by SDS-PAGE. As shown in Fig. 1 (lane 5), SDS-PAGE revealed that a protein of about 30 kDa was strongly expressed after 4 h induction. After induction, the bacteria were pelleted and the soluble recombinant protein scFv–E-tag was isolated (Fig. 1, lanes 2, 3). Western blotting results (Fig. 1, lane 1) showed that only one band of about 30 kDa protein was observed, indicating scFv–E-tag was successfully expressed and prepared.

**Synthesis of deoxynivalenol–HRP conjugate**

To establish scFv-based deoxynivalenol detection, first deoxynivalenol–HRP conjugate was synthesized for enzyme immunoassay. HRP was first added with protected sulfhydryls to amines or its sulfhydryl group was deprotected by reacting with SATA. Then the -OH groups of deoxynivalenol–PMPI reacted with -SH groups of HRP–SATA at pH 6.5–7.5, forming stable thioether linkages. Deoxynivalenol–HRP conjugates were analyzed by SDS-PAGE and ELISA. SDS-PAGE analysis was carried out using HRP and deoxynivalenol–HRP, and the result indicated that conjugated HRP formation was
evident resulting in a higher molecular weight band (Fig. 2a, lane 2) than that of unconjugated HRP (Fig. 2a, lane 1). But the degree of hapten loading could not be determined by SDS-PAGE due to limitations in resolution and the large protein size relative to that of the haptenylated form.

ELISA was also carried out to analyze the bifunctional activity of the conjugate deoxynivalenol–HRP. As is shown in Fig. 2b, positive blue color was given out when deoxynivalenol–HRP conjugate with some deoxynivalenol (lane 2) or without deoxynivalenol (lane 1) was added to the scFv-coated wells, whereas color was not developed when HRP with deoxynivalenol (lane 4) or without deoxynivalenol (lane 3) was added to the same coated wells. This confirmed that deoxynivalenol–HRP was successfully formed with dual function activity, having the deoxynivalenol binding specificity to antideoxynivalenol scFv and HRP activity to form color.

**Cross activity of scFv to deoxynivalenol analogs compared with monoclonal antibody**

To test the scFv function, the cross activity and the recovery efficiency of the scFv were compared with its parental monoclonal antibody. The scFv was slightly more sensitive to both deoxynivalenol and its analogs 3-AC-deoxynivalenol and 15-AC-deoxynivalenol compared with its parental monoclonal antibody (Table 1). However, it retained non-cross reactivity with other trichotecene members such as nivalenol and T2 toxin as in its parent monoclonal antibody. This suggested that scFv retained the specificity of its parental monoclonal antibody and could be a possible replacement of the monoclonal antibody.

**Detection of deoxynivalenol by CD-ELISA in spiked wheat samples**

CD-ELISA based on scFv was used to detect deoxynivalenol in spiked contaminated wheat samples by comparison with the Neogen Varatox 5/5 kit. The results showed that the recovery of spiked deoxynivalenol from wheat samples by scFv and its monoclonal antibody has no significant difference (Fig. 3). Especially when spiked deoxynivalenol is between 0.5 and 4 p.p.m, the recovery of both scFv and monoclonal antibody is almost the same. To further study the scFv applicability in the detection of natural contaminated wheat, correlation R-squared value out of 21 samples tested is 0.9596.

**The detection result of real wheat samples**

To know the deoxynivalenol contamination status in Chinese wheat-producing areas, the scFv-based detection method was further applied to detect deoxynivalenol contents in 55 real wheat samples collected from five different provinces in China. The result was shown in Table 2. Among 18 samples from Fujian province in China, 94.4% were detected to be contaminated with deoxynivalenol, among which 61.1% samples were higher than the standard state, and the highest amount of deoxynivalenol reached 5.2 p.p.m (Data not shown). Heilongjiang province is also a deoxynivalenol-contaminated place, and the detectable ratio of deoxynivalenol was 75%, and the ratio that exceeded the

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**Table 1. Cross-activity of scFv and monoclonal antibody to DON analogs**

<table>
<thead>
<tr>
<th>DON and its analogs</th>
<th>Monoclonal antibody</th>
<th>Cross-activity</th>
<th>scFv</th>
<th>Cross-activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (ng mL^{-1})</td>
<td></td>
<td>IC50 (ng mL^{-1})</td>
<td></td>
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<tr>
<td>Deoxynivalenol</td>
<td>8.3 ± 0.06</td>
<td>100.0</td>
<td>8.2 ± 0.6</td>
<td>100.0</td>
</tr>
<tr>
<td>3-AC-Deoxynivalenol</td>
<td>135.0 ± 18.9</td>
<td>6.1</td>
<td>98.5 ± 12.5</td>
<td>8.3</td>
</tr>
<tr>
<td>15-AC-Deoxynivalenol</td>
<td>175.0 ± 16.1</td>
<td>4.7</td>
<td>153.0 ± 26.3</td>
<td>5.4</td>
</tr>
<tr>
<td>Nivalenol</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>T2</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
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\*Cross-activity defined as (IC50 of deoxynivalenol/IC50 of analog) × 100%.

\*No IC50 and cross reactivity can be detected.

Fig. 3. Correlation of deoxynivalenol detected in wheat samples based on scFv and Neogen Veratox kit. When deoxynivalenol is between 0.5 and 4 ppm, the recovery of both scFv and monoclonal antibody is almost the same. Correlation R-squared value out of 21 samples tested is 0.9596.
acceptable limit was 33.3%. The situation in Henan province was better than that in other places, and no deoxynivalenol was detected.

Discussion

There are growing evidences that indicate that deoxynivalenol can enhance the virulence of plant-pathogenic Fusarium species on plant hosts (Proctor et al., 1995; Yuan et al., 1999), and it causes many diseases in plants, including head and seedling blight of small grains, such as wheat and rye, ear and stalk rot of maize, and seedling blight and root rot in a number of other plant species, including beans, clover and tomato (Eriksen et al., 2003; Schneider et al., 2004). Therefore, more rapid and sensitive methods towards deoxynivalenol detection should be established, and these may lead to prevention of plant and animal diseases associated with this mycotoxin.

In this paper, a method to detect fungal toxins based on scFv through CD-ELISA was tried to develop. Using scFv for immunoassay has several advantages. First, it retains the same specific affinity to the antigen to its monoclonal antibody; second, scFv can be produced in large quantities in bacterial expression systems at low cost; third, it is easy to manipulate and adaptable to different applications, e.g. fusion with marker molecules for detection purposes (Winter et al., 1994). Hence scFv was used to detect deoxynivalenol in this paper.

Fast and accurate detection of mycotoxins is extremely important in management of toxins. Although several scFvs to mycotoxins have been reported to be cloned (Yuan et al., 1999; Choi et al., 2004), few scFv were reported in applications to detect naturally contaminated samples. In this paper, it was demonstrated that antideoxynivalenol scFv–E-tag was applicable in detecting natural contaminated deoxynivalenol in wheat samples. The correlation of detected deoxynivalenol between scFv and its parental monoclonal antibody is high, which suggests that it is possible to replace monoclonal antibodies with scFv in the detection of toxins.

To develop the competitive immunoassay based on scFv, first the generation of sensitive and specific deoxynivalenol-HRP conjugate was described by adding sulphydryl groups through reacting with SATA and acting to the -OH groups of deoxynivalenol by PMPI. The approach of synthesis and characterization of deoxynivalenol–HRP conjugates by SDS-PAGE confirmed that this conjugation method was successful. This provided a simplified but highly efficient way to add carrier protein to small molecules of semi-antigens.

Finally, there are few regular reports regarding the mycotoxin contamination of cereals in China, but the contamination can be assumed to be very common from the preliminary result of this study. Moreover, the deoxynivalenol contamination in wheat samples is coincident with the wheat head blight epidemics in China, this signals to us that low deoxynivalenol wheat should be incorporated in the wheat breeding program, which required an easy way to handle deoxynivalenol detection procedure. The method developed in this paper would facilitate the establishment of an easy and low-cost deoxynivalenol monitoring ELISA kit, which could be used both in regular products detection and in breeding programs.

Acknowledgements

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References


Fazekas B, Hajdu ET, Tar AK et al. (2000) Natural deoxynivalenol (DON) contamination of wheat samples grown in 1998 as

<table>
<thead>
<tr>
<th>Province</th>
<th>Ratio of samples with detectable DON (%)</th>
<th>Ratio of samples with DON ≥ 1 ppm (%)</th>
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<tbody>
<tr>
<td>Fujian</td>
<td>94.44</td>
<td>61.11</td>
</tr>
<tr>
<td>Heilongjiang</td>
<td>75</td>
<td>33.33</td>
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<tr>
<td>Sichuan</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>Jiangsu</td>
<td>54.55</td>
<td>9.09</td>
</tr>
<tr>
<td>Henan</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. DON contamination of wheat samples in various places in China detected by anti-DON scFv based CD-ELISA.


