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A phage-displayed chicken single-chain antibody fused to alkaline phosphatase detects *Fusarium* pathogens and their presence in cereal grains

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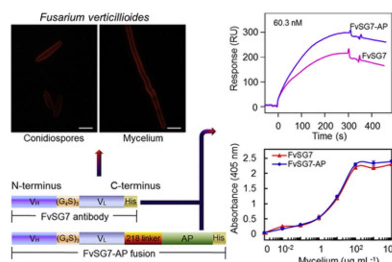
HIGHLIGHTS

- Generation of a highly reactive scFv antibody against *F. verticillioides*.
- Localization of the antibody binding to the surface target of *F. verticillioides*.
- Expression of the antibody–alkaline phosphatase (AP) fusion linked by a 218 linker.
- The antibody–AP fusion has a higher affinity than the parental antibody.
- The antibody–AP fusion detects toxigenic *Fusarium* pathogens in cereal grains.

GRAPHICAL ABSTRACT

A phage-displayed chicken scFv antibody, FvSG7, binds on the surface antigen of conidiospores and the mycelia of *F. verticillioides*. Its fusion with alkaline phosphatase (AP) through a 218 linker displayed a 4-fold higher affinity compared with the parent scFv antibody and efficiently detected toxigenic *Fusarium* pathogens in cereal grains.



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ABSTRACT

Fusarium and its poisonous mycotoxins are distributed worldwide and are of particular interest in agriculture and food safety. A simple analytical method to detect pathogens is essential for forecasting diseases and controlling mycotoxins. This article describes a proposed method for convenient and sensitive detection of *Fusarium* pathogens that uses the fusion of single-chain variable fragment (scFv) and alkaline phosphatase (AP). A highly reactive scFv antibody specific to soluble cell wall-bound proteins (SCWPs) of *F. verticillioides* was selected from an immunized chicken phagemid library by phage display. The antibody was verified to bind on the surface of ungerminated conidiospores and mycelia of *F. verticillioides*. The scFv–AP fusion was constructed, and soluble expression in bacteria was confirmed. Both the antibody properties and enzymatic activity were retained, and the antigen-binding capacity of the fusion was enhanced by the addition of a linker. Surface plasmon resonance measurements confirmed that the fusion displayed 4-fold higher affinity compared with the fusion's parental scFv antibody. Immunoblot analyses showed that the fusion had good binding capacity to the components from SCWPs of *F. verticillioides*, and enzyme-linked immunosorbent assays revealed that the detection limit of the fungus was below

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10^{-2} μg mL^{-1}, superior to the scFv antibody. The fusion protein was able to detect fungal concentrations as low as 10^{-3} mg g^{-1} of maize grains in both naturally and artificially contaminated samples. Thus, the fusion can be applied in rapid and simple diagnosis of *Fusarium* contamination in field and stored grain or in food.

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1. Introduction

Fusarium is an important genus of phytopathogenic fungi that are responsible for devastating diseases such as Fusarium ear rot in maize and Fusarium head blight in wheat and barley [1,2]. *Fusarium* species occur worldwide [3], leading to huge economic losses from decreased crop yield and quality [4]. They additionally produce mycotoxins, which endanger human and animal health [5]. *Fusarium verticillioides* is one of the most common of the *Fusarium* species [6–9] and can produce a chemically diverse array of mycotoxins, including fumonisins and moniliformin. The former is positively linked with porcine pulmonary edema, equine leukoencephalomalacia, and deranged sphingolipid metabolism [5] and is potentially carcinogenic in humans [10–12], while the latter is assumed to interact with fumonisins and produces cardiotoxic effects in a wide range of laboratory and domesticated animals [5]. However, it is difficult to monitor pathogens and predict epidemics because the disease occurrence varies with crop period, season, tillage regime, and even ecological and environmental factors [1,2,13]. Simple and rapid test methods to detect the presence of *Fusarium* pathogens in the field during the growing season may provide farmers with another tool to conveniently assess and forecast disease risk, which would allow taking measures to minimize mycotoxin production and the damage caused by the fungi. Reliable analytical tools are also essential for inspection and quarantine within the entry–exit cereals trade.

Relative to more labor-intensive and costly procedures using other biological or molecular approaches, enzyme-linked immunosorbent assay (ELISA) techniques provide an attractive and promising alternative for conventional mold detection with rapid, safe, convenient, and excellent specificity and sensitivity. For more than two decades, immunoassays have been developed for detection of mold contamination by using polyclonal sera [14–18] and monoclonal [19,20] or single-chain variable fragment (scFv) antibodies [21,22]. For *Fusarium* pathogens, however, only polyclonal antibodies from chicken [16] or rabbit [17,18] have been used in immunological tests and their detection limits in the ELISAs were higher than 1 μg of mold per gram of maize flour, with no affinity data being reported. Unlike polyclonal and monoclonal antibodies, scFv antibodies can be isolated together with their coding sequence by phage display [21,23], expressed with a relatively high yield with a bacterial expression system [24], and readily extracted from the periplasm space [25]. Moreover, affinity of the scFv antibodies can be easily improved by directed molecular evolution in vitro [26,27]. The antibodies may also be engineered as variants [24] or fused genetically to a vast range of molecules, such as toxins, peptides, and enzymes, to achieve additional improved properties [28–30]. Previous research confirmed that the highly reactive chicken-derived scFv antibodies can be selected by phage display technology [31,32]. Constructing a phage library is technically easier using chickens compared with other animals because only one set of primers is required for each antibody chain due to the peculiar mechanism of chicken immunoglobulin gene diversification [33–35].

Conventional immunoassays for detection rely upon primary or secondary antibodies labeled with enzymes or fluorescent dyes. Horseradish peroxidase (HRP) and alkaline phosphatase (AP), conjugated to antibodies by chemically cross-linking, are the most commonly used enzymes in ELISA detection. However, the chemical reaction is a random cross-linking procedure that always leads to reduced or even lost activity and produces many unexpected conjugates, including antibody–antibody and enzyme–enzyme conjugates. Therefore, experienced technicians, a complex procedure, and optimized reaction conditions are essential for the preparation of high-quality conjugates. In contrast, construction of scFv–AP fusions by genetic techniques is considered an attractive alternative for simple and rapid immunological detection. This procedure has been used to detect various antigens, such as beet necrotic yellow vein virus [30], *Bacillus anthracis* [36], *Yersinia pestis* [37], *Entamoeba histolytica* [38], and so on. These fusion proteins can be easily produced and purified in a bacterial expression system, and they accelerate analyte detection in antibody-based diagnostic tests because enzyme-labeled secondary antibodies are not needed [30].

The objective of this study was to generate and characterize *Fusarium*-specific recombinant scFv antibodies by using phage display technology with an immunized chicken antibody library. The localization of the high-affinity fragment to *F. verticillioides* was confirmed by specific binding to the surface of hyphae and conidiospores observed through immunofluorescence microscopy. The scFv–AP fusion displayed a similar antigen-binding capacity compared to the parental scFv antibody as validated by ELISA and immunoblot analyses. Further surface plasmon resonance (SPR) analyses showed that the FvSG7–AP fusion had good binding kinetics, with a $K_D = 3.69 \times 10^{-8}$, which was approximately four times higher than that of the FvSG7 antibody. Thus, the fusion protein can be developed for rapid detection of mold contamination in field and storage samples or in food.

2. Experimental

2.1. Reagents, culture media, and buffers

All chemical reagents were of analytical grade. Complete and incomplete Freund's adjuvant and AP-labeled goat anti-mouse IgG antibody were purchased from Sigma–Aldrich Company (Saint Louis, MO). TRNzol-A+ total RNA extraction kit and anti-His antibody were purchased from Tiangen (Beijing, China). Oligotex® mRNA mini kit, QIAquick® Gel Extraction Kit and Ni-NTA chromatography were from Qiagen (Hilden, Germany). SuperScript® III Reverse Transcriptase Kit was purchased from Invitrogen (Grand Island, NY). *Escherichia coli* strain XL1-Blue MRF' was from Stratagene (La Jolla, CA). The restriction enzymes *Sfi*I, *Not*I, and *Bst*NI were purchased from NEB (Beijing, China). M13KO7 helper phage was from Amersham Biosciences (Piscataway, NJ). Cy3-conjugated affinipure goat anti-mouse IgG (H+L) was from ProteinTech Group (Chicago, IL). Antifade Mounting Medium was purchased from Beyotime (Haimen, China). The poly-L-lysine and BCIP/NBT Color Development Kit were from Boster (Wuhan, China).

The composition, concentration and pH of the employed culture media and buffers were Czapek-Dox Broth, 3% (w/v) sucrose, 0.3% (w/v) $NaNO_3$, 0.1% (w/v) K_2HPO_4, 0.05% (w/v) $MgSO_4 \cdot 7H_2O$, 0.05% (w/v) KCl, and 0.001% (w/v) $FeSO_4$, pH 8.0; Luria–Bertani (LB) medium, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, pH 7.0; 2× TY medium, 1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, and 0.5% (w/v) NaCl, pH 7.0; TYE medium, 1% (w/v) trypton,

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