Food Chemistry 319 (2020) 126546

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Single-chain variable fragment antibody-based immunochromatographic strip for rapid detection of fumonisin B_1 in maize samples

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ARTICLE INFO

Keywords:
Single-chain variable fragment antibody
Immunochromatographic strip
Fumonisin B_1
Rapid detection
Maize

ABSTRACT

A rapid and sensitive immunochromatographic strip (ICS) based on a single-chain variable fragment (scFv) was developed for detecting fumonisin B_1 (FB_1). The ICS was based on a competitive reaction for colloidal gold-labeled scFv between FB_1 and FB_1-BSA, which was used along with sheep anti-mouse IgG as capture reagents immobilized at test and control lines, respectively, on a nitrocellulose membrane of the strip. The limit of detection of the ICS was 2.5 ng/mL (25 μg/kg) FB_1 in buffer, and the sensitivity was eight times higher than that of monoclonal antibodies for the preparation of the scFv. The cross-reactivity of the scFv with common mycotoxins was determined by ICS, the results showed that the scFv were not against other mycotoxins. Eight naturally contaminated maize samples were analyzed with the scFv-based ICS and by LC-MS/MS. The results of analysis obtained with the strip assay showed good agreement with those obtained by LC-MS/MS.

1. Introduction

Maize is one of the most important food crops in the world; it is not only consumed in its original form, but is also processed as feed and other products as part of the agroindustrial supply chain (Galvão, Miranda, Trogello, & Fritsche-Neto, 2014; de Oliveira, de Castro Gomes Vieira, Orlando, & Faria, 2017). Maize is one of the crops that are most significantly affected by fumonisin worldwide. The proportion of fumonisin B_1 (FB_1) contamination among 146 maize samples collected in Gansu, Sichuan, and Guizhou, China in 2012 was 39.7% (Li, Jiang, Zheng, Chen, & Li, 2015). FBs were also detected in 98.1% of 522 maize products from Shandong Province (China) collected in 2014 with an average total level of 369.2 μg/kg (Li, Tao, Pang, Liu, & Dong, 2015), and in 85% of maize samples (n = 120) from three agricultural research institutes in the Tanzania ecoregion (Kamala et al., 2016). Among 102 maize samples from northern Vietnam, 24 (23.5%) contained FBs (Huong et al., 2016). FB is a water-soluble secondary metabolite mainly produced by *Fusarium verticillioides* and *Fusarium proliferatum* (Wentzel, Lombard, Du Plessis, & Zandberg, 2017). More than 28 structural isoforms of fumonisin have been identified and characterized to date; of these, FB_1 has the highest toxicity (Shu et al., 2015). FB_1 can induce global DNA and histone demethylation, causing chromatin instability in human liver that can lead to liver cancer (Chuturgoon, Phulukdaree, & Moodley, 2014); reduce the viability of hemocytes (Zhang, Diao, Li, & Liu, 2018); and cause pulmonary edema in pigs (Harrison & Stein, 1990) and white matter softening in horse. FB_1 is listed as Class 2B carcinogen by the Internationa Agency for Research on Cancer (Li et al., 2018; Loiseau et al., 2015).

Given their many hazards to humans and animals, some countries and organizations have established residual limits for FBs (FB_1, FB_2, and FB_3) in food and animal feed; the United States Food and Drug Administration (FDA) recommends limits of 2–4 mg/kg for human food and 5–100 mg/kg for animal feed (Tang et al., 2017). Currently available FB_1 detection methods are either chromatography- or immunoassay-based; the former includes high-performance liquid chromatography(Li et al., 2015) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Souto et al., 2017), while the latter includes enzyme-linked immunoassay (ELISA) and immunochromatographic assay (Shu et al., 2016; Chen, Liang, Zhang, Leng, & Xiong, 2018; Wang, Bao, et al., 2016; Wang, Fan, et al., 2016; Ling et al., 2014). MS has high accuracy but sample preparation is cumbersome and the instrument is costly. ELISA is suitable for screening a large number of samples, and

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https://doi.org/10.1016/j.foodchem.2020.126546
Received 1 May 2019; Received in revised form 18 August 2019; Accepted 1 March 2020
Available online 02 March 2020

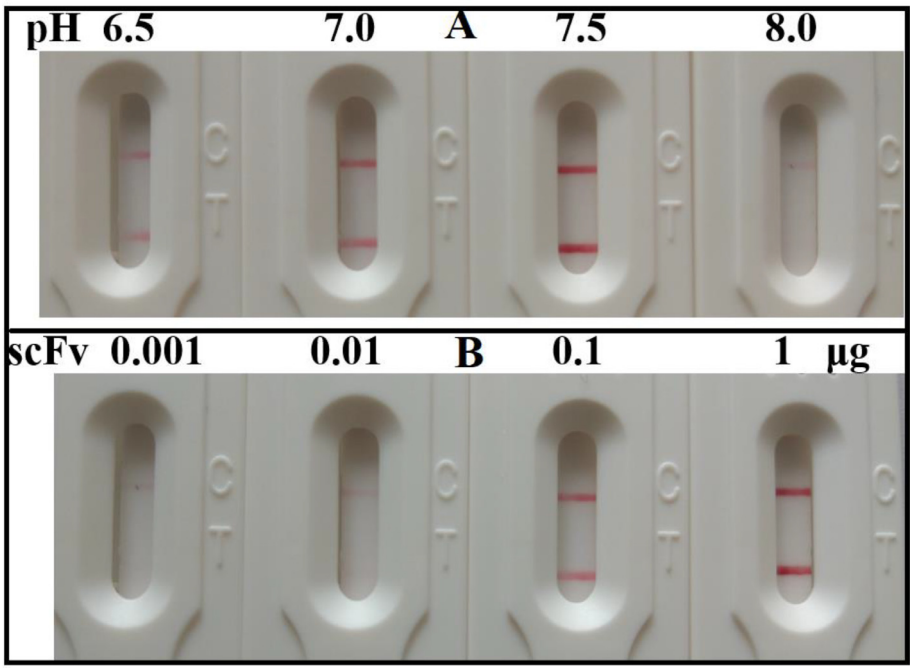

Fig. 1. Optimal pH and quantity of monoclonal antibody of the AuNPantibody probe. (A) Optimal pH. (B) Optimal quantity of scFv.

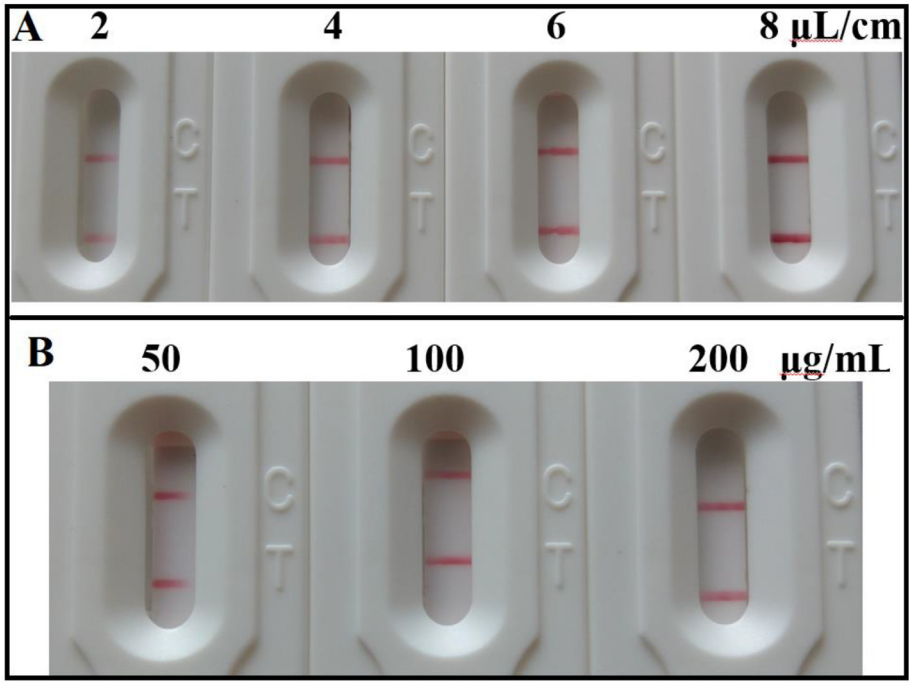

Fig. 2. Quantity of FB1BSA and monoclonal antibody in the AuNPantibody probe. AuNPantibody probe: 2.0, 4.0, 6.0, and 8.0 μL/cm. (A–C) Test antigen: 50.0, 100.0, and 200.0 μg/mL.

has the advantages of high sensitivity and specificity, but is technically demanding and must be performed in a laboratory setting. In contrast, immunochromatographic assay–which is widely used in the detection of FB (Hao et al., 2018; Ling et al., 2015) –is rapid and easy to carry out since it does not require special equipment; it is also suitable for on-site and large-scale sample screening. In addition, various optical biosensors and electrochemical methods have been developed for FB detection (Lu, Seenivasan, Wang, Yu, & Gunasekaran, 2016; Ren et al., 2017; Zhao, Luo, Li, & Song, 2014; Chen et al., 2015; Yang et al., 2015; Ezquerra, Vidal, Bonel, & Castillo, 2015).

Immunological detection involves the use of monoclonal antibodies, single-chain variable fragment (scFv), nanobodies, and aptamers (Shu et al., 2016; Xu, Chen, et al., 2014; Ren et al., 2015; Zou et al., 2014; Wang et al., 2014). The scFv is the most common type of genetically engineered antibody and is formed by linking heavy and light chain variable regions of immunoglobulin by an elastic linking peptide; it is the smallest functional unit (molecular weight: 27–30 kDa) of the parent antibody with complete antigen-binding properties. In addition to the small molecular mass, other advantages of scFv include a strong capacity for penetration, low immunogenicity, and ease of genetic modification and expression (Nisbet et al., 2017; Patel et al., 2014; Badescu, Marsh, Smith, Thompson, & Napier, 2016; Greineder et al.,

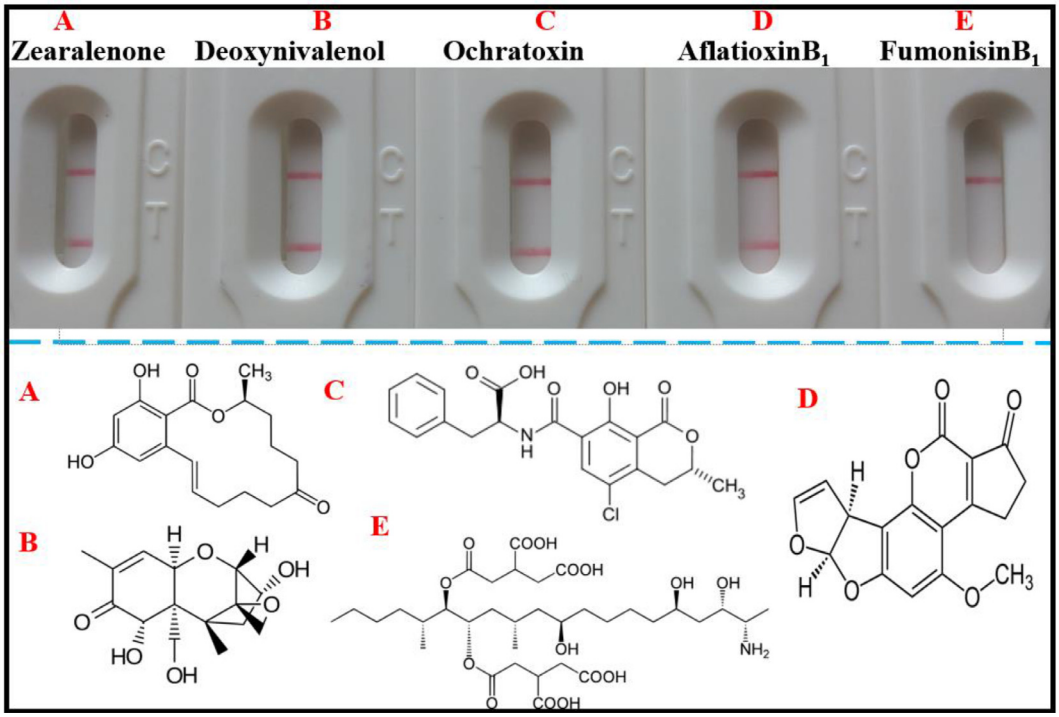

Fig. 3. Cross-reactivity of the ICS with other toxins. (A–E) The mycotoxins ZEN (A), DON (B), OTA (C), AFB_1 (D), and FB_1 (E) were tested at a concentration of 5.0 ng/mL.

2016).

In the present study, we developed a novel immunochromatographic strip (ICS) in which colloidal gold was labeled with scFv. The ICS successfully detected FB_1 in maize samples and can thus be useful for rapid and simple on-site testing for FB_1 contamination.

2. Materials and methods

2.1. Materials and reagents

FB_1, aflatoxin B_1 (AFB_1), ochratoxin (OTA), deoxynivalenol (DON), and zearalenone (ZEN) were purchased from Fermentek (Jerusalem, Israel). Sodium dodecyl sulfate, Tween-20, K_2CO_3, and bovine serum albumin (BSA) were from Bioengineering Biotechnology (Shanghai, China). Chloroauric acid ($HAuCl_4{\cdot}3H_2O$) and trisodium citrate were from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and NC95 nitrocellulose (NC) membrane were from Sartorius (Göttingen, Germany). Polyvinyl chloride (PVC) sheets, sample pads, and absorbent paper were from Shanghai Jieyi Biotechnology Co. (Shanghai, China). Other chemicals (analytical grade) were purchased from Sinopharm Chemical Reagent Co. (Beijing, China). An FB_1 immunochromatography affinity column (PriboFast IAC-050–3) was obtained from Pribolab Bioengineering (Qingdao, China). FB_1-BSA and scFv were prepared in our laboratory. Phosphate-buffered saline (0.01 M PBS, pH 7.4), methanol, and acetonitrile (high-performance liquid chromatography grade) were from Sinopharm Chemical Reagent Co.

2.2. Instruments

Ultrapure water was prepared using a water purification system (67120; Millipore, Billerica, MA). Test strips were prepared using a constant temperature incubator (Sanyo, Tokyo, Japan) to dry the strips along with the XYZ 3000 platform and test strip cutter (BioDot, Irvine, CA). Gold nanoparticles (AuNPs) were characterized with an H-600 transmission electron microscope (Hitachi, Tokyo, Japan) and ultraviolet–visible (UV–Vis) spectrophotometer (Ultrospec-4300; Amersham, Little Chalfont, UK). The magnetic stirring electromantle heater was from Cole-Parmer (Wertheim, Germany). Detection of FB_1 in maize by LC–MS/MS and scanning electron microscopy characterization of AuNPs were performed at the Nanchang University Analytical Testing Center.

2.3. Expression and identification of anti-FB_1 scFv

As described in our previous work (Zou et al., 2014), the cloned recombinant plasmid pMAL-1D11 was transformed into *Escherichia coli* BL21 (DE3) cells and a single colony was inoculated into Luria-Bertani broth as a shaking culture. After inducing protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG), the cells were collected for sonication. The lysate was centrifuged at 10,000 × *g* for 15 min at 4 °C and the precipitated protein was collected for denaturation, purification, and renaturation. The target protein was identified by ELISA.

2.4. Generation of FB_1–BSA

FB_1 artificial antigen was synthesized by the carbodiimide method. Briefly, 1.0 mg FB_1 dissolved in 1.0 mL of PBS (0.01 M, pH 7.4) was combined with 4.0 mg BSA dissolved in 1.0 mL of PBS (0.01 M, pH 7.4). A 200 μL volume of 20.0 mg/mL *N*-ethyl-N'- (3-dimethy-laminopropyl) carbodiimide aqueous solution was added dropwise to the mixture , which was next shaken at room temperature for 2 h, and then allowed to stand at room temperature for 20 min. The synthesized antigen was transferred to a dialysis bag in PBS (0.01 M, pH 7.4) solution and dialyzed at 4 °C for 72 h.

2.5. Preparation of AuNPs

The AuNP solution was synthesized by trisodium citrate reduction. A 1.0 mL volume of 1% (w/w) aqueous chloroauric acid solution was added to 99.0 mL ultrapure water to achieve a final chloroauric acid concentration of 0.1% (w/w). The solution was heated to boiling with

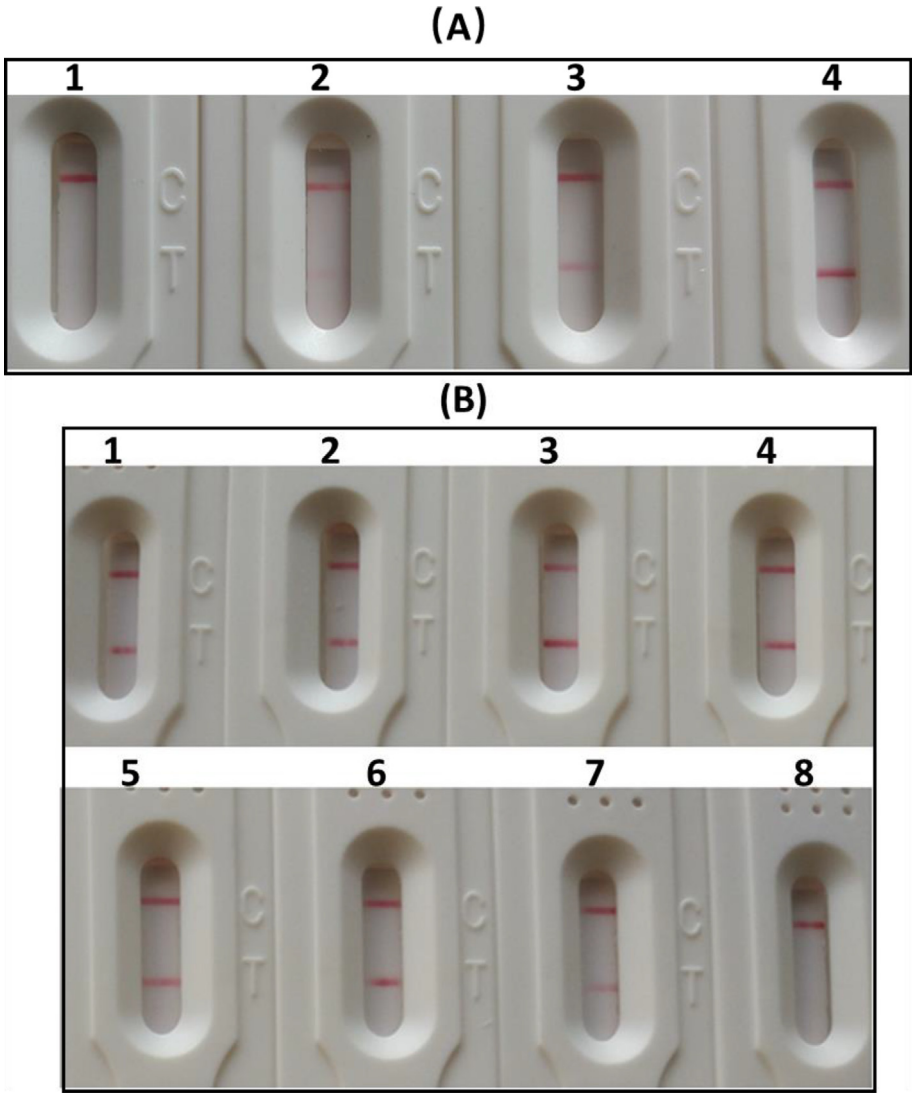

Fig. 4. (A) ICS results for different concentrations of FB_1 standard. FB_1 concentrations (1–4) were 5.0, 2.5, 1.0, and 0 ng/mL, respectively. (B) Detection of FB_1 in maize samples with the ICS. Sample 8 containing over 2.5 ng/mL FB_1 caused the disappearance of the red T line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of the two methods to detect the FB_1 in maize samples.

Maize	LC-MS/MS (μg/kg)	ICS test (n = 3)	Maize	LC-MS/MS (μg/kg)	ICS test (n = 3)
1	5.42	−,−,−	5	4.08	−,−,−
2	5.27	−,−,−	6	5.28	−,−,−
3	0.785	−,−,−	7	606.63	−,−,−
4	2.96	−,−,−	8	1251.36	+,+,+

Note: "+" means positive result, T line vanished; "−" means negative result, both C line and T line appeared clearly.

vigorous stirring; 2.5 mL of 1% (w/w) trisodium citrate solution was added while boiling and the reaction was continuously stirred for 10 min. After the solution turned red and the color did not change further, the heat was reduced and the solution was left to cool to room temperature. The synthesized product was analyzed by UV–Vis spectroscopy and transmission electron microscopy.

2.6. Preparation of the AuNP probe

A 2.0 mL volume of colloidal gold solution was transferred to a small glass flask and cleaned with aqua regia. The flask was stirred at a constant speed and the pH of the solution was adjusted with 0.1 mol/L K_2CO_3. An appropriate amount of the anti-FB_1 scFv was added dropwise and the mixture was stirred for 30 min at room temperature. After blocking by adding 200 μL of 10% BSA solution for 15 min, the labeled colloidal gold solution was transferred to a clean centrifuge tube and centrifuged at 10,000 × *g* for 20 min. The supernatant was discarded, and the precipitate was resuspended in 100 μL colloidal gold solution and stored at 4 °C until use.

2.7. ScFv-based ICS assembly

Different concentrations of FB_1-BSA antigen and anti-mouse IgG (1.0 mg/mL) were sprayed onto the detection and control areas of the NC membrane as test (T) and control (C) lines, respectively, using a spotting machine (XYZ 3000; BioDot) and then dried at 37 °C for 2 h. The pad was immersed in buffer and dried at 60 °C for 2 h. The NC membrane, conjugate and sample pads, absorbent paper, and PVC bottom plate were assembled in order and then cut into 4-mm-wide strips that were placed in a foil bag with desiccant; the bag was sealed and stored at 25 °C.

2.8. Optimal pH and scFv amount for AuNP synthesis

The optimal pH for AuNP synthesis was established with a pH gradient test. A 1.0 mL volume of colloidal gold solution was transferred to a 3.0-mL beaker and the pH was adjusted to 6.5, 7.0, 7.5, or 8.0 with 10% potassium carbonate solution; 2.0 μg scFv was then added to each bottle for labeling, followed by centrifugation and resuspension. A 2.0 μL volume of gold-labeled probe was added to the test strip with an antigen concentration of 200.0 μg/mL, and the optimal pH was determined based on the T line color.

The optimal scFv amount was determined with a protein gradient assay. A 1.0 mL volume of colloidal gold solution was added to a 3.0-mL beaker, and the pH was adjusted to 7.5 with potassium carbonate solution before adding 0.001, 0.01, 0.1, or 1.0 μg scFv antibody for labeling. After centrifugation and resuspension, 2.0 μL of gold-labeled probe were added to a test strip containing 200.0 μg/mL FB_1-BSA. The optimal scFv amount was determined based on the color of the T line.

2.9. Optimization of antigen (FB_1-BSA) and AuNP amounts

The concentration of antigen on the detection line was set to 200.0 μg/mL. The samples were divided into four groups with 2.0, 4.0, 6.0, and 8.0 μL/cm gold-labeled scFv on the colloidal gold pad. The optimal amount of gold-labeled scFv was determined based on the shape and color intensity of the T line. Based on the determined amount of scFv with the greatest amount of gold labeling, three sets of test strips with different antigen concentrations (50.0, 100.0, or 200.0 μg/mL) on the NC membrane were prepared that were distinguished by the shape and color of the T line. The optimal amount of test antigen was confirmed according to the color intensity and shape of the T line.

2.10. Sensitivity and selectivity of scFv-based ICS

Different concentrations of FB_1 standard solution were diluted with loading buffer (0.01 M PBS [pH 7.4] containing 0.5% Tween-20 and 0.1% sodium dodecyl sulfate) to achieve final concentrations of 0.0, 1.0, 2.5, and 5.0 ng/ml. A 100.0 μL volume of standard was applied to the sample pad, and the results were observed for about 10 min. Changes in color intensity of the T line were evaluated by naked eye; the concentration of the standard when the T line completely disappeared was considered as the simplest and most accurate semi-quantitative measurement of test strip concentration. Cross-reactivity with mycotoxins often present with FB_1 including FB_2, FB_3, DON, ZEN, OTA, and AFB_1 was analyzed at a concentration of 5.0 ng/mL.

2.11. Analysis of spiked sample

Crushed maize samples (5.0 g) that were negative for FB_1 by LC-MS/MS were combined with FB_1 standards (0.0, 0.4, and 2.0 mg/kg) and 25.0 mL of PBS (0.1 M, pH 7.4) were added, followed by vigorous shaking for 5 min, sonication for 15 min, and centrifugation at 8000 × *g* for 10 min. The supernatant was passed through a 0.45 μm filter to obtain the sample extract, which was diluted 80 times with loading buffer. Three replicates were used for each concentration. The FB_1 content was determined with the test strip; the color intensity was evaluated visually to evaluate the matrix effect and determine the recovery rate.

2.12. Analysis of natural samples

Eight maize samples were obtained from the local market and stored in the laboratory. Sample extraction and detection were the same for ICS as for the spiked samples. For LC-MS/MS analysis, FB_1 in the sample was extracted, purified, and concentrated according to the FB_1 immunoaffinity column instructions. The samples (5.0 g) were weighed in a 50.0 mL centrifuge tube and 20.0 mL of acetonitrile:water (50:50, v/v) solution were added, followed by shaking and centrifugation. A 2.0 mL volume of supernatant was purified and the residue was dissolved in 1.0 mL acetonitrile:water (20:80, v/v) solution. Each sample was tested with the ICS and analyzed by LC-MS/MS three times. The LC-MS/MS run conditions and MS parameters of FB1 was based on the our previous published method (Shu et al., 2016).

3. Results and discussion

3.1. Generation and identification of scFv and AuNPs

The pET22b-1D11 expression plasmid was transformed into *E. coli* BL21 (DE3) for efficient expression of scFv at an induction temperature of 30 °C and final IPTG concentration of 0.3 mmol/L. The precipitate collected by centrifugation after cell disruption was denatured, purified, and renatured to obtain scFv with antibody activity. The sensitivity of scFv was evaluated by competition ELISA; a standard inhibition curve was established using FB_1 as the competition antigen. The linear fitting range was 2.10–76.45 μg/L, (R^2 = 0.9922) and the half-maximal inhibitory concentration was 12.67 μg/L.

The colloidal gold solution was prepared by trisodium citrate reduction (Fig. S1A). The obtained colloidal gold solution was red and uniformly clear. There were no obvious changes in appearance after storage at 4 °C for 10 months, indicating that the solution was relatively stable. Using a colloidal gold solution of poor quality and with poor performance for rapid detection can result in low stability, sensitivity, and specificity of the ICS results; to ensure that this was avoided in our study, we confirmed the quality of the AuNPs by UV–Vis spectroscopy and transmission electron microscopy analyses. The maximum absorption peak of the AuNPs was 518 nm; the peak area was relatively small, indicating that the particle size distribution was uniform (Fig. S1B). Meanwhile, the electron micrograph revealed that the AuNPs were evenly distributed with no irregularities and an average diameter of 20 nm (Fig. S1C).

3.2. Assembly of scFv-based ICS and principle of FB_1 detection

The competitive inhibition method is generally used to detect mycotoxins. The ICS was composed of five parts: sample pad, AuNP pad, NC membrane, absorbent pad, and bottom plate (Fig. S2B). The sample to be tested was loaded on the sample pad; the AuNP pad consisted of a glass fiber containing a AuNP-labeled FB_1 scFv; the NC membrane was sequentially marked with a T line (FB_1-BSA) and C line (goat anti-mouse IgG); and the absorbent pad was composed of a water-absorbing material that allowed the movement of sample solution from the sample pad. In the absence of FB_1, the gold-labeled scFv bound to and formed a complex with the antigen at the detection line, yielding a red band (T line) (Fig. S2C). However, FB_1 in the sample solution formed a complex with the gold-labeled scFv, preventing its binding to the coupled antigen at the T line, which bound any remaining scFv. Thus, the color intensity of the T line was inversely related to the amount of toxin present in the sample solution. However, the gold-labeled scFv could still bind to the secondary antibody at the C line. Thus, the ICS greatly simplifies the process and time required (10 min) for toxin detection, and can be applied to on-site analyses and simultaneous screening of a large number of samples.

3.3. Optimization of scFv-based ICS for FB_1 detection

The protein was bound to the gold surface by physical adsorption (Fig. S2A) as a result of the following forces: the negative charge of AuNPs and positive charge of amino acids (e.g., lysine) in the protein; hydrophobic interactions between AuNPs and tryptophan on the protein surface; and interaction between AuNPs and the thiol group of cysteine in the protein. The pH of the colloidal gold solution and amount of antibody that is added are critical for the preparation of

AuNPs since they not only affect the stability of the probe but also the sensitivity and stability of the test strip.

The T and C lines had the highest color intensity at pH 7.5 (Fig. 1A), indicating that the labeling was successful; the color intensity remained high after adding AuNPs (optical density = 1) to 1.0 μg scFv (Fig. 1B). In order to ensure probe sensitivity, 1 μg was selected as the minimum amount of stable protein to be added. In the classic competitive immune response, reducing immunoreagent concentration increases the sensitivity of the assay for small-molecule mycotoxins. As such, appropriate amounts of antibody and antigen are key parameters for detection sensitivity. The C lines had a good shape and moderate color intensity (Fig. 2). At a detection antigen concentration of 200.0 μg/mL, the color intensity of the T line increased with the amount of gold-labeled scFv. On the other hand, when the amount of gold-labeled scFv was constant, the color intensity of the T line increased with the amount of detection antigen. In order to improve detection sensitivity, 100.0 μg/mL detection antigen with 6.0 μL/cm gold-labeled scFv was selected as the optimal combination.

3.4. Selectivity and sensitivity of scFv-based ICS

None of the major tested mycotoxins cross-reacted with FB_1 when the concentration of the standard was 5 ng/mL (Fig. 3), indicating that the test strip had high selectivity.

These results indicate that the ICS can specifically detect FB_1 contamination.

The sensitivity and selectivity of the optimized ICS was evaluated using FB_1 standard solutions (0.0, 1.0, 2.5, and 5.0 ng/mL) and other mycotoxins, respectively. As FB_1 concentration increased, the color intensity of the detection line declined (Fig. 4A). At FB_1 concentrations greater than 2.5 ng/mL, the line completely disappeared. Thus, the visible detection limit of the test strip was determined as 2.5 ng/mL.

3.5. Detection of FB_1 in spiked samples by scFv-based ICS

In testing actual samples, the most important factor influencing immunoreactivity is the matrix effect, which can be effectively reduced by increasing the sensitivity of the detection method and the dilution factor of the test sample. The sensitivity of the ICS was 2.5 ng/mL. The FDA recommends FB residue limits of 2.4 mg/kg for human food (maize) and 5–100 mg/kg for animal feed (maize), whereas the European Union has established a maximum residue limit in human maize of 1 mg/kg FB_1 + FB_2. We tested FB_1-negative maize samples spiked with FB_1 to achieve final concentrations of 0.0, 1.0, 5.0 ng/mL, and found that the ICS could accurately detect these levels of FB_1(Table S1).

3.6. Detection of FB_1 in maize samples by scFv-based ICS

Eight naturally contaminated maize samples were analyzed with the scFv-based ICS and by LC-MS/MS and the results were compared. The test strips detected FB_1 in eight maize samples, of which one was contaminated with FB_1 and the remaining seven had FB_1 contents that were lower than the 1000 μg/kg limit for grains (Table 1 and Fig. 4B). LC-MS/MS analysis of the eight samples yielded identical results, supporting the reliability of the ICS.

4. Conclusion

To the best of our knowledge, this is the first study investigating the use of scFv to detect mycotoxins by the ICS. This study demonstrates that the scFv-based ICS can specifically detect FB_1 in maize with a sensitivity that is eight times higher than that of monoclonal antibodies for the preparation of the scFv. Importantly, the results obtained by ICS were consistent with those obtained by LC-MS/MS. The short detection time and simple operation makes the scFv-based ICS a convenient and reliable tool for on-site detection of mycotoxins in real-world samples.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (No. 31471648 and 81660660), and supported the Training Plan for the Main Subject of Academic Leaders of Jiangxi Province (NO. 20172BCB22006), and Major Program of Natural Science Foundation of Jiangxi,China (20152ACB20005), and the Jiangxi Province Key Technology R & D Program(20171BBG70072), and also supported by the Project of the Goal-oriented and the Open Project Program of State Key Laboratory of Food Science and Technology, Nanchang University (No.SKLF-ZZA-201612 and SKLF-KF-201610), and also supported by the National Institute of Environmental Health Science Superfund Research Program (P42ES04699), National Key R&D Program of China (Grants 2018YFC1602203).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126546.

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