Screening of Fumonisin B1 Aptamers and Establishment of a Rapid Quantitative Detection Method Postprint

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Abstract

This study aimed to obtain fumonisin B1-specific aptamers and establish a rapid detection method for fumonisin B1 utilizing their interaction with colloidal gold. Aptamers against fumonisin B1 were screened via Systematic Evolution of Ligands by EXponential enrichment with non-immobilized targets (SELEX), and the affinity and specificity of the obtained aptamers were evaluated using enzyme-linked immunosorbent assay (ELISA) to establish an aptamer-based rapid quantitative detection method employing colloidal gold. The results demonstrated that the dissociation constant of the screened fumonisin B1 aptamer F102 was (42.1±4.0) nmol/L; the established colloidal gold detection method was simple, rapid, and highly specific; the relative error between sample detection results and liquid chromatography results was below 15.7%. Therefore, this study established a rapid quantitative detection method for fumonisin B1 content in grains and feed using the screened aptamer.

Full Text

Screening of Fumonisin B1 Aptamers and Establishment of a Rapid Quantitative Detection Method

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Abstract

This study was conducted to obtain fumonisin B1 (FB1) aptamers and develop a rapid quantitative detection method. Using systematic evolution of ligands by exponential enrichment (SELEX) technology with a non-immobilized target strategy, FB1 aptamers were screened and their affinity and specificity were determined by enzyme-linked immunosorbent assay (ELISA). A colloidal gold-based rapid quantitative detection method was subsequently developed. The results showed that the dissociation constant (Kd) of aptamer F102 for FB1 was (42.1±4.0) nmol/L. The developed colloidal gold detection method was simple, rapid, and specific, with relative error compared to high-performance liquid chromatography (HPLC) results of less than 15.7%. Therefore, the FB1 aptamers obtained in this study can be used for rapid and quantitative detection of FB1 content in cereals and feedstuffs.

Keywords: immobilization-free selection; SELEX technology; fumonisin B1; aptamers; colloidal gold

Introduction

Aptamers are RNA or single-stranded DNA (ssDNA) molecules that fold into stable secondary or tertiary structures—such as hairpins, stem-loops, pseudoknots, pockets, bulges, and G-quadruplexes—through intramolecular hydrogen bonding between bases, enabling spatial matching and binding to target molecules. In theory, aptamers can be screened for virtually any target, including small molecules. Comprising only dozens of nucleotides, aptamers offer several advantages: low molecular weight, efficient cell membrane penetration, high stability, and ease of preparation and modification.

In 2002, O' Sullivan first applied aptamers for biological recognition. Since then, various derived SELEX technologies have continuously evolved, enabling widespread application of aptamers in detecting heavy metals, biotoxins, antibiotics, and illegal additives in food and feed. For instance, Shi et al. developed an FB1 detection method based on signal amplification by gold nanoparticles and graphene, using a probe DNA-FB1 aptamer hybrid as a specific recognition element. This method achieved a linear range of 1–1×10 ng/L with a detection limit of 1 ng/L, demonstrating promising application prospects for aptamer-based detection methods.

Currently, various FB1 detection methods have been established, including thin-layer chromatography, enzyme-linked immunosorbent assay (ELISA), microarray, gas chromatography, gas chromatography-mass spectrometry, and high-performance liquid chromatography. However, these methods are often limited by expensive instrumentation and complex procedures. Colloidal gold methods

offer advantages of simplicity, rapidity, and high sensitivity, leading to rapid development in recent years. While colloidal gold has been applied to FB1 detection primarily through antigen-based assays, no reports exist on aptamer-based colloidal gold methods for FB1.

Based on these considerations, this study aims to combine screened aptamers with colloidal gold detection technology to develop a rapid quantitative detection method for FB1. This will provide an accurate, fast, and convenient detection tool for FB1 content in cereals and feed, while offering a foundation for aptamer applications in agricultural product quality and safety.

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1. Materials and Methods

1.1 Materials and Equipment **Materials:** FB1, aflatoxin B1 (AFB1), T-2 toxin, and zearalenone (ZEN) were purchased from Qingdao Pribolab Biological Engineering Co., Ltd. Taq polymerase and mixed dNTPs were obtained from Beijing ComWin Biotech Co., Ltd. Streptavidin-coated magnetic beads were from Thermo Fisher Scientific (USA). The UNIQ-10 Oligonucleotide Purification Kit and denaturing electrophoresis dye were from Sangon Biotech (Shanghai) Co., Ltd. FB1-BSA conjugate was from Aladdin Reagent (Shanghai) Co., Ltd. AFB1-BSA and T-2-BSA conjugates were from Shanghai Hushi Pharmaceutical Technology Co., Ltd. and Shanghai Yaocao Biotechnology Co., Ltd., respectively. Chloroauric acid (HAuCl ·4H O) was from Sinopharm Chemical Reagent Co., Ltd. The ssDNA library and biotin-modified DNA probes were synthesized by Boshi Biotechnology Co., Ltd.

Equipment: Elga Ultra Bioscience ultrapure water system (UK), DK-8D thermostatic water bath (Shanghai Baixin Instrument Equipment Factory), Eppendorf AG tabletop microcentrifuge (Germany), STANLEY PCR cycler (USA), DYY-11B electrophoresis apparatus (Beijing Liuyi Biotechnology Co., Ltd.), Thermo UV-Vis spectrophotometer (USA), digital magnetic stirring heating mantle (Tianjin Labotery Instrument Equipment Co., Ltd.), SMartGel II gel imaging system (USA), Envision multimode fluorescence plate reader (USA), Implen ultramicro spectrophotometer (Germany), PerkinElmer automated microplate reader (Germany), Hitachi H-7650 transmission electron microscope (Japan), and WATERS 6000 high-performance liquid chromatograph (USA).

1.2 Methods

1.2.1 Immobilization of ssDNA Library Ten microliters of streptavidin-coated magnetic beads were washed three times with 2× Binding Buffer and resuspended in 500 L of 2× Binding Buffer. Twelve microliters of biotinylated aptamer complementary strand B (GTC/ISP 18/GATCGAGCCTCA) were added and incubated at room temperature for 1 hour. The beads were washed three times with 1× Binding Buffer and resuspended in 300 L phosphate-buffered

saline (PBS). The screening ssDNA library (ATACCAGCTTATTCAATT-N -TGAGGCTCGATC-N -AGATTGCACTTACTATCT) was dissolved in PBS buffer, denatured at 95°C for 8 minutes, rapidly cooled at 4°C for 10 minutes, and briefly equilibrated at room temperature. The treated ssDNA library was then added to the bead suspension and incubated overnight to immobilize the library on the magnetic beads.

1.2.2 Screening of FB1 Aptamers The immobilized library beads were washed multiple times with PBS, and the supernatant was removed by magnetic separation. One hundred microliters of 1 mg/mL FB1 solution was added to the beads, with PBS as the control group, and incubated at room temperature for 5 minutes. The supernatant was collected by magnetic separation as the candidate aptamer pool for Round 1. From Round 2 onward, counter-selection was performed: after washing the immobilized library beads, ZEN, AFB1, and T-2 solutions in PBS were added sequentially and incubated at room temperature for 10 minutes to remove sequences binding to non-target toxins. FB1 target solution was then added and incubated at room temperature for 5 minutes, and the supernatant was collected as the candidate pool for Round 2. This process was repeated for a total of 8 rounds. The screening scheme is summarized in .

1.2.3 PCR Amplification PCR primer information is provided in . The PCR reaction conditions were: 10 L Mix, 0.5 L ssDNA, 0.5 L primer RA (10 mol/L), 0.5 L primer FF (10 mol/L), and sterile distilled water to a final volume of 20 L. The thermal cycling program consisted of initial denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 51°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. Amplification products were verified by 2% agarose gel electrophoresis.

1.2.4 Recovery and Purification of Amplified Library PCR products were separated by 8% denaturing polyacrylamide gel electrophoresis, and fluorescent bands containing carboxyfluorescein (FAM) were excised. Gel pieces were crushed and centrifuged at 8,000 rpm for 3 minutes. The crushed gel was boiled for 15 minutes, centrifuged again at 8,000 rpm for 3 minutes, and the supernatant was collected. This process was repeated three times. Four milliliters of guanidine hydrochloride and 8 mL of absolute ethanol were added to the collection tube, boiled for 15 minutes, ice-bathed for 3 minutes, and centrifuged at 3,500 rpm for 10 minutes, followed by 30 minutes at 4°C. The UNIQ-10 Oligonucleotide Purification Kit was used with a peristaltic pump to purify ssDNA, which was stored at -20°C. Enriched sequences obtained by high-throughput sequencing were analyzed for homology using Mega 6.0 software.

1.2.5 Determination of Affinity and Specificity Affinity of candidate aptamer sequences was determined by ELISA. FB1-BSA antigen (5 g/mL) was coated onto fluorescence microplates and incubated at 4°C overnight. After washing three times with PBS, plates were blocked with 5% BSA at 37°C for 1

hour and washed again. FAM-modified aptamers at concentrations of 0–2 mol/L (0, 0.015625, 0.0625, 0.25, 1.0, and 2.0 mol/L) were added (90 L) and incubated at 37°C for 1 hour. After three PBS washes, fluorescence intensity was measured at 490 nm. SigmaPlot 5.0 software was used for non-linear curve fitting to calculate the dissociation constant (Kd) for each sequence. RNA Structure 3.0 was used for secondary structure prediction and binding site analysis.

For specificity determination, microplates were coated with 5 g/mL FB1-BSA, AFB1-BSA, ZEN-BSA, or T-2-BSA antigens at 4°C overnight. After PBS washing and BSA blocking, 90 L of 1 mol/L FAM-modified candidate aptamers were added and incubated at 37°C for 1 hour. Following three PBS washes, fluorescence intensity was measured as described above.

1.2.6 Preparation of Colloidal Gold Solution Colloidal gold solution was prepared by the sodium citrate reduction method. A 50 mmol/L chloroauric acid solution and a 194 mmol/L sodium citrate solution were prepared and stored at 4°C. In a three-neck round-bottom flask with a condenser, 98 mL of pure water and a stirring bar were added, followed by 2 mL of chloroauric acid solution. After heating to boiling, 2 mL of sodium citrate solution was added and boiling was maintained for 20 minutes before cooling to room temperature. The prepared gold nanoparticles were observed by transmission electron microscopy.

1.2.7 Determination of Minimum Aptamer Concentration for Gold Protection and Optimal pH To determine the optimal conditions, orthogonal experiments were performed by adding 0, 10, 20, 30, 40, or 50 L of 0.1 mol/L K CO to 1 mL colloidal gold solution to adjust pH, and using aptamer dilutions at 0, 0.125, 0.25, 0.5, 1.0, and 2.0 mol/L. The minimum aptamer concentration for gold protection and optimal pH were determined based on these results.

1.2.8 FB1 Detection Procedure Based on the method of Lu et al. with slight modifications: 50 L of optimal-concentration aptamer and 50 L of FB1 at various concentrations (0, 0.0625, 0.125, 0.25, 0.5, and 1.0 ng/mL) were incubated in a microplate at room temperature for 1 hour. Fifty microliters of colloidal gold solution were added and left to stand for 5 minutes, followed by addition of 5 L of 2 mol/L NaCl with thorough mixing. After 5 minutes of equilibration, absorption spectra from 450–750 nm were measured using an automated microplate reader.

1.2.9 Spiked Recovery Test Commercial corn samples were ground and tested for FB1 content. Different concentrations of FB1 standard (0, 50, 100, and 150 g/kg) were spiked into the samples, mixed thoroughly, and tested again. Sample preparation and extraction followed the method specified in "Determination of Fumonisins in Feed" (NY/T 1970-2010). Spiked recovery rates were calculated.

1.2.10 Sample Detection Five commercial corn samples with FB1 content between 10–100 g/kg were randomly selected and analyzed using both the developed method and the HPLC method specified in NY/T 1970-2010. The results were compared.

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2. Results

2.1 Optimization of Screening Conditions Non-specific amplification products can arise from nucleic acid libraries under inappropriate PCR conditions. This study optimized the annealing temperature for library amplification, with results shown in [Figure 1: see original paper]. While higher annealing temperatures reduce non-specific products, they also decrease specific product yield. Considering both amplification efficiency and non-specific amplification, the optimal annealing temperature was determined to be 61°C.

2.2 Monitoring of the Screening Process Eight rounds of screening were performed. PCR verification of library enrichment and recovery was conducted for Rounds 2, 5, and 8, with Round 8 results shown in [Figure 2: see original paper] and [Figure 3: see original paper]. A specific 118 bp band was amplified, and fluorescent FAM-labeled bands were obtained by denaturing PAGE, consistent with expected results.

2.3 Enrichment and Alignment of Homologous Sequences High-throughput sequencing was used to evaluate enrichment in each round, yielding 10,000 sequences. Homology alignment using Mega 6.0 software identified 10 highly similar sequences ([Figure 4: see original paper]), with F102, F98, and F92 showing relatively high enrichment levels ().

2.4 Determination of Affinity and Specificity Ten candidate aptamers were evaluated for affinity. lists the sequences and affinity test results of aptamers with measurable binding. [Figure 5: see original paper] shows non-linear fitting analysis for aptamers F102 and F98. Aptamer F102 exhibited a Kd value of (42.1±4.0) nmol/L for FB1. Specificity results for aptamers F102, F98, and F92 are shown in [Figure 6: see original paper]. The relative fluorescence intensity ratio represents the ability of mycotoxin molecules to compete for aptamer binding on the magnetic bead surface, causing conformational changes and release of the complementary fluorescent strand. Therefore, higher fluorescence intensity indicates stronger specificity. Fluorescence recovery rates for F92, F102, and F98 were 43.3%, 80.0%, and 36.6%, respectively, while non-target mycotoxins showed recovery rates around 30%, demonstrating good specificity, particularly for F102. This validates the success of the magnetic competitive SELEX approach using non-target molecules (AFB1, T-2, and ZEN).

2.5 Colloidal Gold Solution Preparation Transmission electron microscopy of the prepared colloidal gold solution revealed uniformly distributed particles with consistent size (~13 nm diameter), transparent wine-red color, and no impurities ([Figure 7: see original paper]), confirming suitability for assay development. Orthogonal experiments determined the minimum aptamer concentration for gold protection to be 0.25 mol/L and the optimal K CO addition to be 20 L, yielding a colloidal gold pH of approximately 7.0 with appropriate color and minimal aptamer consumption.

2.6 FB1 Detection by Aptamer [Figure 8: see original paper], [Figure 9: see original paper], and [Figure 10: see original paper] show absorbance values of colloidal gold at different aptamer and FB1 concentrations. Color changes induced by NaCl resulted in absorbance variations, confirming the aptamer' s utility for FB1 detection. The entire detection process required less than 2 hours, demonstrating simplicity and rapidity.

Specificity was tested using ZEN as an example ([Figure 11: see original paper]). At 0.500 mol/L aptamer concentration, no binding to ZEN was observed, and colloidal gold was fully protected. Absorbance values for ZEN at various concentrations matched those of the 0 mg/mL control, confirming excellent specificity. Similar results were obtained for AFB1 and T-2.

[Figure 12: see original paper] presents calibration curves for FB1 detection. At 520 nm, absorbance decreased with increasing FB1 concentration for both 0.250 and 0.500 mol/L aptamers. Both concentrations showed good linear relationships ($R^2 = 0.9585$ and 0.9875, respectively) across the range of 0.0625–1.0000 ng/mL, with a detection limit of 0.125 ng/mL. These results indicate that 0.250 mol/L aptamer is sufficient for FB1 detection.

2.7 Spiked Recovery Test Results Corn samples were tested for FB1 content, then spiked with FB1 standards at 50, 100, and 150 g/kg. Spiked recovery rates ranged from 97% to 121% ().

2.8 Sample Detection Results Five commercial corn samples were analyzed using both the developed method and HPLC (). Results showed good agreement between methods, with relative error less than 15.7%.

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3. Discussion

FB1 molecules are diester compounds composed of different polyhydric alcohols and tricarboxylic acids, lacking fixed active groups for immobilization. This necessitates conjugation through other active groups such as amino groups, which increases screening difficulty and may affect aptamer specificity. Therefore, this study employed a library-immobilized screening strategy to obtain highly

specific aptamers. Another major challenge was eliminating non-specific interference, which arises from two sources: non-specific adsorption of DNA to magnetic bead surfaces, and DNA sequences that recognize only partial FB1 structures (e.g., individual functional groups or regions). To address this, structurally similar compounds (AFB1, T-2, and ZEN) were introduced for counter-selection. Since AFB1 shares similar binding sites with FB1, this approach enhanced screening efficiency.

Mathematical analysis of SELEX suggests that, theoretically, aptamers with highest affinity should show best enrichment under ideal conditions without interference. However, actual screening results often show that most enriched sequences do not necessarily exhibit optimal affinity, as minor variations in experimental conditions such as pH and temperature can affect outcomes. Yu et al. mathematically analyzed SELEX enrichment levels, demonstrating that under certain conditions, the highest-affinity aptamer may not achieve the highest enrichment. In this study, aptamer F102 did not show the highest enrichment level but exhibited the best affinity, confirming this principle.

Aptamers can selectively bind target molecules with high affinity comparable to antibodies, while offering superior resistance to biodegradation. These properties make them ideal recognition elements for specific target detection. The FB1 aptamer obtained in this study showed a Kd of (42.1±4.0) nmol/L with excellent selectivity—binding to structurally similar compounds (ZEN, AFB1, and T-2) was significantly lower than to FB1. Compared with previously reported FB1 aptamers F39 and F10 [with Kd values of (100±30) nmol/L and (62±5) nmol/L, respectively], our aptamer demonstrated higher affinity. The established colloidal gold method used a lower aptamer concentration (0.250 mol/L), showed low cross-reactivity with other mycotoxins, and required shorter detection time, providing a solid foundation for practical FB1 detection applications. For comparison, Li et al. developed an ultra-high-performance liquid chromatography-tandem mass spectrometry method for FB1 in corn oil with a linear range of 5-200 ng/mL and detection limit of 0.27 ng/mL. Wang et al. developed a fluorescent microsphere-labeled monoclonal antibody immunochromatographic assay for FB1 in corn with a detection limit of 120 ng/L and cross-reactivity rates of 1.5% and 67.3% with fumonisin B2 and B3, respectively. Our method demonstrates superior specificity and performance compared to these approaches.

Through appropriate screening methodology and increased selection pressure, we obtained the high-affinity, high-specificity FB1 aptamer F102. By analyzing its secondary structure and proposed binding mechanism, we established and validated a colloidal gold detection method. This work not only provides a theoretical reference for rapid mycotoxin detection in agricultural product safety but also offers a foundation for developing antibody-alternative biosensors, with broad potential for aptamer applications in this field.

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4. Conclusion

This study successfully screened FB1 aptamers and established a rapid quantitative detection method for FB1 content in cereals and feedstuffs. The method demonstrates good accuracy, specificity, and practicality for field applications.

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Note: Figure translations are in progress. See original paper for figures.