

Engineered Nanobodies Against Aflatoxin B₁ and M₁: Expression, Optimization, and Robust Immunoassay Performance

Heng Zhao¹

Xinjiang Second Medical College

Zhenhua Fang²

Xinjiang Second Medical College

Ruijun Xu²

Xinjiang Second Medical College

Yang Liu³

liuyang_07@126.com

Xinjiang Second Medical College

Article

Keywords: Aflatoxin B₁, aflatoxin M₁, nanobodies, characterizations

Posted Date: July 8th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10218575/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Aflatoxin B₁ (AFB₁) and its hydroxylated metabolite, aflatoxin M₁ (AFM₁), are potent group 1 carcinogens that pose significant risks to food safety, necessitating sensitive and reliable detection methods. Here, we report the development of two novel nanobodies, AFB₁-Nb and AFM₁-Nb, as biorecognition elements for aflatoxin detection. The nanobodies were successfully expressed in *Escherichia coli* BL21(DE3) using pET-22b vectors, and their performance was systematically evaluated using an optimized indirect competitive enzyme-linked immunosorbent assay (icELISA). The assay demonstrated high sensitivity, with half-maximal inhibitory concentrations (IC₅₀) of 1.126 ng/mL for AFB₁ and 1.320 ng/mL for AFM₁, and excellent specificity with minimal cross-reactivity against other mycotoxins. Crucially, the nanobodies exhibited distinct but complementary environmental robustness: AFM₁-Nb retained over 90% binding activity after incubation at 90°C for 75 minutes and showed superior tolerance to organic solvents, while AFB₁-Nb performed optimally under neutral pH and moderate ionic strength. These findings establish a robust prokaryotic expression and purification system for high-affinity anti-aflatoxin nanobodies, and provide the comprehensive characterization of their stability under practical assay conditions. The exceptional thermal and solvent tolerance of AFM₁-Nb, in particular, underscores its potential as a reliable component for developing rapid, on-site diagnostic tools for monitoring aflatoxin contamination in complex food matrices.

Introduction

Mycotoxins are harmful secondary metabolites produced by certain fungi that can significantly taint cereal grains, animal feeds, and various food items throughout their cultivation, storage, and processing stages[1, 2]. These toxins pose serious health risks to both humans and animals, potentially resulting in fatal outcomes. Aflatoxins, a specific group of mycotoxins, are generated by species such as *Aspergillus flavus*, *Aspergillus parasiticus*, or *Aspergillus nominus*. Among them, AFB₁ is recognized as the most prevalent and hazardous type, while AFM₁ is notable for being the only mycotoxin with a defined maximum residue limit in dairy products. Both compounds can lead to severe health issues in humans[3, 4]. AFB₁ is particularly notorious, having been designated as a Group 1 carcinogen by the International Agency for Research on Cancer (IARC)[5, 6]. AFM₁, which is produced when AFB₁ is metabolized in animals, is also highly toxic, though slightly less so than AFB₁. In 2012, the IARC elevated AFM₁'s classification from Group 2 to Group 1 to enhance food safety and protect public health[7, 8]. Consequently, creating efficient, precise, and rapid detection techniques for AFB₁ and AFM₁ is crucial for ensuring food safety and managing associated risks.

Currently, the prevalent techniques for detecting mycotoxins encompass high-performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and enzyme-linked immunosorbent assay (ELISA)[8–10]. While HPLC and LC-MS/MS provide exceptional accuracy and sensitivity, they necessitate costly equipment, skilled personnel, intricate sample preparation, and lengthy analysis times, rendering them impractical for quick on-site testing and extensive sample evaluations. On the other hand, ELISA stands out due to its straightforward procedure, affordability,

efficiency, and specificity, making it one of the most favored rapid detection techniques for mycotoxins[11]. The effectiveness of ELISA hinges on the specific interaction between antigens and antibodies, meaning that the quality of antibodies significantly influences the method's detection capabilities. Traditional polyclonal and monoclonal antibodies face challenges in real-world applications, such as complicated production processes, high expenses, limited stability, and challenges in mass production[12, 13]. In contrast, nanobodies, which are single-domain antibodies sourced from the variable regions of heavy-chain antibodies in Camelidae, weigh only 15–17 kDa, significantly lighter than standard antibodies[14]. Nanobodies offer several advantages over traditional antibodies, including enhanced stability, high specificity and affinity, straightforward expression and purification, reduced immunogenicity, and the ability to target antigenic sites that conventional antibodies struggle to access[15–18]. These distinctive features make nanobodies a promising substitute for traditional antibodies in mycotoxin detection.

Due to their remarkable stability, specificity, and the ease of prokaryotic expression, nanobodies have emerged as a promising substitute for traditional antibodies in mycotoxin detection, leading to significant research into their use for quickly screening food contaminants[19]. Prior investigations have documented nanobody sequences that specifically target AFB₁ and AFM₁, which serve as essential biorecognition components for the immunoassays of these mycotoxins[16, 20–22]. Nonetheless, the majority of these studies have primarily concentrated on sequence identification and initial validation of binding capabilities, with a notable lack of comprehensive research on their expression efficiency within specific prokaryotic systems, optimization of soluble protein yields, and the establishment of standardized parameters for ELISA detection. Furthermore, critical performance indicators necessary for effective detection—such as thermal stability, tolerance to organic solvents, and adaptability to different pH levels and ionic strengths—have not been thoroughly characterized or quantitatively analyzed. These gaps in research hinder the direct application of the identified nanobody sequences as stable and reliable detection tools, thus restricting their broader use in industrial screening. Consequently, this study aims to enhance the recombinant expression conditions for AFB₁ and AFM₁ nanobodies, develop an effective purification method, and systematically evaluate their performance. The goal is to overcome the technical challenges that exist between nanobody sequences and their practical industrial applications, ultimately laying a robust experimental groundwork and standardized protocol for creating sensitive, stable, and cost-effective detection technologies for AFB₁ and AFM₁.

Materials and Methods

Chemicals and reagents

AFB₁, AFM₁, along with their conjugates to bovine serum albumin (AFB₁-BSA, AFM₁-BSA), were sourced from Wuhan Chundu Biotechnology Co., Ltd. The standard solutions for detecting cross-reactivity, including OTA and DON, were also acquired from the same company. Additionally, a mouse monoclonal antibody labeled with HRP against the HA tag was obtained from Wuhan Chundu Biotechnology Co., Ltd. Isopropyl-β-D-thiogalactopyranoside (IPTG), 3,3',5,5'-tetramethylbenzidine (TMB), and skim milk powder

were procured from Sangon Biotech in Shanghai. Other inorganic salts and organic solvents used were of analytical quality and readily available in the market.

Strains, Plasmids and Culture Media

The host strain utilized for the expression of recombinant proteins was *Escherichia coli* BL21 (DE3), obtained from Sangon Biotech in Shanghai. The recombinant plasmids pET-22b, which included nanobody (Nb) sequences (specifically pET-22b-AFB₁-Nb and pET-22b-AFM₁-Nb), were created and confirmed through DNA sequencing by GenScript Biotech Corporation in Nanjing. For the cultivation of bacteria and the induction of recombinant protein expression, Luria-Bertani (LB) medium and Terrific Broth (TB) medium, both containing 100 µg/mL of ampicillin, were employed.

Expression and Purification of Nanobodies

Recombinant plasmids were introduced into competent *E. coli* BL21 (DE3) cells using a heat shock method. Individual positive colonies were selected and grown in 5 mL of LB medium, with shaking overnight at 37°C. The overnight culture was then diluted into 100 mL of fresh LB medium at a 1:100 (v/v) ratio, and shaking continued at 37°C. Once the optical density at 600 nm (OD₆₀₀) reached between 0.6 and 0.8, IPTG was added to achieve a final concentration of 0.1 mM, and the culture was induced at 18°C for 16 to 20 hours.

Cells were collected through centrifugation at 8000 × g for 10 minutes at a temperature of 4°C. Following the removal of the supernatant, nanobodies located in the periplasmic region were isolated via osmotic shock. This involved resuspending the cells in a pre-chilled TES buffer, incubating them on ice for one hour, mixing with TES/4 buffer, and shaking on ice for an additional 45 minutes. The resulting supernatant was then centrifuged to yield the crude periplasmic extract. To purify this extract, a Ni-NTA affinity chromatography column from Sangon Biotech in Shanghai was utilized, and the target protein was eluted using a gradient of 20–300 mM imidazole. The purity and molecular weight of the isolated protein were assessed through SDS-PAGE, employing a 12% separating gel and a 5% stacking gel. The concentration of the protein was measured using a Bradford protein quantification kit, also sourced from Sangon Biotech, Shanghai.

Optimization of icELISA Detection Conditions

The ideal concentration for antigen coating and the dilution ratio for nanobodies were established using a checkerboard titration method. Specifically, AFB₁-BSA or AFM₁-BSA was prepared in carbonate buffer (pH 9.6) at concentrations ranging from 0.125 to 1.0 µg/mL and applied to 96-well microplates, followed by a 12 to 16-hour incubation at 4°C. Afterward, the plates were blocked with PBST buffer (PBS with 0.05% Tween-20) containing 5% skimmed milk powder at 37°C for 1 hour. Nanobodies were then added in serial dilutions from 1:50 to 1:400 and incubated at 37°C for an additional hour. The combination of

antigen and antibody that yielded an absorbance of about 1.0 at 450 nm (OD₄₅₀) was identified as the optimal condition for further experiments.

Sensitivity and Specificity Analysis

The sensitivity of the nanobodies was assessed under optimized icELISA conditions by creating a competitive inhibition curve. Various concentrations of AFB₁ or AFM₁ standards were combined with the nanobodies at their ideal dilution ratio and introduced to the pre-coated and blocked microplates for a competitive reaction. The half-maximal inhibitory concentration (IC₅₀) was determined through four-parameter logistic regression analysis using GraphPad Prism software, allowing for an evaluation of sensitivity based on the IC₅₀ value.

The assessment of specificity involved analyzing the cross-reactivity (CR) of the nanobodies with similar structures and unrelated toxins (AFM₁, AFB₁, OTA, DON). The equation used to calculate cross-reactivity is outlined below:

$$CR(\%) = \frac{IC_{50}(\text{target toxin})}{IC_{50}(\text{competitor})} \times 100\%$$

Stability and Tolerance Analysis

In order to assess the environmental resilience of nanobodies, their ability to bind to antigens was measured across different stress scenarios. The binding performance in standard conditions (PBS, pH 7.4) was established as 100%, and the remaining relative activity for each scenario was computed.

Thermal stability: Nanobodies were subjected to incubation at temperatures ranging from 25 to 95°C for a duration of 5 minutes, or at 90°C for periods between 0 and 75 minutes. Following the incubation, the samples were promptly cooled on ice and allowed to reach room temperature. The remaining binding activity was assessed using icELISA.

Tolerance to pH and ionic strength: Nanobodies were prepared in buffers ranging from pH 5.0 to 10.0, as well as in PBS buffer (pH 7.4) with NaCl concentrations between 5 and 100 mM. Following a 30-minute incubation at room temperature, the binding activity was assessed using icELISA.

Tolerance to organic solvents was assessed by measuring the antigen-binding capability of nanobodies in PBS buffer (pH 7.4) with varying concentrations of methanol, DMF, DMSO, acetone, and acetonitrile, ranging from 10% to 80% (v/v). The evaluation of organic solvent tolerance was based on the remaining relative activity.

Results

Construction and Purification of Nanobody Expression Vectors

The sequences of nanobodies AFM₁ and AFB₁ were effectively inserted into the *E. coli* expression vector pET-22b, leading to the creation of recombinant plasmids (pET-22b-AFM₁-Nb and pET-22b-AFB₁-Nb), which were then introduced into competent *E. coli* BL21 (DE3) cells. PCR analysis and DNA sequencing validated that the target genes were accurately integrated into the vector, free from mutations or frame shifts. To further confirm the successful construction of the recombinant plasmids, double digestion with the restriction enzymes *Mlu* I and *Hind* III was conducted, and the results were analyzed through agarose gel electrophoresis, as shown in Fig. 1. Sequence alignment confirmed a perfect match (100% homology) between the sequencing data and the original nanobody sequences. SDS-PAGE results indicated that the AFM₁ and AFB₁ nanobodies were effectively expressed in *E. coli* BL21 (DE3), with a prominent protein band appearing around 17 kDa in the induced samples, aligning with the expected molecular weight. No target bands were detected in the uninduced samples, supporting the induction's effectiveness.

Optimization of icELISA Conditions

In this study, a checkerboard titration approach was utilized to fine-tune the essential reaction parameters of the icELISA system for detecting AFM₁ and AFB₁. The primary evaluation metric was the OD₄₅₀ value from the positive control wells, with a target signal window established at 1.0. The coating antigens, AFM₁-BSA and AFB₁-BSA, were diluted in four increments (0.125 µg/mL, 0.25 µg/mL, 0.5 µg/mL, and 1.0 µg/mL) for application on 96-well microplates. Concurrently, the purified recombinant nanobodies were diluted at four different ratios (1:50, 1:100, 1:200, and 1:400) to thoroughly assess the compatibility between the antigen coating concentrations and antibody dilution ratios. The findings indicated that the ideal parameters for AFB₁ detection were a coating concentration of 0.5 µg/mL for AFB₁-BSA and a nanobody dilution of 1:50. Under these settings, the OD₄₅₀ value for the positive control wells was recorded at 1.051 ± 0.079 ($n = 3$), aligning closely with the desired signal window, which reflects optimal specific binding efficiency between the antigen and antibody. For AFM₁ detection, the best conditions were found to be an AFM₁-BSA coating concentration of 0.25 µg/mL and a nanobody dilution of 1:100, yielding an OD₄₅₀ value of 1.532 ± 0.106 ($n = 3$) for the toxin-free positive control wells. It is important to note that lowering the AFM₁-BSA coating concentration to 0.125 µg/mL resulted in a significant drop in signal intensity ($OD_{450} < 0.8$), leading to reduced detection sensitivity, thus disqualifying it as an optimal coating condition. These refined icELISA parameters lay a solid experimental groundwork for the future systematic characterization of the nanobodies.

Sensitivity of the Nanobodies

The sensitivity of AFB₁-Nb and AFM₁-Nb was assessed using an enhanced icELISA approach. Inhibition curves were generated through a series of toxin standard dilutions, followed by nonlinear regression analysis conducted with GraphPad Prism (see Fig. 2). The findings revealed that the half-maximal inhibitory concentration (IC₅₀) for AFB₁-Nb against AFB₁ was 1.126 ng/mL, while AFM₁-Nb exhibited an

IC₅₀ of 1.320 ng/mL against AFM₁, indicating a marginally higher sensitivity for AFB₁-Nb compared to AFM₁-Nb. The determination coefficients (R²) for all fitted curves varied between 0.9442 and 0.9859, reflecting a strong fit and high reliability of the data. Both nanobodies successfully enabled the quantitative detection of target toxins at the ng/mL level, fulfilling the criteria for detecting AFB₁ and AFM₁ residue limits in real food samples.

Specificity of Nanobodies

The specificity of AFB₁ and AFM₁ nanobodies was assessed through icELISA, focusing on their cross-reactivity with non-target mycotoxins such as OTA and DON, as well as their structural analogs, AFB₁ and AFM₁. Nonlinear regression analysis was employed to fit the competitive inhibition curves and determine the IC₅₀ values, which are crucial for understanding the specificity of these nanobodies (see Fig. 3). The AFB₁ nanobody exhibited an IC₅₀ of 1.126 ng/mL for its intended target, AFB₁, while showing IC₅₀ values of 17.45 ng/mL for AFM₁, 6.28 ng/mL for OTA, and 4.75 ng/mL for DON, indicating a strong affinity for AFB₁ and minimal cross-reactivity with other mycotoxins. In contrast, the AFM₁ nanobody demonstrated a binding affinity for its target AFM₁ with an IC₅₀ of 1.320 ng/mL, while its IC₅₀ values for AFB₁, DON, and OTA were 14.45 ng/mL, 6.75 ng/mL, and 52.28 ng/mL, respectively, also reflecting low cross-reactivity. All inhibition curves achieved high R² values exceeding 0.95 after nonlinear fitting, confirming the reliability of the results. In summary, both AFB₁ and AFM₁ nanobodies effectively target their respective mycotoxins with minimal interference from other tested compounds, showcasing their specificity.

Environmental Tolerance of Nanobodies

Thermal Stability

The thermal stability of AFB₁-Nb and AFM₁-Nb was assessed by measuring their binding activities after exposure to 90°C for varying lengths of time (0–75 min) and across a temperature range of 25°C to 95°C. In the time-course study at 90°C, AFM₁-Nb demonstrated superior stability, maintaining over 90% of its initial binding activity after 75 minutes (B/B₀ > 0.9). Conversely, AFB₁-Nb showed a notable decline in activity, retaining only around 50% after the same duration. During the temperature-gradient analysis, the OD₄₅₀ readings for AFM₁-Nb remained consistent between 1.1 and 1.2 across all temperatures tested. Interestingly, the OD₄₅₀ for AFB₁-Nb increased with temperature, likely due to temperature-induced conformational changes in the target antigen that improved antibody binding (Fig. 4). AFM₁-Nb exhibited remarkable thermal stability, making it suitable for high-temperature applications, whereas AFB₁-Nb needs further refinement to function effectively in elevated temperature settings.

pH Stability

The stability of pH for the two nanobodies was evaluated by analyzing their binding efficacy and responsiveness at pH levels of 5, 6, 7.4, 9, and 10. Inhibition curves were generated, and IC₅₀ values were determined using nonlinear regression techniques. AFB₁-Nb exhibited notable variations in activity across different pH levels, with peak performance at pH 7.4 (neutral, IC₅₀ = 1.693 ng/mL), followed by pH 9 (weakly alkaline, IC₅₀ = 2.527 ng/mL). Sensitivity showed moderate declines at pH 5 (IC₅₀ = 4.148

ng/mL) and pH 6 (IC_{50} = 5.528 ng/mL), while the least sensitivity was recorded at pH 10 (strongly alkaline, IC_{50} = 8.591 ng/mL). For AFM₁-Nb, the peak activity occurred at pH 9 (weakly alkaline, IC_{50} = 2.234 ng/mL), with moderate sensitivity at pH 5 (IC_{50} = 4.186 ng/mL) and reduced sensitivity at pH 6 (IC_{50} = 5.523 ng/mL) and pH 7.4 (IC_{50} = 5.260 ng/mL). The lowest stability was also noted at pH 10 (IC_{50} = 7.928 ng/mL) (Fig. 5). All inhibition curves demonstrated R^2 values exceeding 0.99, accompanied by narrow 95% confidence intervals for the IC_{50} values. The two nanobodies displayed distinct optimal pH preferences: AFB₁-Nb thrived in neutral conditions, whereas AFM₁-Nb was more effective in weakly alkaline settings. Strongly alkaline conditions significantly impaired both the stability and functionality of the nanobodies.

Ionic Strength Tolerance

The binding activity and stability of the two nanobodies were assessed in buffer solutions with varying ionic strengths (5, 10, 25, 50, 100 mM) to evaluate how ionic strength influences their performance. AFB₁-Nb demonstrated a clear preference for ionic strength, achieving optimal performance at a moderate level of 25 mM (IC_{50} = 1.045 ng/mL), followed by 10 mM (IC_{50} = 1.671 ng/mL). Sensitivity diminished at 5 mM (IC_{50} = 2.432 ng/mL) and 100 mM (IC_{50} = 2.185 ng/mL), with the lowest sensitivity recorded at 50 mM (IC_{50} = 2.784 ng/mL). In contrast, AFM₁-Nb showed enhanced activity at lower ionic strengths, peaking at 5 mM (IC_{50} = 4.439 ng/mL) and 10 mM (IC_{50} = 4.404 ng/mL). Its activity declined at 25 mM (IC_{50} = 4.750 ng/mL) and 50 mM (IC_{50} = 6.100 ng/mL), with the least sensitivity observed at high ionic strength of 100 mM (IC_{50} = 7.948 ng/mL) (Fig. 6). The optimal ionic strengths for the two nanobodies were notably distinct; AFB₁-Nb thrived at 25 mM, while AFM₁-Nb was best suited for 5–10 mM. Elevated ionic strengths of 50–100 mM adversely affected the activity of both nanobodies.

Organic Solvent Tolerance

The tolerance of AFB₁-Nb and AFM₁-Nb was assessed across different concentrations of solvents such as methanol, DMF, DMSO, acetone, and acetonitrile. In methanol, AFM₁-Nb maintained an OD_{450} value consistently between 1.2 and 1.4, indicating strong resistance to methanol. AFB₁-Nb's activity showed a minor decline at lower methanol levels but rebounded at concentrations exceeding 40%, sustaining an OD_{450} value of at least 0.6 even at 80%, which suggests it can adapt to high methanol levels. In the cases of DMF and DMSO, both nanobodies exhibited a continuous decrease in activity as the concentration increased, reaching an OD_{450} of 0.4 or lower at 80%. DMF had the most pronounced inhibitory effect, demonstrating that both nanobodies had limited tolerance to these solvents. In acetone, AFM₁-Nb's OD_{450} value remained stable between 0.8 and 1.0, reflecting good stability. AFB₁-Nb's activity significantly dropped at lower acetone concentrations but gradually improved after reaching 40%, maintaining an OD_{450} value of at least 0.6 at 80%, indicating some level of tolerance to acetone. In acetonitrile, AFM₁-Nb's OD_{450} value decreased gradually but stayed above 0.7 at 80%, showing good tolerance. AFB₁-Nb experienced a slight decline at lower acetonitrile concentrations before stabilizing, with an OD_{450} value between 0.6 and 0.7 at 80%, indicating moderate tolerance. Both nanobodies exhibited a similar pattern in their tolerance to organic solvents, with the highest tolerance observed in methanol and acetone, followed by acetonitrile, and the least in DMF and DMSO. Additionally, AFM₁-Nb demonstrated greater

stability than AFB₁-Nb across all solvents, while AFB₁-Nb showed a distinct recovery in activity at high concentrations of methanol and acetone.

Discussion

This research involved the successful development of prokaryotic expression systems for AFB₁-Nb and AFM₁-Nb, the creation of an enhanced icELISA system for detecting aflatoxins, and a thorough examination of the environmental adaptability of both nanobodies for the first time. In this paper, we explore the efficacy of these nanobodies and their potential use in ensuring food safety.

The enhanced icELISA platform utilizing two specific nanobodies successfully detected target toxins at the ng/mL concentration, achieving a half-maximal inhibitory concentration (IC₅₀) of 1.126 ng/mL for AFB₁-Nb against AFB₁ and 1.320 ng/mL for AFM₁-Nb against AFM₁. This level of sensitivity aligns with leading anti-aflatoxin nanobodies identified in recent studies. For instance, He et al developed a nanobody-based icELISA for AFB₁ that demonstrated an IC₅₀ of 1.08 ng/mL[21], which is comparable to the sensitivity of AFB₁-Nb found in this research; meanwhile, Lee et al reported an anti-AFB₁ nanobody with an IC₅₀ of 2.17 ng/mL[20], indicating that AFB₁-Nb shows slightly better sensitivity. Notably, both nanobodies displayed remarkable specificity for their targets, exhibiting minimal cross-reactivity (CR) with non-target mycotoxins such as ochratoxin A (OTA) and deoxynivalenol (DON), and only a slight cross-reactivity with structural analogs. Specifically, AFM₁-Nb showed a CR of just 9.13% towards the structural analog AFB₁, which is considerably lower than the 15%–20% CR observed in many previously documented anti-AFM₁ antibodies. This suggests that these two nanobodies can effectively mitigate interference from prevalent mycotoxins in complex food matrices, making them promising candidates for incorporation into systems designed for simultaneous detection of multiple toxins.

A key benefit of the nanobodies developed in this research is their exceptional adaptability to various environmental conditions, which is crucial for their reliable use in detecting complex food samples. The AFM₁-Nb demonstrated remarkable thermal stability, maintaining over 90% of its initial binding capacity after being heated at 90°C for 75 minutes, outperforming most traditional monoclonal antibodies and many previously studied nanobodies. In contrast, standard IgG antibodies typically experience a decline of over 80% in activity after just 10 minutes at 90°C, while earlier anti-AFM₁ nanobodies have generally shown stability only at temperatures below 60°C. The impressive heat resistance of AFM₁-Nb allows it to withstand the high-temperature pasteurization processes used in dairy and the elevated pre-treatment temperatures in food processing, preventing the inactivation of antibodies during sample preparation. Interestingly, the OD₄₅₀ value for AFB₁-Nb increased with higher incubation temperatures. This observation is attributed to the heat treatment being applied solely to the free nanobody, not the antigen coated on the microplate, indicating that it is not related to changes in antigen structure. We hypothesize that this effect may result from temperature-induced conformational changes in the complementarity-determining regions (CDRs) of AFB₁-Nb: at room temperature, some AFB₁-Nb exists as loosely associated homodimers, and moderate heating facilitates their conversion into active monomers, thus enhancing the effective concentration of antibodies for specific antigen interaction. Furthermore, the

stable framework region (FR) of the nanobody helps prevent thermal denaturation, while a slight rise in temperature diminishes non-specific hydrophobic interactions in the CDR region, leading to improved binding efficiency.

Regarding adaptability to pH and ionic strength, the two nanobodies demonstrated different optimal conditions for their reactions: AFB₁-Nb showed peak activity at neutral pH (7.4) and moderate ionic strength (25 mM), whereas AFM₁-Nb excelled in slightly alkaline conditions (pH 9) and low ionic strength (5–10 mM). This variation stems mainly from differences in the amino acid makeup and charge distribution within the CDR regions of the nanobodies. AFB₁-Nb's CDR region is richer in neutral polar amino acids, resulting in a more even charge distribution at neutral pH, which aids in forming hydrogen bonds with the antigen. On the other hand, AFM₁-Nb's CDR region contains a higher proportion of negatively charged residues, enhancing the electrostatic interactions with the antigen in weakly alkaline conditions. In tests for tolerance to organic solvents, both nanobodies exhibited the greatest resistance to methanol and acetone, which is particularly relevant since methanol is frequently used for extracting aflatoxins from food samples. Notably, AFB₁-Nb exhibited a distinctive recovery of activity at high concentrations of methanol and acetone: its binding activity slightly diminished at lower solvent concentrations but gradually improved when concentrations surpassed 40%. This suggests that lower organic solvent levels may slightly modify the conformation of AFB₁-Nb's CDR region, leading to decreased binding activity; conversely, higher concentrations may reduce non-specific binding to the microplate surface, enhancing the environment for antigen-antibody interactions and counteracting the partial denaturation caused by the solvent, resulting in restored binding activity. Among all organic solvent systems tested, AFM₁-Nb maintained more consistent binding activity compared to AFB₁-Nb, further supporting its potential use in complex sample preparation systems.

The two nanobodies examined in this research exhibit performance traits that align closely with the practical needs for detecting aflatoxins across various food types. AFB₁-Nb demonstrates exceptional sensitivity towards AFB₁, the predominant contaminant found in cereal grains like corn and wheat, and its ability to withstand high methanol concentrations makes it suitable for the organic solvent extraction process used in cereal sample preparation. On the other hand, AFM₁-Nb showcases remarkable thermal stability and resistance to organic solvents, effectively fulfilling the detection needs for AFM₁ in dairy products, particularly for quick assessments following high-temperature pasteurization. In contrast to traditional monoclonal antibodies, the nanobodies developed in this study offer benefits such as cost-effective large-scale prokaryotic production, enhanced stability, and improved specificity, which can significantly lower the expenses associated with aflatoxin detection reagents and enhance the reliability of detection methods in complex matrices.

This research has certain limitations that need to be explored in future studies. Firstly, the validation of the icELISA system's performance was conducted solely in a buffered environment, and its effectiveness in real food matrices, such as cereal grains and liquid milk, remains untested. Future investigations will include spike-and-recovery tests using actual samples, assess the matrix effects of various food types, and compare the findings with the national standard HPLC method to further validate the method's

accuracy and dependability in real-world applications. Secondly, the thermal stability of AFB₁-Nb is somewhat inadequate, and we plan to enhance its environmental resilience through protein engineering techniques, including site-directed mutagenesis and disulfide bond modifications in upcoming research. Additionally, we aim to create immunochromatographic test strips and fluorescence immunoassay techniques utilizing the two nanobodies for rapid on-site detection of aflatoxins in food products.

Conclusion

This research involved the development of prokaryotic expression systems for nanobodies targeting AFB₁ and AFM₁, resulting in effective soluble expression and high-purity isolation of both nanobodies in *E. coli* BL21 (DE3). Utilizing these nanobodies, we created an enhanced icELISA system that allows for precise and sensitive detection of AFB₁ and AFM₁, while also thoroughly assessing the environmental adaptability of both nanobodies.

The nanobodies identified in this research demonstrate exceptional specificity for their targets and strong stability in various environments. Notably, the AFM₁-Nb shows remarkable resistance to high temperatures and tolerance to organic solvents, making it a dependable component for creating rapid detection methods for aflatoxins. This study lays a strong experimental groundwork for converting aflatoxin-specific nanobodies from sequence data into functional detection instruments, offering significant potential for applications in food safety oversight and monitoring mycotoxin risks.

Declarations

Acknowledgements

We thank the central lab of Xinjiang Second medical college for providing all necessary equipment.

Author contributions statement

H.Z. designed the experiments and wrote the manuscript. Z.F. and R.X. conducted the experiments. Y.L. designed the experiments and provided experimental guidance. All authors reviewed the manuscript.

Funding

The work was supported by the School-level Innovation Team Program of Xinjiang Second Medical College under Grant Number KT202402, the Key Research and Development Program of Karamay of 2023, the Key Talent Program Project of Karamay of 2023, and General Program of Natural Science Foundation of Xinjiang Uygur Autonomous Region under Grant Number 2022D01A48.

Additional information

Accession codes

Not applicable.

Competing interests

The authors declare no competing interests.

References

1. Bennett, J. W., Klich, M. & Mycotoxins *Clin. Microbiol. Rev.* **16**, 497–516 (2003).
2. Vila-Lopez, M. V., Pallares, N., Ferrer, E. & Tolosa, J. Mycotoxin determination and occurrence in pseudo-cereals intended for food and feed: a review. *Toxins Basel* **15**, (2023).
3. Wu, H. et al. Lactoferrin alleviated AFB1-induced apoptosis in intestinal NCM 460 cells through the autophagy pathway. *Foods* **11**, (2021).
4. Li, J. et al. Probiotic-derived extracellular vesicles alleviate AFB1-induced intestinal injury by modulating the gut microbiota and AHR activation. *J. Nanobiotechnol.* **22**, 697 (2024).
5. Zhu, Y., Xu, Y. & Yang, Q. Antifungal properties and AFB(1) detoxification activity of a new strain of lactobacillus plantarum. *J. Hazard. Mater.* **414**, 125569 (2021).
6. IARC monographs on the evaluation of carcinogenic risks to humans. Ingested nitrate and nitrite, and cyanobacterial peptide toxins. *IARC Monogr. Eval Carcinog. Risks Hum.* **94**, v–vii (2010).
7. Ostry, V., Malir, F., Toman, J. & Grosse, Y. Mycotoxins as human carcinogens-the IARC monographs classification. *Mycotoxin Res.* **33**, 65–73 (2017).
8. Maggira, M., Ioannidou, M., Sakaridis, I. & Samouris, G. Determination of aflatoxin M1 in raw milk using an HPLC-FL method in comparison with commercial ELISA kits-application in raw milk samples from various regions of Greece. *Vet Sci* **8**, (2021).
9. Maphaisa, T. C. et al. Advances in mycotoxin detection techniques and the crucial role of reference material in ensuring food safety. A review. *Food Chem. Toxicol.* **200**, 115387 (2025).
10. Anfossi, L., Giovannoli, C. & Baggiani, C. Mycotoxin detection. *Curr. Opin. Biotechnol.* **37**, 120–126 (2016).
11. Alshannaq, A. & Yu, J. H. Occurrence, toxicity, and analysis of major mycotoxins in food. *Int J. Env Res. Public. Health* **14**, (2017).
12. Zhao, Y., He, B., Li, D., Gao, L. & Ren, W. Nanobody and CuS nanoflower-Au-based lateral flow immunoassay strip to enhance the detection of aflatoxin B(1). *Foods* **13**, (2024).
13. Li, H. et al. Time-resolved fluorescence immunochromatography assay (TRFICA) for aflatoxin: aiming at increasing strip method sensitivity. *Front. Microbiol.* **11**, 676 (2020).
14. Lecocq, Q. et al. Theranostics in immuno-oncology using nanobody derivatives. *Theranostics* **9**, 7772–7791 (2019).
15. Li, Y. et al. Detection of AFB(1) by immunochromatographic test strips based on double-probe signal amplification with nanobody and biotin-streptavidin system. *Foods* **13**, (2024).
16. Wang, T. et al. Determination of aspergillus pathogens in agricultural products by a specific nanobody-polyclonal antibody sandwich ELISA. *Sci. Rep.* **7**, 4348 (2017).

17. Meng, N. et al. Screening, expression and identification of nanobody against monkeypox virus A35R. *Int. J. Nanomed.* **18**, 7173–7181 (2023).
18. Sun, S. et al. Nanobody: A small antibody with big implications for tumor therapeutic strategy. *Int. J. Nanomed.* **16**, 2337–2356 (2021).
19. He, T. et al. Nanobody technology for mycotoxin detection in the field of food safety: current status and prospects. *Toxins Basel* **10**, (2018).
20. Lee, Y. C. et al. Development of anti-aflatoxin B1 nanobodies from a novel mutagenesis-derived synthetic library for traditional chinese medicine and foods safety testing. *J. Biol. Eng.* **17**, 30 (2023).
21. He, T. et al. Nanobody-based enzyme immunoassay for aflatoxin in agro-products with high tolerance to cosolvent methanol. *Anal. Chem.* **86** **17**, 8873–8880 (2014).
22. Hu, Z. Q. et al. An affinity improved single-chain antibody from phage display of a library derived from monoclonal antibodies detects fumonisins by immunoassay. *Anal. Chim. Acta.* **867**, 74–82 (2015).

Figures

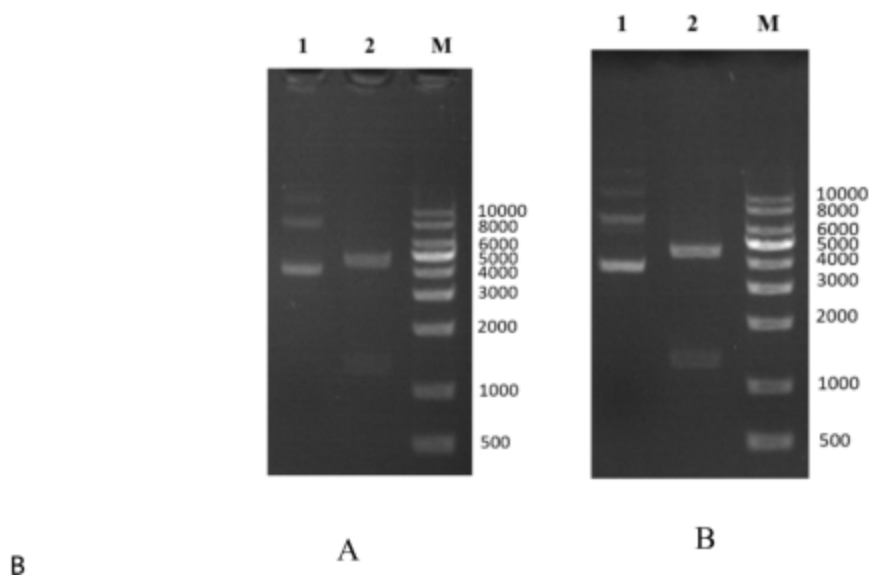


Figure 1

Analysis of recombinant plasmids by agarose gel electrophoresis

A pET-22b-AFM₁-Nb; B pET-22b-AFB₁-Nb M: DNA Ladder; Lane 1: Undigested pET-22b-AFM₁-Nb plasmid or pET-22b-AFB₁-Nb; Lane 2: pET-22b-AFM₁-Nb or pET-22b-AFB₁-Nb plasmid digested with *Mlu*I and *Hind*III.

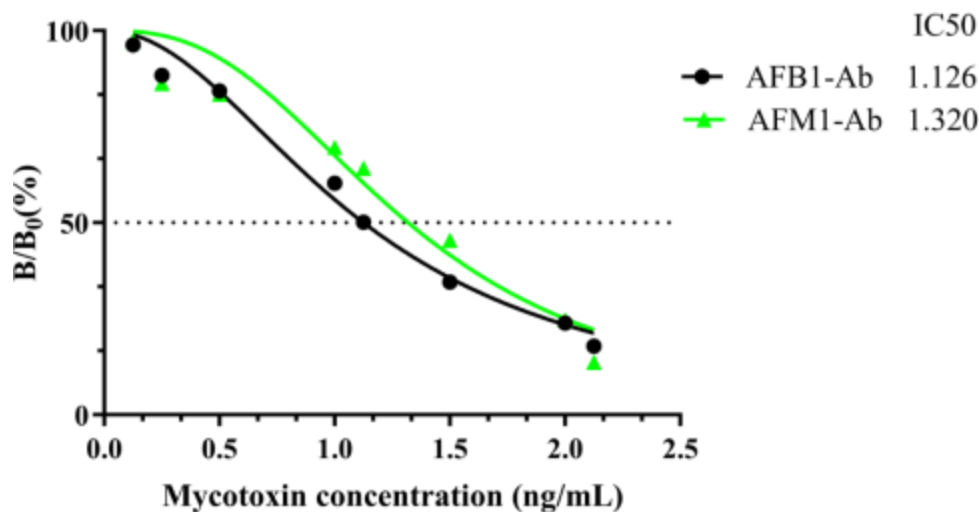


Figure 2

Competitive inhibition curves of AFB₁-Nb and AFM₁-Nb for their target mycotoxins.

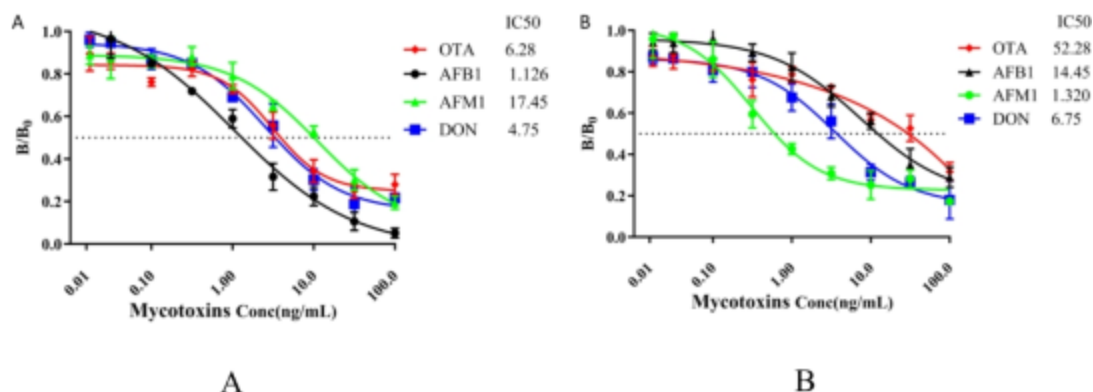


Figure 3

Specificity analysis of AFB₁-Nb and AFM₁-Nb against different mycotoxins. (A AFM1 nanobody B AFB1 nanobody)

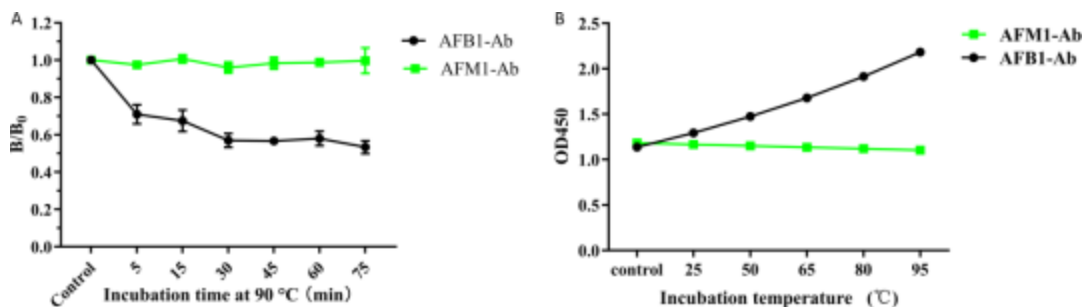


Figure 4

Thermostability analysis of AFB₁-Nb and AFM₁-Nb. (A) Residual binding activity (B/B₀, %) after incubation at 90 °C for different durations. (B) Binding activity (OD₄₅₀) after incubation at different temperatures.

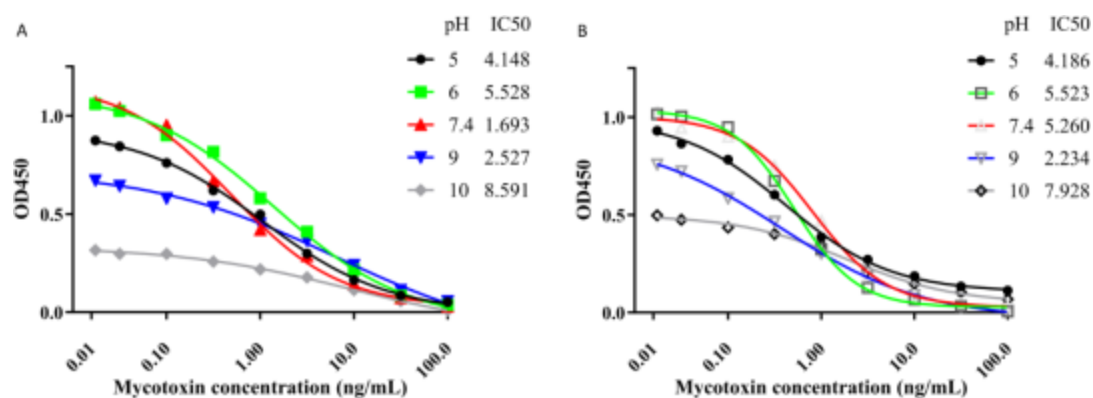


Figure 5

pH stability analysis of AFB₁-Nb and AFM₁-Nb. (A) pH stability of AFB₁-Nb , (B) pH stability of AFM₁-Nb

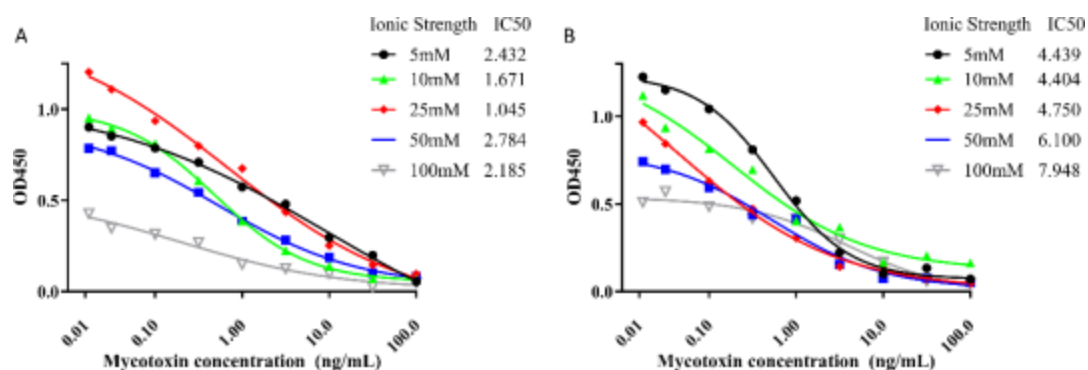


Figure 6

Ionic strength stability analysis of AFB₁-Nb and AFM₁-Nb. (A) Ionic strength stability of AFB₁-Nb ,(B) Ionic strength stability of AFM₁-Nb

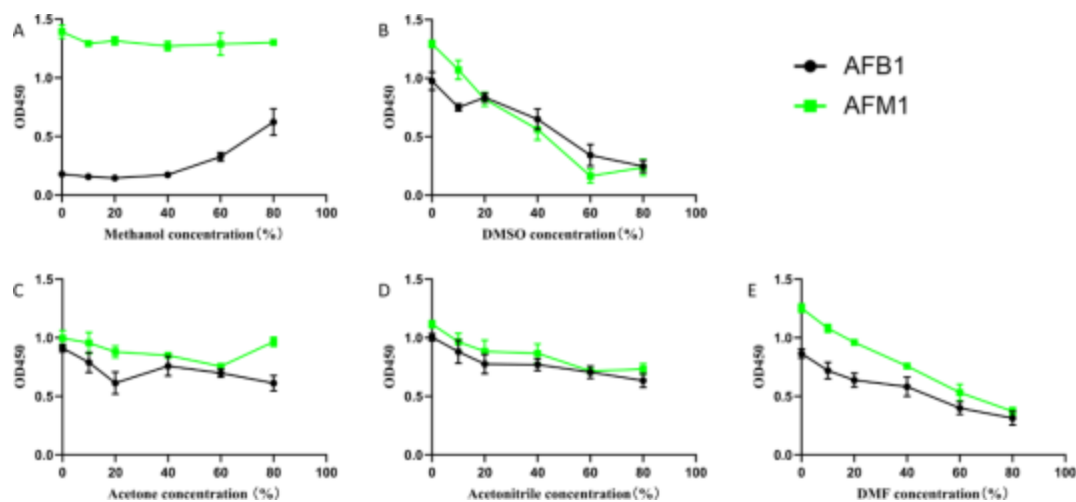


Figure 7

Organic solvent tolerance analysis of AFB₁-Nb and AFM₁-Nb.

(A) Binding activity (OD_{450}) in the presence of different methanol concentrations.(B) Binding activity (OD_{450}) in the presence of different DMSO concentrations.(C) Binding activity (OD_{450}) in the presence of different acetone concentrations.(D) Binding activity (OD_{450}) in the presence of different acetonitrile concentrations.(E) Binding activity (OD_{450}) in the presence of different DMF concentrations.