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Development of a nanobody-alkaline phosphatase fusion protein and its application in a highly sensitive direct competitive fluorescence enzyme immunoassay for detection of ochratoxin A in cereal

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Abstract

A rapid and sensitive direct competitive fluorescence enzyme immunoassay (dc-FEIA) for ochratoxin A (OTA) based on a nanobody (Nb)-alkaline phosphatase (AP) fusion protein was developed. The VHH gene of Nb28 was subcloned into the expression vector pecan45 containing the AP double mutant gene. The Nb28-AP construct was transformed into *E. coli* BL21(DE3)plysS and soluble expression in bacteria was confirmed by SDS-PAGE and Western blot. Both the Nb properties and AP enzymatic activity were validated by colorimetric and fluorometric analysis. The 50% inhibitory concentration and the detection limit of the dc-FEIA were 0.13 ng/mL and 0.04 ng/mL, respectively, with a linear range of 0.06–0.43 ng/mL. This assay was compared with LC-MS/MS, and the results indicated the reliability of Nb-AP fusion protein-based dc-FEIA for monitoring OTA contamination in cereal.

Keywords

Nanobody; ochratoxin A; alkaline phosphatase; fusion protein; fluorescence enzyme immunoassay

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Supporting Information Available

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Ochratoxin A (OTA) is a secondary metabolite primarily produced by *Aspergillus* and *Pencillium* species,[1, 2] which can contaminate cereal and cereal products around the world[3–6]. Many researches have revealed the diverse toxicities of OTA, including teratogenic, mutagenic, carcinogenic, hepatotoxic, immunosuppressive, and nephrotoxic effects.[7–9] In 1993, the International Agency for Research on Cancer (IARC) classified OTA in group 2B as a possible human carcinogen.[10] To regulate the content of OTA in food products, maximum limits of OTA have been set in cereals and cereal products at 5 μg/kg and 3 μg/kg in the European Union (EU), respectively.[11]

In order to minimize the risks of OTA exposure to consumers, many studies have been performed to develop methods for detection of OTA in cereal and cereal products, including gas chromatography, high-performance liquid chromatography, and immunoassays.[12–15] The instrumental methods are sensitive and specific, but they are laborious, expensive, and time-consuming, which are not suitable for routine analysis of large numbers of samples. In contrast, immunoassays have a unique ability to routinely handle a large number of samples and do not require time-consuming procedures and sophisticated equipment. They also lend themselves to point of use formats for rapid feedback of analytical data.

Most of the previously reported immunoassays for OTA are based on a monoclonal antibody or a polyclonal antibody, and are carried out with primary or secondary antibodies which are chemically labeled with enzymes, such as horseradish peroxidase (HRP).[16–18] However, it has been reported that the chemical conjugation of enzymes to antibodies may result in the unstable and randomly cross-linked molecules.[19,20] With the rapid development of antibody engineering and molecular cloning techniques, construction of single chain fragment of variable antibody region (scFv)-alkaline phosphatase (AP) fusions is considered an attractive alternative for simple and rapid immunoassay analysis, which can avoid the chemical conjugation of enzymes to antibodies and the use of a second antibody. It has been confirmed that the bivalent nature of AP contributes to the increased binding affinity of scFv-AP fusions to target antigens while retaining enzymatic activity.[21,22] Many studies on the detection of small molecular weight compounds using scFv-AP fusions have been reported, such as ractopamine[23] and *O,O*-diethyl organophosphorus pesticides[24]. However, scFvs are often observed to have poor solubility and stability which require complicated genetic engineering to improve.[25,26] As an alternative to conventional antibodies, variable domain of heavy chain antibodies (VHHs), also known as nanobodies (Nbs), that are derived from the variable region of heavy chain antibody existing in camelids and sharks,[27] is more attractive than scFv. Compared to scFv, Nbs have a smaller molecular weight of 15 kDa, high solubility and high expression in microbial systems.[28] Furthermore, it has been demonstrated that Nbs can maintain their functional binding affinity and specificity by refolding to their native structure after heat or chemical denaturation.[29,30] It has been reported that the fusion of AP to Nbs can broaden the application of this powerful immunodiagnostic reagent, such as enhancing the binding affinity compared to unfused Nbs.[31] Recently, a study on the AP activity of scFv-AP fusion showed that the fluorometric assay allowed for more sensitive detection than the chemiluminescence assay, revealing the potential of AP fusions in developing a highly sensitive fluorescence enzyme

immunoassay.[32] Up till now, there are few reports on the application of Nb-AP fusions in detection of toxic small molecular weight compounds.[28,31]

In our previous work, we reported on the panning of binders specific for OTA from an immunized alpaca VHH-derived Nb library, and sixteen binders with four different amino acid sequences were selected.[33] In the research described here, the binder VHH-28 which showed the highest sensitivity in a phage ELISA was used to construct the Nb-AP. The Nb gene of phage isolate VHH-28 was cloned into the expression vector pecan45 which contained the AP gene to produce the Nb28-AP fusion protein. After the characterization by SDS-PAGE and Western blotting, the purified fusion protein was used to develop a rapid, simple, and sensitive direct competitive fluorescence enzyme immunoassay for detection of OTA in cereal.

MATERIALS AND METHODS

Chemicals and reagents

T4 DNA ligase and restriction enzyme *Sfi* I were purchased from New England Biolabs, Inc. (Beverly, MA, USA). PfuTurbo Cx Hotstart DNA Polymerase was from Agilent Technologies Inc. (Santa Clara, CA, USA). Standards (ochratoxin A, aflatoxin B$_1$, zearalenone, deoxynivalenol), isopropyl-β-D-1-thiogalactopyranoside (IPTG), and p-nitrophenyl phosphate (pNPP) substrate were from Sigma-Aldrich (St. Louis, MO, USA). Standard ochratoxin B was from Bioaustralis (Smithfield, NSW, AUS). AttoPhos AP fluorescent substrate system was purchased from Promega (Madison, WI, USA). Chemically competent cells of *E. coli* TOP10F′ strain and *E. coli* BL21(DE3)plysS strain, B-PER bacterial protein extraction reagent, HisPur Ni-NTA resin, NuPAGE 12% Bis-Tris gel, and SYPRO Ruby protein gel stain were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Primers AP-F and AP-R (Table S-1 in Supporting Information [SI]) were purchased from Integrated DNA Technologies (Coralville, IA, USA). BCIP/NBT phosphatase substrate (1-component) was from KPL Inc. (Gaithersburg, MD, USA). The vector pecan45 containing an AP double mutant gene was a generous gift from Dr. Jinny L. Liu and Dr. Ellen R. Goldman (Naval Research Laboratory, Center for Bio/Molecular Science and Engineering, Washington, DC, USA).

Construction of the recombinant plasmid pecan45-Nb28-AP

The recombinant plasmid encoding the Nb-AP fusion protein pecan45-Nb28-AP was constructed as shown in Figure 1. Briefly, primers AP-F and AP-R were used to amplify the Nb gene and add two Sfi I restriction enzyme sites flanking the 5′ and 3′ termini of the VHH coding sequence from the plasmid pHEN1-VHH28. The VHH gene PCR products were purified with QIAquick PCR Purification Kit (Chatsworth, CA, USA) and digested with *Sfi* I restriction enzyme. The purified VHH fragment was then ligated into a similarly digested vector pecan45 containing the AP gene at a 3:1 molar ratio using T4 DNA Ligase, followed by transforming the ligation products into the chemically competent cells of the *E. coli* TOP10F′ strain by heat shock (42 °C, 90 s). The transformed bacteria were spread on Luria-Bertani (LB)-agar plates containing 50 μg/mL carbenicillin and positive clones were picked for plasmid extraction and DNA sequencing.

Expression and identification of Nb-AP fusion proteins

The recombinant plasmid was transformed into *E. coli* BL21(DE3)plysS cells, and the colony was cultured in LB medium with 50 μg/mL carbenicillin at 37 °C until the OD_{600} reached approximately 0.6–1.0. The culture was then induced with 0.5 mM IPTG at 30 °C by shaking at 250 rpm overnight. The bacteria cells were collected by centrifugation at 5000 × *g* for 10 min and the fusion protein was extracted by the B-PER method according to the manufacturer's instruction. In brief, 4 mL of B-PER reagent containing DNase I and EDTA-free protease inhibitors were added into 1 g of cell pellet. The cells were suspended by pipetting up and down, followed by incubation at room temperature for 10 min. Then the suspension was centrifuged at 15000 × *g* for 5 min to separate soluble Nb-AP fusion protein from the insoluble proteins. The presence of Nb-AP fusion protein was determined by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot with the single component BCIP/NBT phosphatase substrate.

Purification of the anti-OTA Nb-AP fusion protein

The extracted Nb-AP fusion protein which contains a 6×Histidine tag was filtered through a 0.22 μm sterile filter (Millipore, USA), followed by loading onto a high-capacity nickel-IMAC resin column for purification. After washing with six resin-bed volumes of wash buffer (10 mM PBS containing 25 mM imidazole, pH 7.4), the Nb-AP fusion protein was eluted with 6 mL of elution buffer (10 mM PBS containing 100 mM imidazole, pH 7.4). After dialyzing with PBS (10 mM, pH 7.4) at 4 °C for 48 h, the purified Nb-AP fusion protein was stored at −20 °C until use, and the purity of Nb-AP fusion protein was evaluated by SDS-PAGE[15].

Measurement of the AP activity of the Nb-AP fusion protein

Colorimetric analysis—Serially diluted Nb-AP fusion protein (50 μL) was added into a 96-well microplate, followed by adding 100 μL of AP buffer (pH 10.4) containing 3.8 μM, 0.1 M glycine, 50 mM $MgCl_2$ and 50 mM $ZnCl_2$. The plate was mixed and incubated at 37 °C for 30 min. The reaction was stopped with 50 μL/well of 4 M NaOH. The well absorbance at 405 nm was measured on a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

Fluorometric analysis—The AttoPhos AP fluorescent substrate (pH 10) that contains 1 μM 2´-(2-benzothiazoyl)-6´-hydroxybenzothiazole phosphate (BBTP), 2.4 M diethanolamine (DEA), 57 μM $MgCl_2$, and 0.005% NaN_3 (100 μL) was added into a black opaque 96-well microplate, in which a serial dilution of Nb-AP fusion protein (50 μL) had been placed. The plate was mixed, followed by incubation at room temperature for 15 min. Then the fluorescence was measured on a microtiter plate reader (Molecular Devices, Sunnyvale, CA), in which the fluorescence excitation spectrum (435 nm) was collected with an emission wavelength of 575 nm.

Fluorescence enzyme immunoassay for OTA based on Nb-AP fusion protein

For this assay, a black opaque 96-well microplate was incubated with 100 μL/well of 0.5 μg/mL OTA-ovalbumin (OVA) conjugate[33] in PBS (5 mM, pH 7.4) at 4 °C overnight. After

blocking with 3% (w/v) skimmed milk powder in PBS (300 μL/well) at 37 °C for 1 h, the plate was washed 3 times with PBST (PBS, pH 7.4, 10 mM, containing 0.05% Tween-20). Subsequently, 50 μL/well of Nb-AP fusion protein diluted in PBS was added and incubated with 50 μL/well of serial concentrations of OTA standards (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1 ng/mL in 5% methanol-PBS) at 37 °C for 30 min. After five washings with PBST, the plate was incubated with 100 μL/well of the AttoPhos AP fluorescent substrate at room temperature for 15 min. The fluorescent signal was measured as described above and standard curves were established by plotting the value of F/F_0 (%) against the OTA concentration, where F is the fluorescence intensity in the presence of OTA or OTA-related analytes and F_0 is the fluorescence intensity in their absence.

Sample preparation for dc-FEIA

Rice, oats and barley samples used for the spiking and recovery study were collected from local markets in Davis, California. Naturally contaminated cereal samples were obtained from different markets and supermarkets in the US. Sample preparation and extraction were performed as described by Wang.[18] Briefly, 5 g of finely ground sample were weighed and spiked with 1, 2.5, 5, and 10 μg/kg OTA. The spiked samples were mixed with 10 mL of 50% methanol in water (v/v) and subjected to ultrasonic extraction for 20 min. The mixture was centrifuged ($10000 \times g$) at 4 °C for 10 min, and the supernatant was diluted 1 in 9 with PBS for FEIA analysis.

LC-MS/MS analysis of OTA

Chromatographic analysis was performed using an Acquity UPLC™ system (Waters Corp., Milford, MA) equipped with a binary solvent pump, an autosampler and column heater. The separation was carried out on a UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm, 1.7 μm particle size, Kinetex, Phenomenex). The temperature of the sample plate was kept at 4 °C and 10 μL samples were injected on the column. The temperature of the column heater was maintained at 40 °C. Aqueous phase A was deionized water containing 0.1% formic acid as a modifier. Organic phase B was methanol containing 0.1% formic acid as a modifier. Starting gradient conditions were 10% B at 0.25 mL/min flow rate. The following gradient program was used: 0–1 min 10% B; 1–5 min 100% B; 5–8 min 100% B; 8–8.5 min 90% B; 8.5–10 min 90% B.

The Quattro Premier tandem mass spectrometer (Waters) was operated in positive electrospray (ESI) mode under the following conditions: 25 L/h cone gas flow, 750 L/h desolvation gas flow, 120 °C source temperature, 350 °C desolvation temperature and 3.00 kV capillary voltage. Argon was used as a collision gas at 2.2×10^{-3} mbar and flows 0.16 mL/min. Precursor and corresponding product ions for the multiple reaction monitoring (MRM) detection are shown in Table S-2. The data were acquired using Masslynx 4.1 software with Instrument and data processing was performed using MassLynx 4.1 with TargetLynx.

The extraction procedure was performed as described by Soleimany.[34] Briefly, a ground cereal sample (5 g) was mixed with 20 mL of solution 1 (acetonitrile/water/acetic acid, 79:20:1) by shaking 1 h on an orbital shaker. The mixture was centrifuged at 10000 rpm for

10 min, and 0.5 mL of the supernatant was diluted with the same volume of solution 2 (acetonitrile/water/acetic acid, 20:79:1), followed by passing through a 0.22 μm filter before LC-MS/MS analysis.

RESULTS AND DISCUSSION

Expression, purification and characterization of the Nb-AP fusion protein

The positive recombinant plasmid confirmed by colony PCR (Figure S-1 in SI) and DNA sequencing was transformed into *E. coli* BL21(DE3)plysS cells. One colony was picked and cultured in LB medium with carbenicillin at 37 °C until the OD_{600} reached 0.8. The expression of the Nb-AP fusion protein was induced by 0.5 mM IPTG at 30 °C for 12 h. The periplasmic protein was extracted by the B-PER method and characterized by SDS-PAGE (Figure 2A) and Western blot (Figure 2B). A band of approximately 65 kDa was detected from the induced cell culture while this band was absent in the non-induced cell culture. The Western blot results indicated that the Nb-AP fusion protein was present as a single 65 kDa band. No degradation products were shown and no endogenous AP was detected, indicating the stability of the Nb-AP fusion protein.

AP enzyme activity and anti-OTA reactivity of Nb-AP fusion protein

The AP enzyme activity of Nb-AP fusion protein was evaluated using colorimetric and fluorometric analysis, respectively. As shown in Figure 3A, signal intensity decreased as the amount of Nb-AP fusion protein decreased, and the lowest amount of Nb-AP fusion detected by fluorometric analysis was approximately 20 times lower than the Nb-AP detected by colorimetric analysis, indicating the higher sensitivity of fluorometric analysis. In order to evaluate the anti-OTA reactivity of Nb-AP fusion protein, one-step direct competitive ELISA was carried out. Briefly, 50 μL of serial concentrations of OTA standards (0, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25 ng/mL in 5% methanol-PBS) were added into a 96-well microplate that had been coated with 2 μg/mL of OTA-OVA conjugate, followed by addition of an equal volume of Nb-AP fusion protein (0.5 μg/mL in PBS) and was mixed. The plate was incubated at 37 °C for 30 min and washed five times with PBST. Subsequently, pNPP substrate (100 μL) was added and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 4 M NaOH (50 μL), and the absorbance of wells at 405 nm was measured. As shown in Figure 3B, a standard competitive inhibition curve using the Nb-AP fusion protein was established by plotting B/B_0 (%) against logarithm of OTA concentrations with Origin version 8.5 (OriginLab Corp., Northampton, MA), where B is the absorbance at 405 nm in the presence of OTA or OTA-related analytes and B_0 is the absorbance at 405 nm in their absence. It is obvious that the binding between the Nb-AP fusion protein and OTA-OVA conjugate can be inhibited by free OTA (Figure 3B), showing the good anti-OTA reactivity of Nb-AP fusion protein.

FEIA for OTA using Nb-AP fusion protein as the probe

The performance of the purified Nb-AP fusion protein was tested by direct competitive FEIA with two parameters of IC_{50} and the ratio of the maximum relative fluorescence unit (RFU_{max}) to IC_{50} (RFU_{max}/IC_{50}). To monitor the low concentrations of OTA, a highly sensitive detection scheme is the required with optimization of the concentration of coating

antigen and antibody, ionic strength, pH value, and organic solvent. The working concentration of OTA-OVA conjugate (0.5 μg/mL) and Nb-AP fusion protein (0.02 μg/mL) were first determined by a checkerboard titration. Methanol is a commonly used solvent in OTA analysis, since OTA is a highly lipophilic analyte. It has been reported that the interaction between antigen and antibody can be influenced by methanol[35], so we evaluated the effect of different concentrations of methanol on the assay (Figure 4A). Both the RLU_{max} and the IC_{50} increased as the concentration of methanol increased, indicating the methanol can influence the activity of Nb-AP fusion. The lowest IC_{50} and the highest RLU_{max}/IC_{50} was observed at 2.5% methanol-PBS, so the assay buffer containing a final concentration of 2.5% methanol was chosen for further optimization. It has been reported that both ionic strength and pH can influence immunoreactions.[15] Different concentrations of PBS (5, 10, 25, 50 mM) were tested (Figure 4B), and it was obvious that the fluorescence intensity decreased greatly as the ionic strength increased. The lowest IC_{50} and the highest RFU_{max}/IC_{50} was observed at 5 mM PBS. The influence of the assay buffer with different pH on the FEIA was evaluated from 4.0 to 11.0 (Figure 4C). The FEIA was more likely to be influenced at low pH (6.0), since negligible RFU_{max} was detected at pH 4.0 and 5.0 (32 and 31, respectively) and a high IC_{50} (4.3 ng/mL) was shown at pH 6.0, which may be due to the denaturation of Nb-AP fusion protein caused by protein protonation at low pH.[36] The RFUmax decreased as the pH increased from 7.4 to 11.0, while the IC_{50} varied in a narrow range of 0.2–0.6 ng/mL, indicating the feasibility of this assay to be performed at a pH ranging from 7.4 to 11.0. Taking account of the IC_{50} and RFU_{max}/IC_{50}, the best performance was obtained at pH 7.4. A direct competitive Nb-AP fusion protein-based FEIA standard curve was established using the optimal conditions (Figure 5). The standard curve exhibited a good limit of detection of 0.04 ng/mL and a linear correlation coefficient of 0.998. The assay has a linear range (IC_{20}-IC_{80}) of 0.06–0.43 ng/mL and an IC_{50} of 0.13 ng/mL.

Cross reactivity

To determine the selectivity of the assay, cross-reactivity (CR) of the Nb-AP fusion protein with OTA analog (OTB) and some other mycotoxins (deoxynivalenol, zearalenone, aflatoxin B_1) which are commonly detected in contaminated cereal samples was evaluated by dc-FEIA. The cross-reactivity was calculated as: CR (%) = [IC_{50} (OTA)/IC_{50} (tested analytes)] × 100. As shown in Table 1, negligible CR was observed with any analyte, showing the good selectivity of Nb-AP fusion protein in dc-FEIA for OTA. Interestingly, in our previous work, a low CR(%) of 3.5% was detected with OTB for phage clone VHH28 in VHH phage-based competitive real-time immuno-PCR.[33] Since the AP has a larger size (50 KDa) than Nb (15 KDa), it can be inferred that the steric hindrance between Nb and fused AP may partly contribute to no CR with OTB for Nb-AP fusion protein in dc-FEIA.

Matrix effect

Matrix effect is a very common challenge of immunoassay for food analysis, which can cause false positive or negative results, lower sensitivity and reduce the specificity of the assay.[37] The matrix effect can be reduced in a number of ways, such as dilution of sample extract or removal of interferences by sample cleanup procedures using solid-phase extraction. Dilution is a common way to reduce the matrix effect, but it can cause lower

sensitivity with a high dilution. Three sample matrixes (rice, oats, barley) were selected to evaluate the matrix effect, and each sample was confirmed to be free of OTA contamination by LC-MS/MS (LOD = 0.01 ng/mL). Sample extracts were diluted 10, 20, and 40 times and were used to prepare serial concentrations of OTA standards containing 5% methanol for the dc-FEIA. As shown in Figure 6, no significant reduction of maximum fluorescent intensity was observed, and the sensitivity was not affected, indicating the Nb-AP fusion protein was resistant to matrix effects. Taking the dilution into account, the final sensitivity of the FEIA was 0.8 μg/kg, with a linear range of 1.2–8.6 μg/kg, which is suitable for monitoring OTA contamination under current regulatory limits for OTA (5μg/kg) in the EU. Higher sensitivity could be obtained by improved clean up methods or additional assay optimization.

Validation study

To evaluate the effectiveness of the assay for OTA analysis, the Nb-AP fusion protein-based FEIA was performed to detect OTA in the spiked cereal samples. Before the spiking and recovery study, all samples were confirmed to be free of OTA contamination by LC-MS/MS. Cereal samples spiked with four different concentrations of OTA (1, 2.5, 5, 10 μg/kg) were prepared for intra- and inter-assay precision analysis. As shown in Table 2, the average recoveries for intra-assay ranged from 72% to 121%, with the coefficient of variation ranging from 0.02 to 0.13. Meanwhile, the results for the inter-assay ranged from 73% to 109% and 0.05 to 0.09, respectively. In addition, eight breakfaset cereal samples naturally contaminated OTA were analyzed. These samples were made from rice, oats, or barley, and previously tested for OTA by HPLC with fluorescence detection.[3] In this work, samples were analyzed in a blind fashion with dc-FEIA and LC-MS/MS. As shown in Table 3, results obtained from the two methods were in good agreement with each other. The content of OTA in two oats samples (1 and 4) were beyond the maximum limit for OTA in cereal (5 μg/kg). These results indicate the acceptable accuracy and precision of the developed Nb-AP fusion protein-based dc-FEIA for OTA detection in cereal samples.

CONCLUSIONS

Thus we have developed a sensitive and selective immunoassay for ochratoxin A in a cereal matrix. Since these assays can be performed in a parallel rather than sequential fashion, they complement chromatographic assays in being able to handle large numbers of samples over a short period of time. The immunoassays offer a second advantage in allowing development of portable methods that can be run where the samples are collected and the data needed. From a technological standpoint the work illustrates the value of nanobodies (Nb, VHH) in small molecule analysis. Nb offer the advantages of monoclonal technology while yielding binding agents that are far less expensive to develop and to produce. Nb are more thermally stable than classical antibodies and, as demonstrated here, they are easily modified by simple recombinant DNA technology. We have also demonstrated that the alkaline phosphatase fusion method is applicable to small molecule analysis. Not only does the fusion technology speed the assay procedure and reduce variability by removing a step, but the formation of the alkaline phosphatase dimer increases the avidity and thus the sensitivity of the procedure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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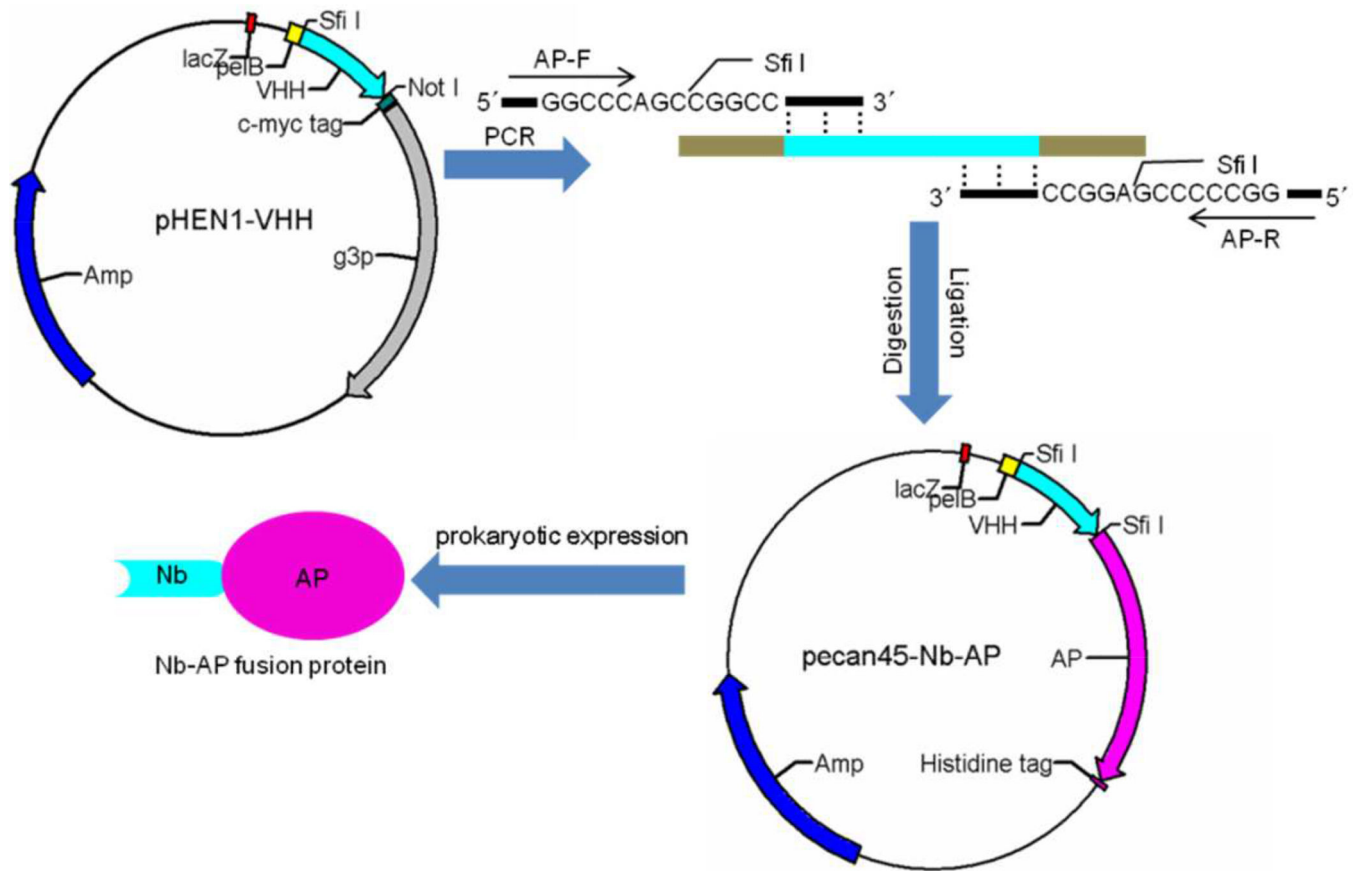
Figure 1.
Schematic diagram of the construction of the expression plasmid for the Nb-AP fusion protein.
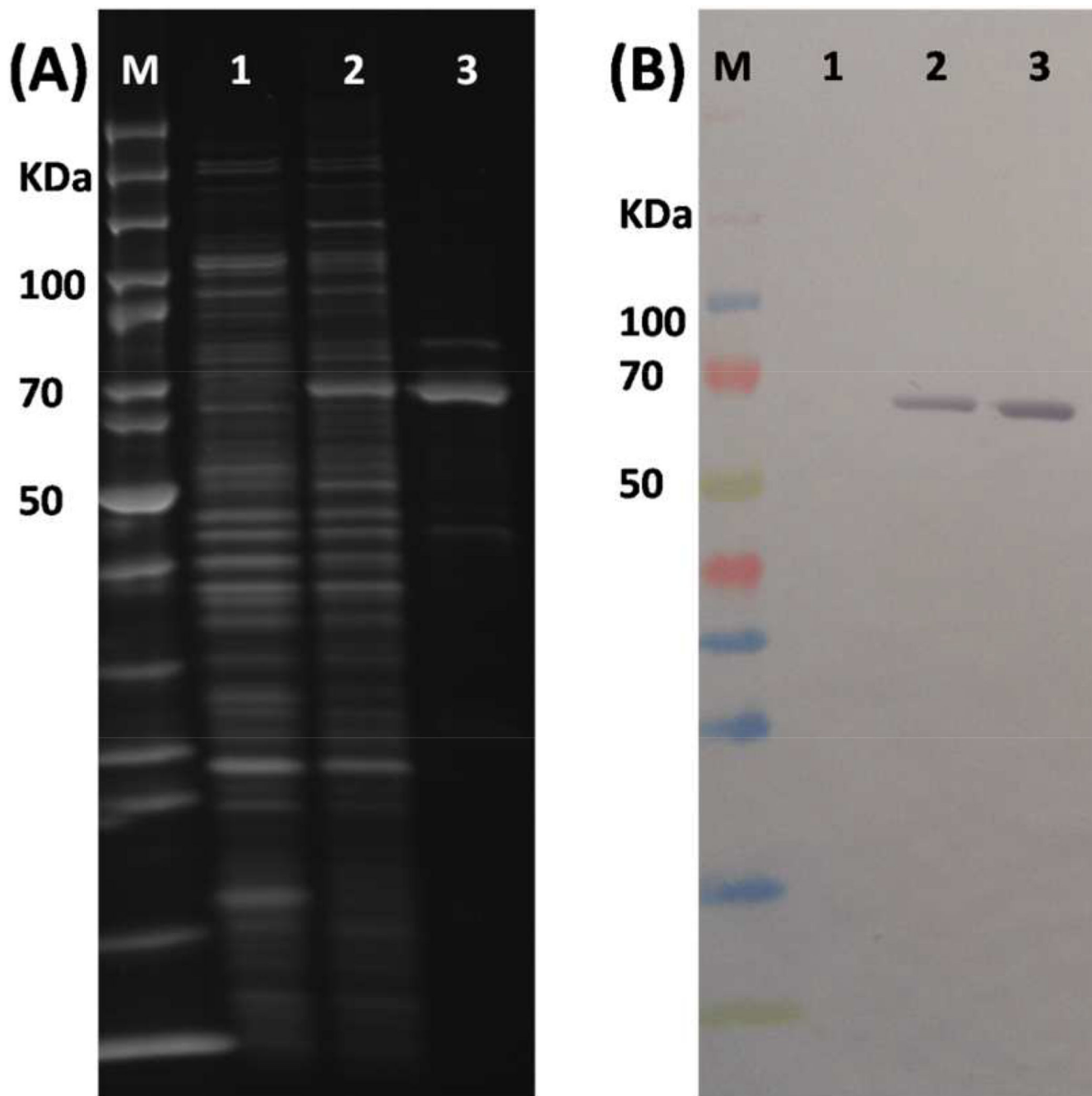
Figure 2.
(A) SDS-PAGE and (B) Western blot analysis of expression of the Nb28-AP fusion protein. Blots were stained with SYPRO Ruby protein gel stain or incubated with BCIP/NBT phosphatase substrate. Lane M, PageRuler unstained protein ladder (A) and spectra multicolor broad range protein ladder (B); Lane 1, whole-cell extract under non-induced conditions; Lane 2, whole-cell extract under induced conditions; Lane 3, purified Nb28-AP fusion protein by high-capacity nickel-IMAC resin.
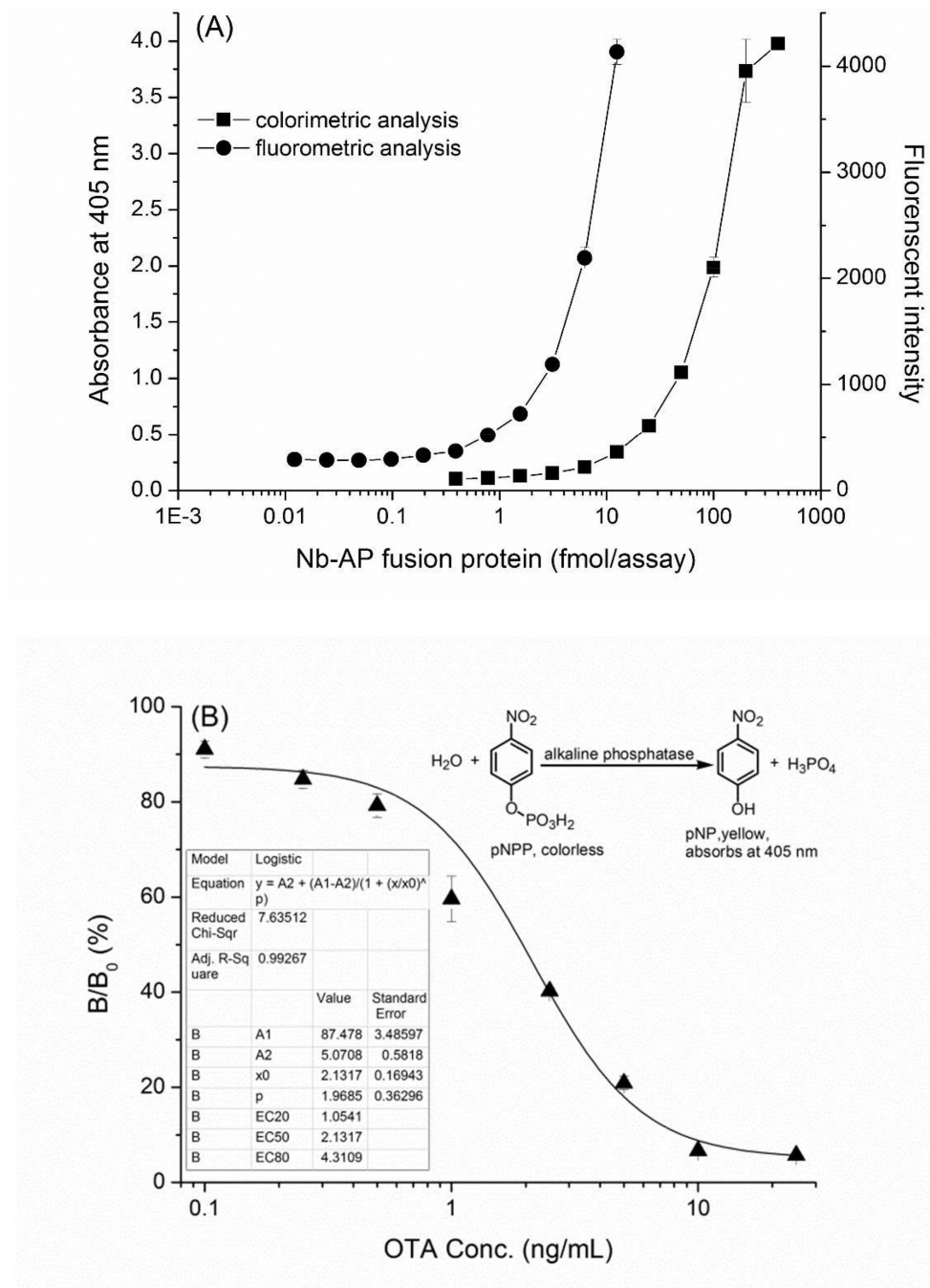
Figure 3.
(A) Dose-response curves for AP enzyme activity of the purified Nb28-AP fusion protein determined by colorimetric (■) and fluorometric (•) analysis. Error bars represent the standard derivation (n = 3). (B) Competitive binding curve (▲) based on Nb28-AP fusion protein by colorimetric analysis. The mixture of Nb28-AP fusion protein and free OTA was added into the 96-well microtiter plate coated with OTA-OVA conjugate (2 μg/mL), and then the binding was detected by incubation with pNPP substrate. Error bars represent the standard derivation (n = 3).
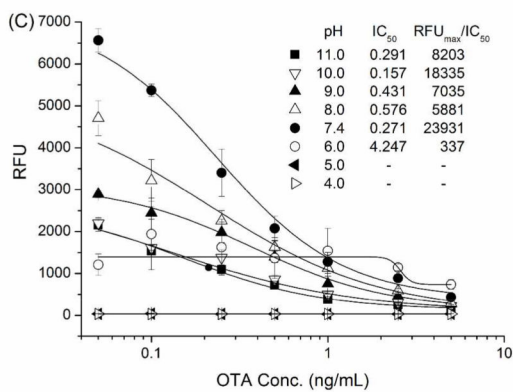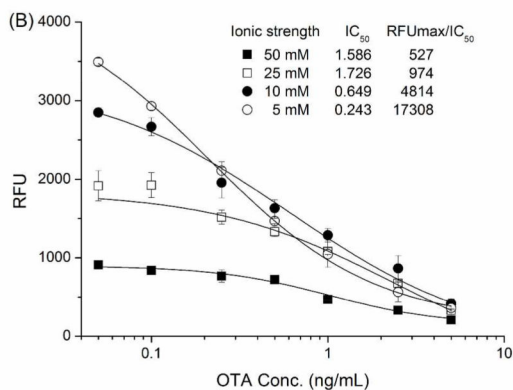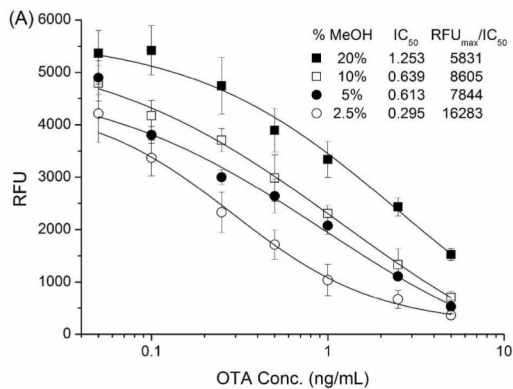
Figure 4.
Effects of (A) methanol, (B) ionic strength, and (C) pH on the performance of dc-FEIA for OTA probed with Nb28-AP fusion protein. The vertical bars indicate the standard deviation (n = 3).
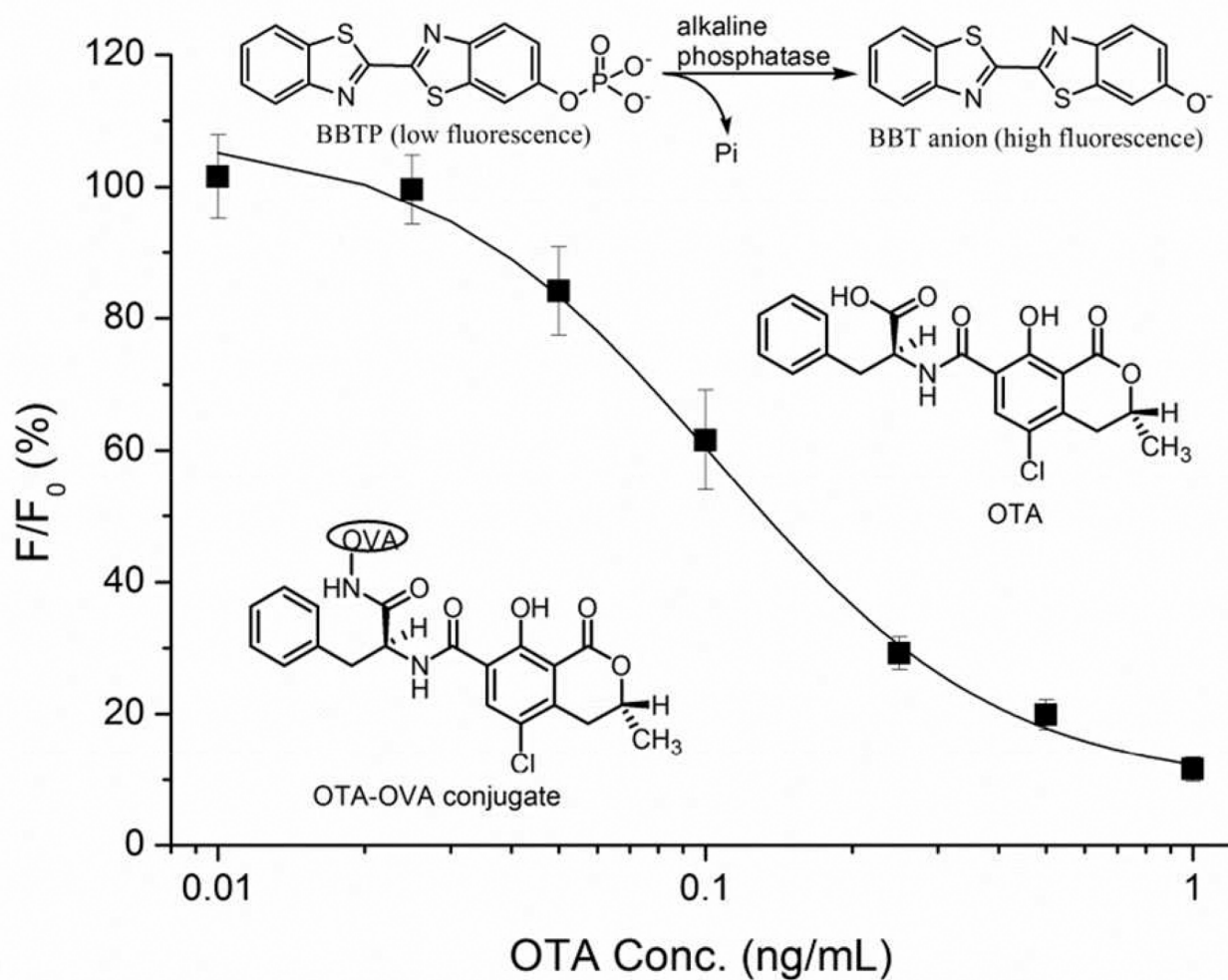
Figure 5.
Standard competitive binding curve of Nb28-AP fusion protein-based dc-FEIA for OTA under optimized paramaters. The curve shows the inhibition of the Nb28-AP fusion protein binding to OTA-OVA conjugate with increasing concentrations of OTA standard. The optimized concentration of coating antigen and purified fusion protein in the assay was 0.5 μg/mL and 16 ng/mL, respectively. Each point was tested in triplicate.
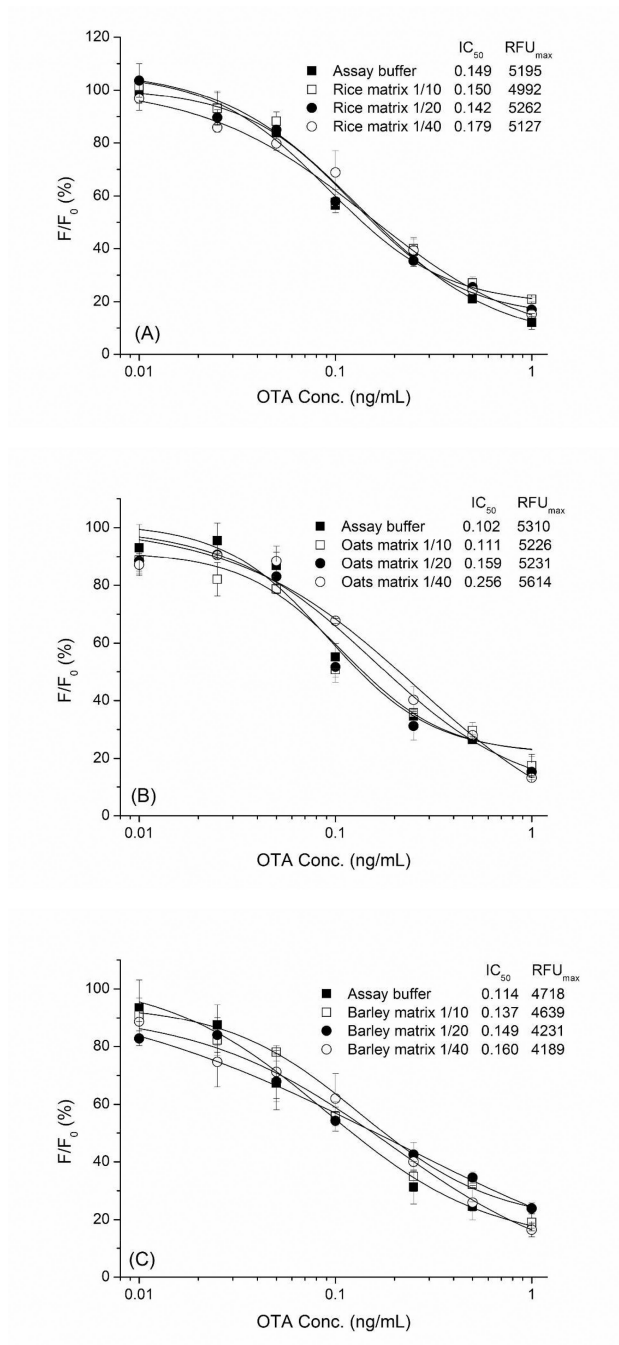
Figure 6.
Assay standard curves of Nb28-AP fusion protein in 2.5% methanol-PBS, rice (A), oats (B) and barley (C) matrix. Each assay was performed in triplicate.

Table 1

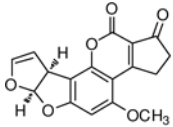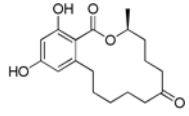Cross reactivity of Nb-AP fusion protein with OTA structural analogues
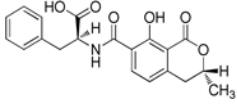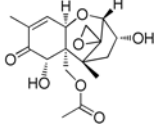
Analytes	Chemical structure	Cross-reactivity (%)
ochratoxin B		< 0.01
deoxynivalenol		< 0.01
aflatoxin B$_1$		< 0.01
zearalenone		< 0.01

Table 2

Recoveries of OTA from the spiked cereal samples by dc-FEIA

Sample Matrix	OTA spiked (μg/kg)	mean ± SD (μg/kg)	Recovery (%)	CV (%)
Intra-assay (n = 3)[a]				
Rice	1	1.18 ± 0.02	118	2
	2.5	2.35 ± 0.15	94	6
	5	3.60 ± 0.10	72	3
	10	9.90 ± 0.75	99	8
Oats	1	1.21 ± 0.06	121	5
	2.5	2.13 ± 0.28	85	13
	5	4.50 ± 0.48	90	11
	10	7.30 ± 0.02	73	3
Barley	1	0.85 ± 0.09	85	11
	2.5	2.45 ± 0.18	98	7
	5	3.75 ± 0.23	77	6
	10	9.10 ± 0.43	91	5
Inter-assay (n = 6)[b]				
Rice	1	1.09 ± 0.06	109	6
	2.5	2.28 ± 0.17	91	7
	5	3.65 ± 0.31	73	9
	10	9.78 ± 0.52	98	5

[a]Each assay was carried out in three replicates on the same day.

[b]The assays were carried out on six different days.

Table 3

Analysis of OTA content in cereal samples

Sample	FEIA (µg/kg ± SD, n = 3)	CV (%)	LC-MS/MS (µg/kg ± SD, n = 3)	CV (%)
	Rice			
1	1.64 ± 0.10	6	1.45 ± 0.18	12
2	1.15 ± 0.11	10	1.80 ± 0.17	9
3	1.14 ± 0.09	8	2.03 ± 0.09	4
	Oats			
1	5.77 ± 0.23	5	7.53 ± 0.11	2
2	1.27 ± 0.04	3	0.82 ± 0.10	12
3	1.56 ± 0.11	7	1.22 ± 0.04	3
4	11.2 ± 1.1	10	12.0 ± 0.3	3
	Barley			
1	1.17 ± 0.10	9	1.83 ± 0.14	8