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Determination of Ochratoxin A with a DNA Aptamer

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This work describes the identification of an aptamer that binds with high affinity and specificity to ochratoxin A (OTA), a mycotoxin that occurs in wheat and other foodstuffs, and a quantitative detection method for OTA based on the use of this aptamer. Aptamers are single-stranded oligonucleotides selected in vitro to bind to molecular targets. The aptamer selected in this work exhibited a dissociation constant in the nanomolar range and did not bind compounds with structures similar to OTA such as *N*-acetylphenylalanine or warfarin. The aptamer bound with a 100-fold less affinity to ochratoxin B. The selected aptamers could be used for the determination of ppb quantities of OTA in naturally contaminated wheat samples. Further work is ongoing to broaden the application demonstrated here with the development of sensors, affinity columns, and other analytical systems for field and laboratory determination of this toxin in food and agricultural products.

KEYWORDS: Ochratoxin; aptamers; mycotoxins; toxins; antibodies; sensors; affinity columns; wheat; analysis

INTRODUCTION

Ochratoxin A {*N*-[(3*R*)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl)carbonyl]-L-phenylalanine}, also referred to as OTA, is a mycotoxin that has been identified as a contaminant in grains, coffee, and wine throughout the world (*1, 2*). OTA is a nephrotoxic toxin, with strong carcinogenic effects on rodents, as well as documented teratogenic and immunotoxic effects in humans (*3, 4*). At present, regulatory limits for OTA exist in many countries, and testing of products is carried out at central testing laboratories. The next step in the development of an improved quality control process for agricultural products would be the development of a testing process simple enough to be performed by grain handlers and agricultural product manufacturers themselves. Currently accepted testing methods rely on the use of immunoaffinity columns followed by high-performance liquid chromatography (HPLC) (*5, 6*).

We report here on the identification of an aptamer for OTA and the development of a quantitative detection method for OTA based on the use of this aptamer. Aptamers are synthetic, single-stranded oligonucleotides that bind with high affinity and specificity to molecular targets. To our knowledge, this work represents the first aptamer identified for the detection of any mycotoxin. Aptamers provide several advantages over antibodies as the basis for analytical test kits. Aptamers can be chemically synthesized in vitro in a few minutes, while antibody production requires several months and takes place in vivo, with concomitant variation in product performance. Aptamers are much more stable than antibodies under a broad array of conditions and can resist physical and chemical denaturation with little or no loss of activity. Aptamers can also be modified with a variety of fluorescent dyes or other tags, providing an extraordinary flexibility in assay development (*7, 8*)

In this study, we used the SELEX technique (*9, 10*) for the selection of aptamers that bind OTA. The selected aptamers exhibited high affinity and binding specificity to OTA with a dissociation constant in the nanomolar range. We demonstrated that the selected aptamers can be used for the determination of ppb quantities of OTA in naturally contaminated wheat samples. Further work is ongoing to broaden the application demonstrated here with the development of sensors, affinity columns, or other analytical systems for field and laboratory determination of this toxin in food and agricultural products.

EXPERIMENTAL PROCEDURES

Reagents. OTA and a ground wheat reference standard containing OTA with a concentration of 2.7 ± 1.0 μg/kg were supplied by Romer Laboratories (Union, MO). A series of ground wheat samples containing a range of OTA concentrations were shared by the Grain Research Laboratory (Canadian Grain Commission, Winnipeg, Canada) as blind test material. A ground wheat sample certified as not containing OTA (BCR-certified reference material) was obtained from Sigma. Diaminodipropylamine agarose (DADPA), streptavidin-agarose, and sulfo-NHS acetate were acquired from Pierce (Rockford, IL). All synthetic oligonucleotides were supplied by Sigma-Genosys. The rest of the reagents were purchased from Sigma.

Selection of Aptamers. We adapted the SELEX protocol for the selection of aptamers. Briefly, each selection cycle consisted of loading a solution containing a library of single-stranded oligonucleotides with an internal random sequence onto an affinity column containing immobilized OTA. The column was then washed with binding buffer (BB), and an enriched fraction with putative binding ability to OTA was eluted through the addition of free OTA. This enriched library was polymerase chain reaction (PCR) amplified, the sense strands were recovered through the use of a biotinylated antisense primer, and the library was reapplied to fresh immobilized OTA columns.

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Table 1. Summary of the Selection Protocol

cycle	selection with negative resin	time the library was incubated in the column with positive resin	pre-elution with free competitors[a]
1	yes	1 h	no
2	no	1 h	no
3	no	1 h	yes
4	no	1 h	yes
5	no	1 h	yes
6	no	1 h	yes
7	no	1 h	yes
8	no	1 h	yes
9	no	10 min	yes
10	no	5 min	yes
11	yes	1 min	yes
12	yes	1 min	yes
13	no	1 min	yes

[a] Free competitors were warfarin, *N*-acetylphenylalanine, or an aqueous extract from wheat grains.

For the immobilization of OTA on an agarose-based resin (positive resin), OTA, 5 μmol, in 200 μL of DMSO was mixed with 500 μmol of 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) in 1 mL of distilled water. Immediately, 5 mL of DADPA slurry in 20 mM phosphate buffer, pH 5.0, was added, and the mixture was rotated for 1 h at room temperature. To quench unreacted amine groups, the resin was equilibrated with 0.2 M carbonate buffer, pH 8.5, and 300 μmol of sulfo-NHS acetate was added to the slurry. The mixture was rotated for another hour, after which the resin was washed extensively with selection buffer (SB), composed of 10 mM HEPES, pH 7.0, 120 mM NaCl, 5 mM KCl, and 5 mM $MgCl_2$. The presence of OTA in the resin was corroborated by the intense fluorescence of the resin when irradiated with UV light at 366 nm.

Simultaneously, a resin that did not contain immobilized OTA (negative resin) was prepared by adding 75 μmol of sulfo-NHS acetate to 1 mL of resin equilibrated in carbonate buffer, pH 8.5, and packed into a column. The library of oligonucleotides was incubated with the negative resin for 10 min in selection cycles 1, 11, and 12 to select against oligonucleotides that bound to the resin. The flow-through from this negative selection step was loaded onto the column containing the resin with immobilized OTA (positive resin). The library was designed with 30 random nucleotides, flanked with two constant regions for primer annealing. The primers were as follows: reverse, Biotin-CGTTGTCCGATGCTC; and forward, Cy3-TGGTGGCTGTAGGTCA.

An aliquot consisting of 10^{15} random oligonucleotide sequences was heated to 90 °C for 5 min and allowed to stand at room temperature for half an hour before loading onto a column containing either the negative or the positive resin. The columns were prepared by loading 250 μL of the slurry containing the resin in a disposable microspin column (BioRad); this yielded a column volume (CV) of approximately 200 μL. The resin was washed extensively with SB before the oligonucleotide library was loaded onto the column and incubated for the specified time (**Table 1**). The column was then washed with 12 CV of SB. After cycle 3, these washes were followed by washes with solutions of the SB containing 2.5% dimethylsulfoxide (DMSO) and an aqueous extract from wheat grains. This grain extract was prepared by mixing 1 g of ground wheat grain with 10 mL of SB and rotating the mixture for 1 h. This mixture was centrifuged, and the supernatant was filtered through a 0.45 μm filter. The extract was mixed in a 1:10 ratio with SB containing 2.5% DMSO. After cycle 5, we did two washes with grain extract in each cycle. The column was then washed with 2 mM warfarin and 5 mM *N*-acetyl-L-phenylalanine (NAP). These solutions were incubated in the column for 10 min each. After this, the column was washed with 6 CV of SB, and the oligonucleotides bound to OTA were eluted with three incubations of 10 min each with 100 μL of 2 mM OTA in SB containing 2.5% DMSO. DMSO was used to predisolve OTA. The first two eluates of 100 μL were combined and used to estimate the proportion of oligonucleotides eluted with OTA. For this, 5 μL of each collected fraction (washes and elution) was mixed with 100 μL of water in a 96 well black microplate, and the fluorescence was measured using $\lambda_{exc} = 540$ nm and $\lambda_{em} = 580$ nm. The proportion of oligonucleotides in the elution fraction was calculated by dividing the fluorescence in this fraction over the total fluorescence of all of the washes and the elution. The third eluate was then pooled with the other two eluates before concentrating and washing the DNA with PCR buffer in a 10 kDa microcon filter (Millipore). The selected oligonucleotides were amplified for 12 PCR cycles using four PCR reactions containing 5 μL of concentrated eluate, 5 units of Taq polymerase, 1 μM each primer, and 200 μM dNTPs in a 100 μL total volume. After cycle 9, the reverse primer was used at a concentration of 0.2 μM to prevent the formation of primer dimers. After PCR amplification, the double-stranded DNA was incubated at room temperature with an agarose resin containing immobilized streptavidin for 30 min with rotation, the mix was heated at 95 °C for 5 min to denature the DNA and spun, and the supernatant was collected to yield a single-stranded DNA labeled with Cy3 and ready to use in the next selection cycle. In the last selection cycle, the PCR was performed using unlabeled primers, so the product could be ligated to pGEM-T vector (Promega) and cloned. The sequence of the cloned fragments was determined by standard methods with an Applied Biosystems 3730 Analyzer at Robarts Research Institute (London, Canada). Oligonucleotides corresponding to these sequences were synthesized (Sigma Genosys) for affinity testing. **Table 1** summarizes the procedure used in each selection cycle.

Binding Assays. Synthesized aptamers were screened for binding to OTA by equilibrium dialysis. Microequilibrium dialyzers (Harvard apparatus) were loaded with SB in the receiving chamber and SB containing 200 nM OTA and 20 μM aptamers in the loading chamber. Dialysis was performed for 48 h at room temperature. Two replicates were tested for each aptamer. The affinity of the aptamers to OTA was estimated by mixing 65 μL of solution from each chamber with 200 μL of carbonate buffer, pH 9.6, in a 96 well black microplate (Corning) and measuring the intrinsic fluorescence of OTA in the loading (F_l) and receiving (F_r) chambers using $\lambda_{exc} = 375$ nm and $\lambda_{em} = 430$ nm. Mixing the dialysis solution with an alkaline buffer maximized the quantum yield of OTA. The fraction of bound OTA (f) was determined as:

$$f = \frac{F_l - F_r}{F_l} \tag{1}$$

and the dissociation constant (K_d) was estimated as:

$$K_d = \frac{[A_0]}{f} - [A_0] \tag{2}$$

where $[A_0]$ is the total concentration of the aptamer. As the concentration of the putative aptamer is in a hundred fold excess to that of OTA, there was not a significant difference between total and free aptamer concentration. This method was corroborated in the case of the aptamer 1.12 through the use of a number of dialyzers that were prepared with 200 nM OTA and a changing concentration of the putative aptamer in the loading chamber. We assumed the stoichiometry between OTA and the putative aptamer was 1:1. The K_d was determined by fitting the experimental data to the eq 3 using Marquardt−Levenberg algorithm and SigmaPlot (SPSS Inc., Chicago, IL).

$$f = \frac{([A_0] + [OTA_0] + K_d) - \sqrt{([A_0] + [OTA_0] + K_d)^2 - 4[A_0][OTA_0]}}{2[OTA_0]} \tag{3}$$

where $[OTA_0]$ is the total concentration of OTA.

Equilibrium dialysis was used as well to determine the effect of magnesium and calcium ions and pH on the affinity between OTA and aptamer 1.12.2. In this case, the concentration of the aptamer was set at 2 μM and OTA concentration at 20 nM. The specificity of the aptamer to OTA was evaluated through the use of a similar dialysis assay where 100 nM OTA and 10 μM aptamer were incubated in the

loading chamber in the presence of either 20 μM warfarin or 20 μM NAP. To determine if the aptamer binds ochratoxin B (OTB), a solution of 100 nM OTB and 10 μM aptamer was loaded in the loading chamber.

Preparation of an Aptamer Affinity Column and Determination of OTA. The aptamer 1.12.2 was conjugated through the 5′-phosphate group to DADPA according to a protocol from the manufacturer. The resin (400 μL) was washed three times with distilled water and one time with 0.1 M imidazole, pH 6. After the washes, 200 μL of the imidazole solution, 32 nmol of aptamer 1.12.2 in water, and 400 μL of 156 mM EDC in 0.1 M imidazole, pH 6, were added. The reaction was incubated for 3 h at room temperature with rotation. The concentration of DNA remaining in the supernatant was estimated through acrylamide gel electrophoresis. The resin was washed several times with BB (10 mM TRIS, pH 8.5, 120 mM NaCl, 5 mM KCl, and 20 mM $CaCl_2$), and 100 μL aliquots of resin were packed in a column made with a pipet tip (Sorensen barrier tips). To test that the conjugated aptamer was active, 1 mL of a 100 nM OTA solution was passed through the column, and the presence of OTA in the solution was measured by its fluorescence before and after passage through the column.

To determine the OTA concentration within wheat grain, 10 g of finely ground wheat grain containing known concentrations of OTA was mixed with 40 mL of 60% methanol in distilled water in a 50 mL Falcon tube. The flour was mixed well with the solvent and shaken by hand for 5 min. The tube was centrifuged in a Centra CL2 centrifuge (Thermo ECL) at 2500*g* for 5 min. A 3.5 mL aliquot of the grain extract was mixed with 10.5 mL of BB, whereupon a translucent suspension was observed. This mixture was spun for 5 min at 12000*g*. Experiments were performed demonstrating that OTA was not coprecipitated with this suspension. The supernatant, 12 mL, was run through the affinity column with the aid of a syringe at a rate of 1 mL per min. The column was washed with 6 mL of BB, and OTA was eluted from the column with 2 mL of 20% methanol in TE buffer (10 mM TRIS, 1 mM EDTA), pH 9. The concentration of OTA in the eluate was determined fluorometrically, by comparing the intrinsic fluorescence of OTA with that of a calibration curve made in the same buffer ($\lambda_{exc} = 375$ nm and $\lambda_{em} = 430$ nm). Using the concentration of OTA in the eluates from the aptamer affinity column, we calculated the quantity of OTA in the original wheat sample. The analysis was replicated with four independent samples in the case of the Romer laboratory sample and with two independent samples in the case of the GRL and Sigma samples. The concentrations of OTA in the samples from the GRL were unknown to us until after completion of the analysis.

Fluorescence Spectroscopy. Steady state fluorescence and fluorescence excitation and emission spectra were recorded with a Microplate reader Sapphire II (TECAN, Switzerland) using 96 well black microplates with a low-binding surface (Corning).

RESULTS AND DISCUSSION

Selection of Aptamers. To separate oligos that bind OTA, we immobilized OTA on an agarose resin through the carboxyl group. This design favored the selection of aptamers specific for the isocoumarin moiety, as the phenylalanine part of the molecule would be inaccessible. It was crucial to block the remaining free amino groups with sulfo-NHS acetate; failure to do so would lead to a considerable nonspecific binding of DNA to the resin.

OTA bound weakly to random sequences of DNA, as indicated by the fact that more than 20% of the library was present in the elution fraction in only the second cycle of selection (**Figure 1**). To increase the stringency of the selection, we washed the column of immobilized OTA and the bound library with SB-containing compounds with structural similarities to OTA from cycle 3 onward. The OTA structure comprises a polyketide-derived 3,4-dihydro-3-*R*-methyl-isocoumarin moiety linked via its 12-carboxyl group by an amide bond to L-β-phenylalanine. We used a 5 mM solution of NAP to remove

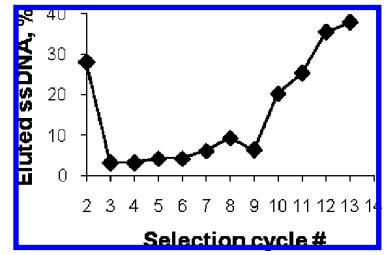

Figure 1. Percent of eluted oligonucleotides after each selection cycle.

Table 2. Sequences and Dissociation Constants (K_d) between OTA and Selected Aptamers[a]

aptamer	sequence	K_d (μM)
1.12	GCATCTGATC**GGGTGTGGGTGGC**GTAAAGG	0.36
1.13	G**GGGTG**AAACG**GG**TCCCG	6.7
1.14	GCAGTCCTAGATC**GGGTGTGGC**T**GGC**TTGG	0.99
1.4	GCACGATGGGGAAA**GGGT**CCCCC**TGG**GTTG	19.3
2.2	ACTGTCCGTC**GGGT**TTA**GGGTGGC**GTTCGG	1.6
2.3	TCAGTCCCGATCA**GGTGTGGGTGGC**ATTGG	1.7
2.4	CCAAATGCGACG**GGG**CCTGTTTTAATGGGG	19.5
2.6	CGTACGGT**GGG**AACG**GT**TCCTCTTAGGGT	7.1
2.9	CAGGTGGCAGATC**GGGTGTGGGTGGC**CTGG	0.96
2.10	ACATGCGACTGA**GG**C**T**CGGTTT**T**ATTGAGGG	4.3
2.11	CCTGACGATC**GGGTGTGGGTTGGC**TTGAGG	2.5
2.12	CCTTGTAGATC**GGGTGTGG**TTT**GGC**GTAGG	0.97
2.13	GCAGTACGATCGG**GGGTGGGTGG**ATGTAGG	1.9

[a] K_d values are the average of two independent experiments.

aptamers that bind only the phenylalanine part of OTA. Additionally, we washed the column with a 2 mM warfarin solution. This is a hydrophobic molecule that contains a coumarin heterocycle and a phenyl group; similar structures are present in OTA, so washes with warfarin increased the stringency of the selection by removing sequences that bound those structures but were not specific for OTA.

Finally, in view that our goal was to analyze OTA in wheat grain, we performed washes with an aqueous grain extract, thus providing a counter selection for the removal of oligonucleotides with binding affinity to soluble compounds present in wheat grain. The proportion of oligonucleotides present in the counter selection eluates relative to the positive selection eluates decreased over selection cycles. By cycle 13, the proportion of oligonucleotides present in the positive selection eluates was higher than that of the combined counter selections.

Binding Assays. A number of putative aptamers were cloned, sequenced, and synthesized. A list of all of the sequences of the selected aptamers and the binding affinity determined by equilibrium dialysis is provided in **Table 2**. The sequence of the primers was omitted for simplicity. The aptamer that exhibited the highest affinity for OTA was 1.12. We measured the K_d for this aptamer by loading a series of dialyzers with a range of aptamer concentrations (**Figure 2**). The calculated K_d was not different from the K_d calculated using a single point measurement. The curve fitting also showed that the stoichiometry between the aptamer and OTA is 1:1.

A multiple sequence aligment analysis demonstrated a high degree of consensus among the aptamers that exhibited a high level of OTA binding affinity (**Table 2**), possibly indicating that only a narrow range of sequences provide strong binding affinity to this target. To further determine the specific portion of the aptamer that was responsible for OTA binding, we removed the primers from the aptamers 1.12 and 1.13. The resulting short oligo from aptamer 1.12 (1.12S, 30 mer) did not bind OTA ($K_d = 640$ μM), while the short version of 1.13,

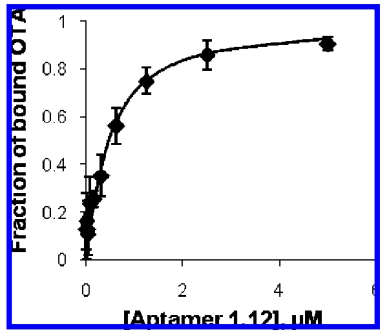

Figure 2. Equilibrium dialysis binding assay of OTA and aptamer 1.12. The concentration of OTA was 200 nM. The K_d was calculated to be 360 ± 60 nM. Each point is the mean of three independent experiments. Bars represent standard deviations.

Table 3. Modifications on Aptamer 1.12 and Dissociation Constants[a]

name	sequences	K_d (μM)
1.12	TGGTGGCTGTAGGTCAGCATCT GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACAACG	0.36
1.12.5	GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACAACG	0.8
1.12.6	GATCGGGTGT**C**GGTGGCGTAAAGGGAGCATCGGACAACG	NB
1.12.2	GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGGACA	0.2
1.12.8	GATCGGGTGTGGGTGGCGTAAAGGGAGCATCGG	0.2
1.12.11	GATCGGGTGTGGGTGGCGTAAAGGGAGCATCG	0.4
1.12.12	GATCGGGTGTGGGTGGCGTAAAGGGAGCATC	0.5
1.12.9	GATCGGGTGTGGGTGGCGTAAAGGGAGCAT	1.6
1.12.10	GATCGGGTGTGGGTGGCGTAAAGGGAG	NB
1.12S	GATCGGGTGTGGGTGGCGTAAAGG	NB
1.12.13	ATCGGGTGTGGGTGGCGTAAAGGGAGCATCGG	NB
1.12.14	TCGGGTGTGGGTGGCGTAAAGGGAGCATCGG	NB
1.12.S22	ATCGGGTGTGGGTGGCGTAAAG	NB

[a] K_d values are the average of two independent experiments. NB, no binding was detected.

designated 1.13S18 (18-mer), exhibited a K_d of 24 μM. Removing the forward primer in 1.12 and a section of the 5′-nonconsensus sequence, however, resulted in a 39-mer aptamer (1.12.5) that bound OTA with slightly less affinity that 1.12. Further removal of three bases from the 3′-end, (aptamers 1.12.2 and 1.12.8) resulted in a significant increase in binding affinity (**Table 3**). It is clear that the consensus sequence, shown in bold in **Table 2**, is responsible for OTA binding. In support of this, we found that changing the guanine-11 in aptamer 1.12.5 for a cytosine (aptamer 1.12.6) resulted in a complete loss of binding affinity (**Table 3**).

In addition to a high level of binding affinity, the aptamers identified herein also exhibited a high level of binding specificity to OTA as revealed by a lack of affinity to NAP or warfarin (**Figure 3**). Equilibrium dialysis of OTA with aptamer 1.12 in the presence of 100-fold molar excess of NAP and warfarin (relative to OTA) was demonstrated to not have a significant effect on the partitioning of OTA between the two chambers of the dialysis tubes. This result contrasts with the fact that OTA and warfarin both occupy the same binding pocket in HSA, the main carrier of OTA in the body under natural exposure (*11, 12*), so the structural or conformational basis of OTA-DNA binding differs substantially from that of the OTA-protein binding. A comparison between OTA and OTB, a structural analogue of OTA that lacks the chlorine atom in the isocoumarin ring, supports this. Equilibrium dialysis of OTB with the same aptamer (**Figure 3**) resulted in an estimated K_d of 30 μM. This indicates that OTB presented 100-fold less affinity to aptamer 1.12.2 than OTA, while the difference in reactivity between OTA and OTB with a monoclonal antibody developed for OTA

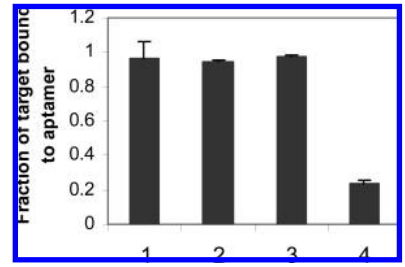

Figure 3. Specificity of aptamer 1.12 for OTA. Equilibrium dialysis of 100 nM OTA in SB shows that OTA bound almost completely to aptamer 1.12 at an aptamer concentration of 10 μM (1); adding 20 μM NAP (2) or 20 μM warfarin (3) to this mix did not significantly affect the binding of OTA to the aptamer. A dialysis of 100 nM OTB with 10 μM aptamer 1.12 (4) carried out in the same conditions as for OTA shows that OTB binds much more weakly to the aptamer. Each data point is the mean and standard deviation of two independent experiments.

Table 4. Effect of Magnesium and Calcium Concentration on the Binding Affinity between OTA and Aptamer 1.12.2 in 10 mM HEPES, pH 7.0, 120 mM NaCl, and 5 mM KCl[a]

	K_d (nM)	
concentration (mM)	Mg^{2+}	Ca^{2+}
0	NB	NB
5	197 ± 74	112 ± 12
10	148 ± 8	54 ± 8
20	118 ± 46	49 ± 3

[a] NB, no binding was detected.

is only 3-fold (*5*), and there is less than 20% difference between these toxins in the case of polyclonal antibodies (*13*).

Binding of OTA to the DNA aptamers depended on the presence of divalent cations (**Table 4**). Similar dependence on magnesium for an aptamer/target binding was documented with an aptamer for ATP (*14*). In the aptamer/ATP case, it was suggested that the reliance on Mg was due to a bridging interaction mediated by magnesium between the target and the oligonucleotide. Surprisingly, including calcium in the buffer instead of magnesium resulted in a lower K_d. Presumably, OTA forms a coordination complex with magnesium or calcium with the aid of both the carboxyl and the 8-hydroxyl groups in OTA, and this complex enhances binding to the aptamer. In the absence of these cations, negatively charged OTA did not exhibit binding to these aptamers. This is expected as DNA is also highly negatively charged. On the other hand, divalent cations affect the conformation of the DNA, which in turn could play an important role in the interaction with other molecules (*15*). However, the affinity between OTA and the aptamer was not affected significantly by the concentration of sodium or potassium (data not shown), to the point that complete removal of sodium or potassium did not affect the affinity as long as magnesium or calcium were present. This indicates the ionic strength of the solution had little effect on the interaction between OTA and the DNA aptamer.

Equilibrium dialysis experiments showed that an acidic pH reduced the binding affinity considerably. For example, at a calcium concentration of 20 mM, the K_d increased from 50 to 700 nM when the pH was reduced from 7.0 to 5.5. The effect of pH corroborates the crucial role of the complex with magnesium or calcium in binding to DNA. At lower pH, the 8-hydroxyl group in OTA is protonated, and thus, the complex formation was inhibited, resulting in a lack of binding to the aptamer.

Table 5. Concentration of OTA in Naturally Contaminated Wheat Samples Determined with an Aptamer Affinity Column and with Current Standard Methods[a]

sample	certified concentration (μg/kg)	concentration determined with an aptamer affinity column (μg/kg)
Sigma blank	<0.6	-0.4 ± 0.8
Romer laboratories	2.7 ± 1.0	2.2 ± 0.4
GRL sample #1	1.8 ± 0.6	2.3 ± 1.1
GRL sample #2	5.0 ± 1.5	6.8 ± 3.4
GRL sample #3	7.4 ± 1.9	9.5 ± 2.0
GRL sample #4	61.9 ± 7.5	56.1 ± 9.0
GRL sample #5	<1.0	0.47 ± 0.09

[a] Data are the means $\pm$ SDs.

These experiments led us to the development of buffer conditions where the $K_d$of the aptamer 1.12.2 and OTA was reduced to 50 nM. This buffer (BB) was subsequently used for the purification of OTA with an aptamer affinity column and the determination of OTA in wheat samples.

Determination of OTA in Wheat Grains. To demonstrate the potential use of the aptamers in the quantitative determination of OTA in agricultural commodities, we prepared an affinity column with the aptamer 1.12.2. Taking into account that no DNA was detected in the supernatant after the conjugation reaction, we assumed that the resin contained 8 nmol of aptamer per 100 μL of the slurry. This quantity of aptamer represents more than a 20-fold excess to the maximum expected quantity of OTA in the grain samples. To test the performance of the aptamer affinity column, we used a solution of OTA made in BB. The column removed more than 97% of OTA from 1 mL of a 100 nM OTA solution.

The performance of the aptamer affinity column and its use for OTA analysis was tested with samples of naturally contaminated wheat flour with a certified concentration of OTA. The samples were processed similarly to a protocol used by Neogen Co. (*16*) for the determination of OTA with antibodies. One of the advantages of the use of aptamers is that we only had to dilute the extraction solvent 4-fold with the BB, whereas for the antibody based assay the extraction buffer has to be diluted 10 times to minimize the effect of methanol or other organic solvents on the binding between the antibody and the analyte. In preliminary experiments, we found that the binding affinity between OTA and the aptamer 1.12.2 was not affected significantly with concentrations of methanol up to 20%. The resulting mix of extraction solvent and BB in this experiment contained 15% methanol. The fluorometric determination of OTA in the eluate generated concentrations of OTA not significantly different than the value provided by the suppliers of the standard samples (**Table 5**).

To corroborate that the measured fluorescence was due to OTA and not to other compounds in the grain extract, we scanned excitation and emission spectra of the eluate and confirmed that they coincided with that of OTA. At alkaline pH, OTA has a characteristic excitation spectrum with a peak at 375 nm and a broadband below 300 nm (*12*). The excitation spectra of the eluates from the affinity column exhibited a spectral pattern that coincided with the excitation spectra of standard solutions of pure OTA in the same solvent (**Figure 4**). This confirms that the source of the measured fluorescence was OTA. The emission spectra of both eluates and standard solutions also exhibited a single peak with maximum between 430 and 440 nm (data not shown). OTA fluorescence can not be measured directly in contaminated samples because many natural compounds fluoresce at wavelengths close to that of

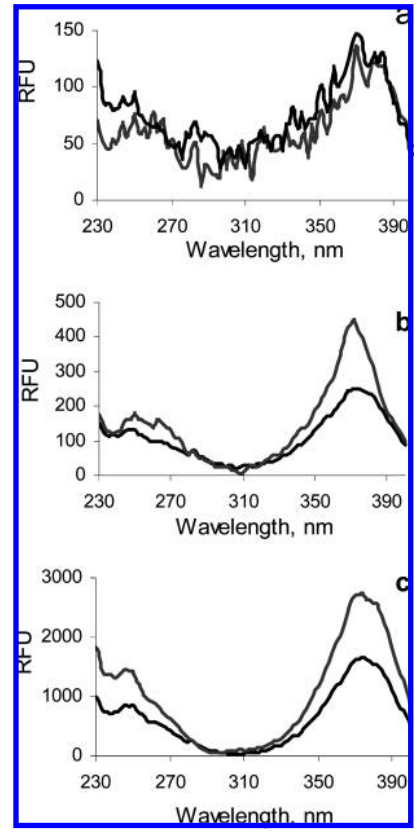

Figure 4. Excitation spectra of representative wheat grain sample extracts after elution from the aptamer affinity column (black lines) and of standard solutions with known concentrations of OTA in the same buffer (gray lines). (a) Extract from Romer laboratory sample and a solution containing 1.56 nM OTA, (b) GRL sample #2 and a solution containing 12.5 nM OTA, and (c) GRL sample #4 and a solution containing 100 nM OTA. The excitation spectra were scanned with $\lambda_{em} = 440$ nm. RFU, relative fluorescence units.

OTA. In the present study, with the use of an aptamer affinity column, we concentrated and separated OTA from other grain components and determined the concentration of OTA fluorometrically. The high specificity of the aptamer affinity column to OTA allowed the direct determination of OTA by its fluorescence with no need for other separation methods, as all other fluorescent contaminants were removed during the washing steps. The concentration of OTA in naturally contaminated wheat samples determined by this method did not differ from that determined by current standard procedures involving immunoaffinity columns and HPLC.

This work shows the first application of aptamers for the quantitative analysis of mycotoxins and, as far as we know, the first time that aptamers have been used in the analysis of food contaminants. The identification of an aptamer-based test for quantitative determination of a mycotoxin in food samples opens the way to significant improvements in quality control and food safety with regard to more dynamic testing of agricultural products.

ABBREVIATIONS USED

OTA, ochratoxin A; OTB, ochratoxin B; SB, selection buffer; BB, binding buffer; CV, column volume; DMSO, dimethylsulfoxide; NAP, *N*-acetyl-L-phenylalanine; DADPA, diaminodipropylamine agarose; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; GRL, Grain Research Laboratories, Winnipeg, Canada.

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