FCS-Based Sensing for the Detection of Ochratoxin and Neomycin in Food

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Abstract: In this work, we present an advanced fluorescence assay for the detection of traces of ocratoxin A and neomycin in food. The described assay is based on measurement of the fluctuations of the fluorescein-labeled analytes by a focused laser beam in the absence and in the presence of the specific antibodies anti-analytes. A competitive assay based on the utilization of unlabeled analytes was developed. The obtained results indicated that the combination of high-avidity IgG antibodies together with an innovative fluorescence immunoassay strategy resulted in the detection limit of 0.0078 ng and 0.0156 ng for ochratoxin A and neomycin, respectively.

Keywords: Food safety, Micotoxins, FCS, Biosensors, Antibodies.

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INTRODUCTION

Food contaminants, as ochratoxin and neomycin, are substances that for effect of environmental contamination, cultivation practices, production processes and storing processes can be present in certain foodstuffs. The presence of elevated levels of these compounds in human's food was been demonstrated to be dangerous for the human health [1].

Ochratoxins are a group of mycotoxins produced as secondary metabolites by several fungi of the *Aspergillus* or *Penicillium* families that often grow in agriculture products prior to harvest or during the storage. Ochratoxins are organic acids composed of an isocumarin moiety linked to L-phenylanine. The family of ochratoxins consists of several members in which the ochratoxin A (OTA) is the more toxic. In fact, several studies in animals have shown that OTA is a nephrotoxin, hapatotoxic, carcinogen and teratogen and it enters in alimentary chain by different sources [2]. In particular OTA is found in different kind of cereals as wheat, barley, corn, oats, and rice and also in different products as coffee, dried fruits, grape juice, wine, beer, pork kidneys and blood. The identification of the effect of mycotoxins on human health has increased the attention on the detection of these compounds in human and animal food. In fact, mycotoxin traces usually have been identified in range from nanogram to micrograms per gram of foodstuff, and the detection and identification, therefore, require a highly and sensitive methodology. In the case of OTA different traditional methods are used, including thin-layer chromatography (TLC), gas chromatography, high-pressure liquid chromatography (HPLC) and mass spectroscopy (MS)]. Recently also an ELISA assay has been developed [3-5].

As concern the neomycin determination, it is important to say that it an antibiotic that belong to the aminoglycosides class. This class includes a group of water-soluble broad-spectrum antibiotics (neomycin B, kanamycin B, amikacin and paromomycin I) that have been widely used in both human and veterinary medicine for their bactericidal activity against Gram-positive and Gram-negative organisms [6]. The aminoglycoside antibiotics act by interfering with bacterial protein synthesis, resulting in the death of bacteria, which are sensitive to the antibiotic. For this reason in veterinary medicine, neomycin is used for the treatment of disease and as dietary supplements of many species of animals destined for human feeding to prevent bacterial contamination during the manufacturing process [7]. Neomycin is contained in a variety of veterinary preparations including topical creams/ointments, eye drops/ointments, oral tablets, oral suspensions, and intra-mammary preparations for use in food-producing and non food-producing animals. It has been reported that the use of neomycin is frequently associated with oto-toxicity and nephro-toxicity (the main reason for kidney damage). Therefore it is of high importance a careful monitoring of the presence of this antibiotic in food destined to feeding [8]. At the moment various methods are available for the determination of aminoglycoside antibiotics, such as microbiological methods, enzyme immunoassays and chromatographic methods [9].

In this work, we show a new advanced fluorescence assay for the detection of traces of ocratoxin A and neomycin

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in food. The described assay is based on measurement by a focused laser beam of the fluctuations of the fluorescein-labeled analytes both in the absence and in the presence of the specific antibodies anti-analytes.

1. MATERIALS AND METHODS

1.1. Materials

Standard chemicals, solvents, buffers, Ochratoxin A and Neomycin Sulphate were purchased from Sigma-Aldrich. Fluorescein-5-isothiocyanate (FITC) and Rhodamine 110 chloride were from Invitrogen, Eugene, OR.

1.2. Preparation of Glutamine Binding Protein

The Glutamine Binding Protein (GlnBP) from *E. coli* was prepared and purified according to Staiano *et al.* [10]. The protein concentration was determined by the method of Bradford [11] with bovine serum albumin as standard on a double beam Cary 1E spectrophotometer (Varian, Mulgrade, Victoria, Australia).

1.3. Conjugation Glutamine Binding Protein-Ochratoxin / Neomycin

A solution of ochratoxin at concentration 0,005 mg was incubated in presence of 0,0075 mg of 1,1-carbonyl-diimidazole were dissolved in 100 µL of dry DMF.

The mixture was added drop-wise with mixing to 1 mL of solution of 5 mg GlnBP in 0.1 M sodium bicarbonate pH 8.5. The mixing was done at room temperature for 2 h in dark condition. After mixing the mixture was dialyzed over night against 0.1 M Sodium Bicarbonate 0,1 NaCl pH 8,5 . The same procedure was done for the Neomicyn conjugation.

1.4. Glutamine Binding Protein-Ochratoxin and Neomicyn (GlnBP-OTA and GlnBP-Neo) Labeling

A solution of GlnBP a concentration of 2.0 mg/mL in 1.0 mL of 0.1 M bicarbonate buffer, pH 9.0, was mixed with 10 µL of FITC (Molecular Probes) solution in N,N- dimethyl-formamide (DMF; 1.0 µg of FITC/100 µL o DMF). The reaction mixture was incubated for 1 h at 30 °C, and the labeled molecules were separated from unreacted probe by a dialysis procedure against 50 mM phosphate buffer,100 mM NaCl, pH 7.0, by using dialysis tubes with a cutoff of 500 Da (Spectrum Labs) overnight, at 4 °C. The same procedure was done for the labeling of GlnBP-Neo.

1.5. Generation of Polyclonal Anti-BSA-OTA and Anti-BSA-Neo Antibodies

Antibodies against cthe compounds BSA-Ochratoxin and BSA-Neomicyn were prepared. Two rabbits were immunized following a standard protocol by intradermal inoculation of a mixture of antigens A and B (0.5 mg each per rabbit). After the immunization period, the rabbits were sacrificed and the blood was centrifuged to separate blood cells from serum (SI1 from rabbit 1 and SI2 from rabbit 2).

1.6. IgG Purification

A 2.0 mL sample of antiserum (SI1 and SI2)was applied to a protein A column of the PURE1A Protein A Antibody Purification Kit, Sigma, and the IgG fraction was purified according to the manufacturer's instructions. Elution of proteins was monitored by absorbance at λ =278 nm. The IgG fraction was eluted with glycine (0.1 M) at pH 2.8 and immediately buffered in Tris (1.0 M) at pH 8.0. Concentration on an Amicon XM50 membrane and dialysis against PBS (20 mM), pH 7.0, NaCl (50 mM) resulted in 4.0 mL of IgG1 and 4.0 mL of IgG2.

1.7. FCS Measurements

The measurements were performed on Alba FCS, the dual-channel fluorescence correlation instrument form ISS (Urbana-Chaimpaign, IL U.S.A.).

The instrument combines a confocal scanning microscope with FCS and uses avalange photodiodes as detectors.

A confocal epi-illuminated fluorescence microscope (Eclipse TE300, Nikon) was used for these measurements and a single Argon Ion laser at 488-nm (Argon Ion laser IMA 100, Melles Griot).

The laser with 34 µW power, out of objective at, 488 nm was focused into the sample by with oil immersion objective 60 × 1.2 (Zeiss, Jena). The resulting fluorescence was collected through the same objective and separate from the laser light by dichroic mirror (Croma). A 50 µm pinhole was used in the confocal detection channel.

All pinhole adjustments, shutters, optics, filter wheels, XYZ-fine positioning of the stage and the positioning of the objective are computer controlled through Vista**,** the FCS software package.

1.8. Data Analysis

All obtain data are fitting with Vinci Analysis program and we use the equation 1 photon 1 components 3D Guassian. The equation is the sequent:

$$G(\tau)=\left(\frac{1}{\pi\sqrt{\pi}w_0^2 z_0 < C >}\right)\frac{1}{\left(1+\frac{4D\tau}{w_0^2}\right)\sqrt{1+\frac{4D\tau}{z_0^2}}}\exp\left[\frac{-(V\tau)^3}{w_0^2 z_0\left(1+\frac{4D\tau}{w_0^2}\right)\sqrt{1+\frac{4D\tau}{z_0^2}}}\right]$$

In this equation, $G(\tau)$ represents the autocorrelation function, C is the concentration of the fluorescence molecule, W_0 is the beam waist, Z_0 is the beam height, D is the diffusion coefficient, V is the excitation volume, π is Archimede s'constant, and τ is the time diffusion. For this analysis, the diffusion times of individual molecules (R6G, glutamine-binding protein, bovine serum albumin) were measured, and this values were used as fixed parameters.

2. RESULTS AND DISCUSSION

The Fig. (**1**) shows the absorption and emission spectra of the fluorescein-labeled GlnBP conjugate with OTA. The spectra, normalized to 0.1 OD, were recorded at room temperature. The ratio of the labeling of the conjugate protein was calculated from the absorption spectra and its value was 90%. The same procedure was performed for the fluorescein-labeled GlnBP conjugated to neomycin. The calculated labeling ratio resulted to be about 85 % (data not shown). These two labeled biomolecules, GlnBP-OTA-F and GlnBP-

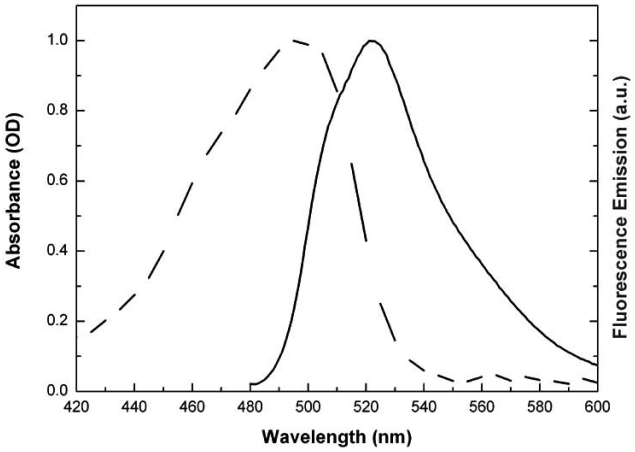

Figure 1. Absorption and emission spectra of fluorescein-labeled glutamine/binding protein-OTA complex.

Neo-F were used to develop a new fluorescence-based assay for the detection of OTA and neomycin in food, respectively.

The study of the interaction between the fluorescein-labeled GlnBP-OTA and the unlabeled antibodies anti-BSA-OTA was carried out in a three-step procedure. First, we studied the diffusion of GlnBP-OTA-F alone and the registered diffusion coefficient value was 128.71 $\mu m^2/s$. Second, we studied the formation of the complex fluorescein-labeled GlnBP-Neo/unlabeled Ab-anti-OTA.

In this case the formation of the complex was accompanied by a drastic variation of the diffusion coefficient from 128.71 $\mu m^2/s$ to 74.41 $\mu m^2/s$ as shown in Table **1**. The last step was the addition of different concentrations of unlabeled OTA to the complex fluorescein-labeled GlnBP-OTA-F/unlabeld Ab-anti-OTA. The result of the addition of increasing concentrations of unlabeled OTA was variation of the diffusion coefficient of the complex. In particular this value returned to the value of 102.66 $\mu m^2/s$.

The results are shown in Fig. (**2**), panel B. In particular, in panel B are reported three different curves related to GlnBP-OTA-F, or GlnBP-OTA-F/unlabeld Ab-anti-OTA, or GlnBP-OTA-F/unlabeld Ab-anti-OTA in the presence of 0.0078 ng of OTA.

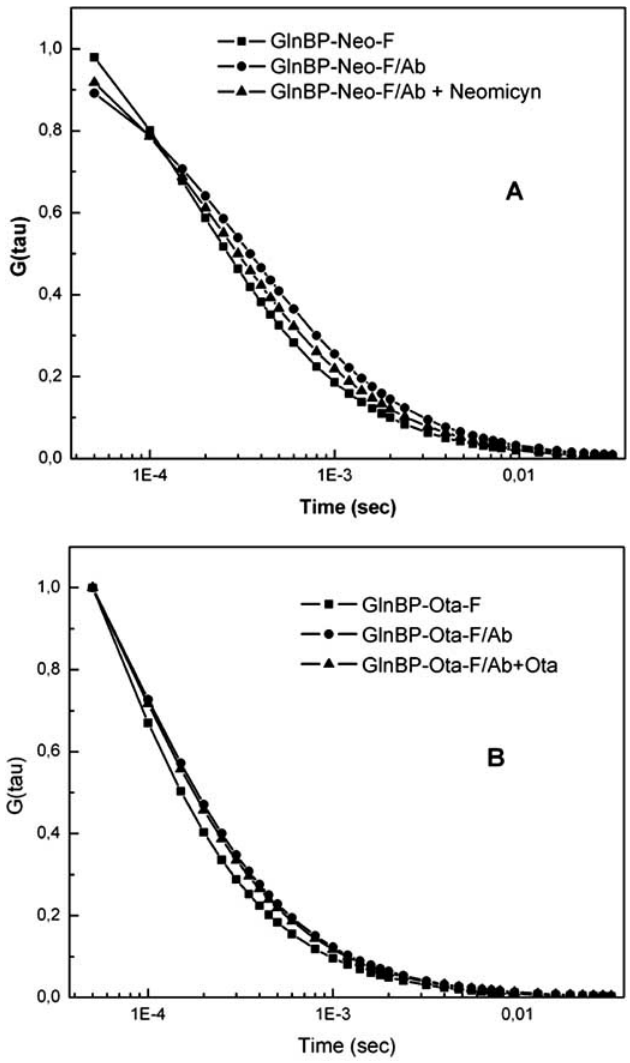

Figure 2. Fluorescence correlation spectroscopy experiments in the absence and in the presence of OTA and neomycin. The experiments were performed at 25°C.

The same experimental procedure was performed for GlnBP-Neo-F. The results showed a diffusion coefficient

Table 1. Effect of OTA Additions on the Coefficient of Diffusion

	D ($\mu m^2/s$)	W_0 (μm)	Z_0 (μm)	CPS	$\chi 2$
GlnBP-OTA-F	128.71	0.157723	5.64927	42373	1.03
GlnBP-OTA-F + Ab	74.41	0.157723	5.64927	54697	1.56
GlnBP-OTA-F + Ab + 0.0078 ng OTA	88.55	0.157723	5.64927	51182	1.42
GlnBP-OTA-F + Ab + 0.0156 ng OTA	102.66	0.157723	5.64927	50123	0.50
GlnBP-OTA-F + Ab + 2.0 ng OTA	99.70	0.157723	5.64927	54618	1.07
GlnBP-OTA-F + Ab + 6.0 ng OTA	93.51	0.157723	5.64927	56253	0.73
GlnBP-OTA-F + Ab + 8.0 ng OTA	103.78	0.157723	5.64927	56034	0.52
GlnBP-OTA-F + Ab + 12 ng OTA	110.42	0.157723	5.64927	57338	0.59

Table 2. Effect of Neomycin Additions on the Coefficient of Diffusion

	D ($\mu m^2/s$)	W_0 (μm)	Z_0 (μm)	CPS	$\chi 2$
GlnBP-Neo-F	137.9	0.317849	5.17311	18725	0.75
GlnBP-Neo-F + Ab	75.0	0.317849	5.17311	42865	5.3
GlnBP-Neo-F + Ab + 0.0156 ng Neo	102.2	0.317849	5.17311	30351	4.3
GlnBP-Neo-F + Ab + 0.0312 ng Neo	92.0	0.317849	5.17311	32932	3.7
GlnBP-Neo-F + Ab + 0.0624 ng Neo	113.1	0.317849	5.17311	23618	1.17
GlnBP-Neo-F + Ab + 0.1248 ng Neo	134.3	0.317849	5.17311	21961	0.96
GlnBP-Neo-F + Ab + 0.2496 ng Neo	102.0	0.317849	5.17311	23444	2.455
GlnBP-Neo-F + Ab + 0.5 ng Neo	108.0	0.317849	5.17311	23221	1.61
GlnBP-Neo-F + Ab + 1.0 ng Neo	112.0	0.317849	5,17311	22837	1.566
GlnBP-Neo-F + Ab + 2.0 ng Neo	101.0	0.317849	5.17311	22138	1.03

change from 137.9 $\mu m^2/s$ to 75 $\mu m^2/s$ in the presence of an antibody against Neo. In addition, we also registered the competition process between the complex and the unlabeled neomycin resulting in a variation of the coefficient of diffusion (Table **2** and Fig. **2**, panel A).

Taken together, the obtained results indicate that the combination of high-avidity IgG antibodies and the utilization of the fluorescence correlation spectroscopy methodology [12] has resulted in the detection limit of 0.0078 ng and 0.0156 ng for OTA A and neomycin, respectively, suggesting the application of this experimental strategy for analyses in which a high sensitivity detection is required.

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