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Peptide conjugated chitosan foam as a novel approach for capture-purification and rapid detection of hapten – Example of ochratoxin A

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ABSTRACT

A novel bioassay for the detection and monitoring of Ochratoxin A (OTA), a natural carcinogenic mycotoxin produced by Aspergillus and Penicillium fungi, has been developed and applied for the screening of red wine. Here we report the immobilization and orientation of NOF4, a synthetic peptide, onto 3-D porous chitosan supports using a N-terminal histidine tag to allow binding to M^{++} ions that were previously adsorbed onto the high surface area biopolymer. Three divalent cations ($M^{++}=Zn^{++}$, Co^{++}, Ni^{++}) were evaluated and were found to adsorb via a Langmuir model and to have binding capacities in the order $Zn^{++} > Co^{++} > Ni^{++}$. Following Zn^{++} saturation and washing, C-terminus vs. the N-terminus His-tagged NOF4 was evaluated. At 1000 $\mu g\,L^{-1}$ OTA the N-terminus immobilization was more efficient (2.5 times) in the capture of OTA. HRP labeled OTA was added to the antigen solutions (standards or samples) and together competitively incubated on biospecific chitosan foam. The chemiluminescence substrate luminol was then added and after 5 min of enzymatic reaction, light emission signals ($\lambda_{max}=425$ nm) were analyzed. Calibration curves of $\%B/B_0$ vs. OTA concentration in PBS showed that half-inhibition occurred at 1.17 $\mu g\,L^{-1}$, allowing a range of discrimination of 0.25 and 25 $\mu g\,L^{-1}$. In red wine, the minimum concentration of OTA that the system can detect was 0.5 $\mu g\,L^{-1}$ and could detect up to 5 $\mu g\,L^{-1}$. Assay validation was performed against immunoaffinity column (IAC) tandem reversed-phase high pressure liquid chromatography with fluorescence detection (HPLC–FLD) and provided quite good agreement. The association of chitosan foam and specific peptide represents a new approach with potential for both purification–concentration and detection of small molecules. In the future this assay will be implemented in a solid-sate bioelectronic format.

1. Introduction

Haptens are low molecular weight chemicals that comprise a major percentage of the universe of pesticides, herbicides, toxins, metals or allergens. Haptens then are small molecules that can elicit an immune response only when attached to a large carrier such as a protein; the carrier may be one that, by itself, also does not elicit an immune response. Therefore, it is very difficult to obtain affine antibodies to such molecules. Moreover, there has been increasing demands for ultrasensitive detection for these small target analytes that can potentially induce disease. This mycotoxin, Ochratoxin A, (a secondary fungal metabolite produced mainly by several *Aspergillus ochraceus* and *Penicillium verrucosum*) is a powerful nephrotoxic, teratogenic, immunosuppressive agent that has been implicated as an endocrine disruptor as well as a contributor to increased cancer risk. The International Agency for Research on Cancer (IARC) has classified OTA in Group 2B (possibly carcinogenic agent). OTA is mainly found in improperly stored foods such as cereals, dried fruit, nuts, and beverages such as beers and wine (Blesa et al., 2004a; Varga and Kozakiewicz, 2006; Zimmerli and Dick, 1995). Several authors have already reviewed wine contamination by OTA (Blesa et al., 2004b; Varga and Kozakiewicz, 2006). Wine is a major source of daily OTA intake for the population as it is widely consumed (Jørgensen, 2005). A level of 2 $\mu g\,kg^{-1}$ of OTA in wines has been established by the Regulatory Commission of the European Community (European Commission Commission Regulation, 2005; European Commission Regulation, 2010).

There is a standardized method for the determination of this mycotoxin in food matrices(Lerda, 2011); however, food and

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beverage producers are constantly looking for simple, less expensive and faster bioanalytical solutions. Indeed, validated standard methods for the detection of OTA are based on chromatographic techniques with fluorescence detection due to the fact that OTA possesses natural fluorescence (Sofie et al., 2010; Tessini et al., 2010; Aresta et al., 2006; Visconti et al., 1999). However, recent studies have proposed new lower cost assays with higher rapidity and sensitivity compared to the methods already on the market (Nasir and Pumera, 2014). These assays belong to three broad classes of analytical techniques: enzyme linked immunosorbent assays (ELISAs), test strips and biosensors.

First, OTA detection has been widely done by competitive ELISA in the past few years. Morgan et al. (1986), reported an assay in barley. Later, the sensitivity of this assay in barley samples was ca. 5 $\mu g\ kg^{-1}$ (Ramakrishna et al., 1990). Second, immunochromatographic strip (ICS) or the test strip, also called lateral flow device based on immobilized antibodies on a membrane, has also been used for the detection of OTA. These are semi-quantitative and give faster results (2–15 min) than ELISA assays (ca. 30–40 min). Test strips display different visual limits of detection (LOD) as a function of the nature of the sample (Krska and Molinelli, 2009; Shim et al., 2009). The LOD was initially set at ca. 500 $\mu g\ L^{-1}$ of OTA (Cho et al., 2005; Rusanova et al., 2009), but, nowadays, the Food and Drugs Administration has allowed a maximum of 1 $\mu g\ L^{-1}$, so the cutoff level was dropped down this the lower limit. Finally, immuno-biosensors are the last technique used to detect OTA in various samples, but their efficiency depends of the nature of the bioreceptor and biotransducer. A variety of immuno-biosensors were developed (e.g. electrochemical, impedimetric or conductimetric immunosensors) against mycotoxins like OTA (Alarcón et al., 2006; Liu et al., 2009; Prieto-Simón et al., 2008; Radi et al., 2009a, 2009b). Indeed, antibodies show high selectivity and affinity towards mycotoxins and so are configured into immuno-biosensors.

The use of antibodies in a detection system has some advantages (high affinity and selectivity) but also several major disadvantages depending the different sample matrix or experimental conditions of the assay. Among the disadvantages are denaturation and loss of structure-based activity in organic solvents, elevated temperatures or increased ionic strength. To overcome these drawbacks, diverse strategies have emerged. Alternatives to the use of OTA antibodies are being developed, including molecularly imprinted polymers (MIP) (Ali et al., 2010; Yu and Lai, 2010), peptides from phage display libraries (Giraudi et al., 2007) and DNA aptamers (Cruz-Aguado and Penner, 2008a, 2008b). DNA aptamers and synthetic receptor(s) (MIP) have some advantages compared to antibodies for the recognition of target molecules (He et al., 2013, 2012; Ma et al., 2013). We previously introduced a novel approach based on a peptide-based enzyme-linked immunosorbent assay (peptide-based ELISA) (Bazin et al., 2013). We have shown that the NF04 peptide allowed the detection of OTA in red wine in a sensitive manner. The use of peptides for the development of biosensors offers a number of attractive benefits. Peptides represent the simplest biological recognition elements for binding of some small molecules. There are examples of highly selective metal-binding peptide motifs available from the protein literature (Chow et al., 2005), for the recognition of OTA (Giraudi et al., 2007) and very recently for the binding of BPA (Yang et al., 2014). Chitosan is the second most abundant biopolymer on Earth and its ability in the sorption of divalent metal ions from aqueous solutions has been widely demonstrated; different authors have reported that chitosan is an effective low-cost sorbent due to its easy availability (Demey et al., 2014).

In the present study, we report a new bioanalytical format for the peptide-based ELISA assay. This new format allows the rapid capture, concentration and detection of hapten molecules such as OTA using NOF4. (Fig. SM1, Supplemental materials). The histidine tagged labeled peptide was immobilized and oriented on chitosan foam through adsorbed divalent zinc ions. Following washing step, OTA–HRP conjugate was added to antigen containing buffer solutions or wine samples and chemiluminescence was measured. As this approach is a based on a competitive ELISA format, the concentration of antigen is inversely proportional to the chemiluminescence light intensity measured.

2. Material and methods

2.1. Materials and chemicals

Solutions of metal ions were prepared from $ZnSO_4 \cdot 6H_2O$; $CoCl_2 \cdot 6H_2O$ and $Ni(NO_3)_2 \cdot 6H_2O$ reagents provided by Panreac (France). Chitosan was supplied by Aber Technologies (France) and its molecular weight (125,000 $g\ mol^{-1}$) determined using size exclusion chromatography (SEC) coupled with light scattering and refractometry was previously reported (Ruiz et al., 2001). The degree of deacetylation, determined by Fourier Transform infrared (FTIR) spectroscopy, was found to be 87% (Guibal et al., 1999). OTA was obtained from Sigma-Aldrich (France) from which a solution was prepared (1 $mg\ ml^{-1}$) in methanol at RT. Polyethylene glycol (PEG8000) and polyvinylpolypyrrolidone (PVPP) were obtained from Promega (France). Luminol was obtained from Pierce (France) and the peptide, NFO4, was synthetized by Smartox (France).

2.2. Preparation of chitosan adsorbent and chitosan foam

Chitosan foam, which served as a support for the immobilized NOF4 peptide, was prepared by dissolving chitosan to a final concentration of 2% (w/w) in 2% aqueous acetic acid solution under continuous agitation. The solution was poured into cylindrical molds (6 mm diameter/ height varies from 1 mm to 10 mm) and frozen for 3 h at 193 K. Then, the frozen solution was freeze-dried during 24 h (using a freeze-dryer Fisher Bioblock Scientific, France) at 223 K and 0.01 mbar. The resulting foam was put in contact with 1 M NaOH solution for 4 h and then was rinsed sequentially with 98% (w/w) and 50% (w/w) ethanol/water solution until pH 7. The resulting chitosan foam was used as sorbent of divalent metal ions (Zn^{+2}, Ni^{+2}, Co^{+2}).

2.3. Equilibrium sorption

In this work, the effectiveness of the chitosan foam to serve as a support for the immobilization of NOF4 via chelation with divalent metal ions was evaluated. In order to provide a material that was easy to handle, the metal ions Zn^{+2}, Co^{+2} and Ni^{+2} were separately introduced to the polymeric matrix of chitosan using a batch sorption system. The ability of chitosan to strongly interact with divalent metal ions through primary amine groups is well documented (Wan et al., 2010). Mono-component isotherms were obtained by mixing 0.2 g of sorbent (chitosan foam) at room temperature (20 °C) with a constant volume (0.2 L) of aqueous metal ion solution (Zn^{+2}, Co^{+2} and Ni^{+2}) at different concentrations (ranging from 50 to 6000 $mg\ L^{-1}$). The pH of the solutions was adjusted to 5.0 with a buffer solution (consisting of sodium acetate). After 72 h of agitation, the residual metals concentration was analyzed with an Inductively Coupled Plasma Atomic Emission Spectrometer ICP-AES (HORIBA JOBIN YVON, France) at the wavelength 221.6 nm for Ni^{+2}, 238.8 nm for Co^{+2}, and 213.8 nm for Zn^{+2}.

The Langmuir and Freundlich models were used to describe the experimental adsorption isotherm data. These models are