Food Chemistry 212 (2016) 688–694

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

A highly sensitive impedimetric label free immunosensor for Ochratoxin measurement in cocoa beans

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 12 April 2016
Accepted 12 June 2016
Available online 14 June 2016

Keywords:
Immunosensor
Ochratoxin A
Electrochemical Impedance Spectroscopy
Atomic Force Microscopy

ABSTRACT

In this work the development and optimization of an impedimetric label free immunosensor for the detection of Ochratoxin A (OTA) is reported.

Two antibody immobilization methods (oriented and not oriented) were compared highlighting a lower limit of detection (5 pg/ml) for the not oriented immobilization but a closer linear range in contrast to oriented anti-OTA immunosensors which showed linearity in the range of 0.01–5 ng/mL OTA. The analysis of the Atomic Force Microscopy (AFM) images showed two different nanostructures indicating that the use of oriented immobilization created a more ordered and highly dense antibody surface. Finally the oriented immunosensor was used to quantify OTA in spiked cocoa bean samples and the results were compared with those registered with competitive ELISA kit. The immunosensor was sensitive to OTA lower than 2 μg/kg that represents the lower acceptable limit of OTA established by European legislation for the common food products.

1. Introduction

Mycotoxins are toxic secondary metabolites responsible for the contamination of approximately 25% of the world's crops, causing spoilage of agricultural products. In pollutants risk assessment, experts consider these contaminants as the most important chronic dietary risk factor (Prieto-Simon, Campas, Marty, & Noguer, 2008).

Ochratoxin A (OTA) is a secondary metabolite produced by several species of *Aspergillus* and *Penicillium* fungi. The toxin, which is a nephrotoxic and nephrocarcinogenic compound, has mainly been found in cereals but significant levels of contamination may also occur in coffee, cocoa beans, wine, dried fruits, beer and grape juice spices. OTA is a proven carcinogen in animals and is classified as a class 2B, possible human carcinogen by the International Agency for Research on Cancer (Reddy & Bhoola, 2010). The National Toxicology Program (NTP) has designated OTA as "reasonably anticipated to be a human carcinogen" based on sufficient evidence of carcinogenicity in experimental animals (Clark & Snedeker, 2006). Regulations relating to mycotoxins have been established in many countries to protect the consumer from the harmful effects of these compounds. In several countries, these contaminants are subject to legislation that is based on the establishment of an Acceptable Daily Intake (ADI) or Tolerable daily intake (TDI). In the European Union the acceptable limits established for OTA in various foodstuffs are listed in Commission Regulation (EC) No 1881/2006 and ranged from 10 μg/kg for instant coffee and dried vine fruits to 0.5 μg/kg for dietary foods intended specifically for infants. OTA is a heat-stable molecule within the range of conventional food processing temperatures and no destruction occurs under normal cooking conditions such as boiling and frying, or even following pasteurization. Thus the accurate knowledge of OTA contamination level in food products represents a key factor in the food safety at worldwide level. Research studies have been conducted to develop appropriate methods for the detection of OTA in food and feed samples (Kaushik, Arya, Vasudev, & Bhansali, 2013). Traditional methods include gas chromatography, thin layer chromatography, capillary electrophoresis and high-performance liquid chromatography. However, these techniques require expensive equipments as well as complicated and time-consuming solvent cleanup steps. Owing to their high sensitivity, good specificity and less dependence on sample cleanup, electrochemical sensors based on immunological procedures seem most promising, thanks to their low cost, compatibility with miniaturization and portability (Muchindu et al., 2010). Therefore, immunosensors have aroused a very great interest with expectations of providing fast and highly sensitive detection of proteins,

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http://dx.doi.org/10.1016/j.foodchem.2016.06.034
0308-8146/

peptides, toxins, viruses and bacteria or part of these, finding widespread applications in clinical diagnostics, food safety and environmental monitoring.

In the field of food safety some studies have been focused on the development of electrochemical immunosensor for OTA determination; Bonel, Vidal, Duato, and Castillo (2010), Liu, Yang, Zhang, and Yub (2013) and Prieto-Simon et al. (2008) studied indirect competitive enzyme-linked immunosorbent assays (ELISA) strategies, developing labelled immunosensors for wine, wheat and corn samples analysis respectively. All measurements were conducted by differential pulse voltammetry technique. In these cases, the immunosensors required a label attached to the target: during readout the amount of label is detected and assumed to correspond to the number of bound targets. However, labelling a biomolecule can drastically change its binding properties and the yield of the target-label coupling reaction is highly variable (Daniels & Pourmanda, 2007). Moreover, the use of labels is also a source of higher costs and analysis times (Ricci, Volpe, Micheli, & Palleschi, 2007).

For these reasons, in the last years, the potential use of Electrochemical Impedance Spectroscopy (EIS) technique has been examined in the immunosensor development; it is, in fact, a powerful, nondestructive and informative technique, which can be used to study the electrical properties of the sensing device interface and to trace the reactions (Ciania et al., 2012). The application of EIS on modified electrodes on which antibodies have been immobilized let to develop label free immunosensors based on the impedimetric change that occurs when the immunocomplex occurred on the electrodes surface. Muchindu et al. (2010) and Radi, Munoz-Berbel, Latesc, and Martyc (2009) reported the development of impedimetric immunosensors for the detection of OTA in a linear range of 2–10 and 1–20 ng/mL respectively. Studies on the development of electrochemical aptasensor for detection of OTA have been recently published (Mishra, Hayat, Catanante, Istamboulie, & Marty, 2015). Even if aptamers offer many advantages in contrast to antibodies, i.e. they are easier and more economical to produce, the analysis of food samples require clean up procedures increasing time and cost analysis and thus reducing the advantages of the biosensors.

An important aspect that has to be considered during the fabrication of an immunosensor is the orientation of the sensing molecules on solid phase for improving sensitivity, specificity and analyte-binding capacity.

From the above the aim of this work is the development of a highly sensitive label-free impedimetric immunosensor for the detection of OTA in food samples. A Self Assembled Monolayer (SAM) procedure coupled with the oriented and not oriented immobilization of the monoclonal anti-OTA was used for the construction of the immunosensors and the EIS with Cyclic Voltammetry were used to characterize the immobilization steps and the performance of the immunosensors. The sensitivity and the topography by Atomic Force Microscopy (AFM) of oriented and not oriented immunosensors was also investigated. Finally the oriented immunosensor was used to quantify OTA in spiked cocoa bean samples and the results compared with competitive ELISA kit.

2. Materials and methods

2.1. Reagents

4-Mercaptobenzoic acid (MBA, 99%), 2-(*N*-morpholino) ethanesulfonic acid (MES >99.5% purity), N-Hydroxysuccinimide (NHS, 99%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, >99%), Sulfuric acid (H_2SO_4, 99.9%), Ethanolamine ($NH_2CH_2CH_2OH$, >99.5%), Potassium hexacyanoferrate (III) ($[Fe(CN)_6]^{3-}$, >99%), Tween 20, Ethanol (>99.8%) and Ochratoxin A were purchased from Sigma-Aldrich (Milano, Italy). Potassium hexacyanoferrate (II) ($[Fe(CN)_6]^{4-}$) was obtained from Carlo Erba reagent (Milano, Italy). Anti-Ochratoxin A antibody (anti-OTA) (1 mg/mL) was purchased from Abcam (Cambridge, United Kingdom), while Protein A/G (5 mg/mL, 59.7 kDa, >98%) was obtained from BioVision Inc. (San Francisco, USA). NaH_2PO_4, Na_2HPO_4, NaCl and KCl used in the preparation of phosphate buffered saline (PBS: 0.1 M KCl, pH 7.4) were received from Sigma Aldrich (Milano, Italy).

2.2. Apparatus

The electrochemical measurements were carried out with a computer-controlled Autolab PGSTAT 204 Potentiostat and Nova software. Au thin-film single-electrodes were obtained from Micrux Technologies (Oviedo, Spain). The electrodes incorporate a conventional three-electrode configuration, with an Au working (diameter 1 mm), reference and counter electrodes.

2.3. Immunosensor manufacturing

Before modification, gold electrodes were cleaned by applying 13 potential cycles between −1.0 and +1.3 V with 100 mV/s scan rate in 0.05 M sulfuric acid. SAM was carried out on the surface of the electrode using an ethanol solution containing MBA 30 mM under a constant potential of 1.2 V for 20 min.

The terminal carboxylic groups on gold electrode surface were activated by dropping on the Au modified electrode a solution of 75 mM EDC and 15 mM NHS in 100 mM MES buffer (pH 7.4) for 2 h.

Thereafter, the immobilization of anti-OTA was carried out in oriented and not oriented way. In the oriented immobilization method 20 μL of Protein A/G 5 mg/mL were dropped on the modified electrode and left to react for 1 h. After incubation, 100 μL of 1 M ethanolamine (pH 8.5) solution was dropped onto the modified surface and incubated for 15 min to block unreacted active sites. After thorough rinsing with PBS, the modified electrode was covered with 10 μL of anti-OTA solution at four different concentrations (0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL) for 30 min at room temperature. Finally the electrode was rinsed in PBS to remove unbound antibodies. In the not oriented construction of the immunosensor, the anti-OTA solution was added after the activation of carboxylic groups with EDC/NHS. Then the electrode was rinsed in PBS to remove unbound antibodies and finally the unreacted active sites were blocked with 1 M ethanolamine.

The schematic diagram of the immunosensors fabrication is presented in Fig. 1.

2.4. Experimental measurement

EIS measures the response of an electrochemical system to an applied oscillating potential as a function of the frequency resulting in an impedance spectra (Nyquist plot) where the complex impedance is displayed as the sum of the real and imaginary components (Z^I and Z^{II} respectively).

For electrochemical impedance studies, a sinusoidal AC potential (10 mV) in the frequency range from 0.1 to 10^5 Hz was super imposed to 0.00 mV (vs. reference electrode) DC potential. The measurements were performed in a solution of 1 mM ferri/ferrocyanide redox couple ($[Fe(CN)_6]^{4-/3-}$, 1:1) in PBS, pH 6.8, as background electrolyte at room temperature.

The CV was also used to characterize each step of electrode modification and anti-OTA immobilization. The measurements were performed from −0.6 to 0.6 V vs. reference electrode with a scan rate of 0.05 V/s; the redox couple used for the CV was the