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Enzyme immunoassay (ELISA/immunosensor) for a sensitive detection of pork adulteration in meat

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ABSTRACT

ELISA/immunosensor were developed for a sensitive detection of pork adulteration in meat. Two formats of ELISA were performed. First, an extracted IgG was directly immobilized in the microplate. This assay allowed an identification of 0.01% as level of pork adulteration in 14 h15 min. In order to decrease the time of the assay, a competitive ELISA was developed by immobilizing IgG standard, which compete with the extracted IgG. This assay allowed a detection of 0.1% of pork adulteration in 45 min.

Furthermore, two formats of electrochemical immunosensors were elaborated using the electro-entrapment of IgG in polymer modified graphite paste electrode. First, a direct immunosensor was capable of identifying 0.1 and 1% in raw and cooked meat respectively in 2 h. The second format was based on a competitive immunosensor, which was able to detect 0.01% of pork adulteration within 20 min.

Both competitive immunoassays revealed high sensitivity, good specify and reduced analysis time.

1. Introduction

Consumers are concerned by a variety of issues, such as food authenticity and adulteration (Kamruzzaman, Makino, & Oshita, 2015). Food choice normally reflects aspects of lifestyle, culture, religion, diet and health concerns (Lubis, Mohd-Naim, Alizul, & Ahmed, 2016). In most countries, food manufacturers choose the lard as a suitable ingredient for oil due to its cheap cost and its disponibility. In the view of some religious (Islam and Judaism) pork and lard are serious matters. Biological complications and health risks may be associated with daily intake (Kim, Seo, Yum, Jeong, & Yang, 2017).

Immunoglobulins (Ig), are found in sera from all species of vertebrates and are a family of globular proteins with a range of protective bioactivities that primarily assures an immune protection (Nollens et al., 2008). They form an essential component of the humoral immune system and are therefore sufficiently conserved to allow a comparison across a broad range of species (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). Depending on their structure, Ig are subdivided into three main classes, namely, IgG, IgM and IgA. The ability of IgG antibodies to diffuse easily throughout the extracellular fluid to the peripheral muscles make them the principal neutralizing antibodies for toxins found in tissues (Janeway, Travers, Walport, & Shlomchik, 2001, Flessner and Lofthouse, 1997). IgG is the most abundant type of antibody, is found in all body fluids and can be easily extracted and purified by the proposed method. Furthermore, IgG-species (immunoreactants), the anti-species IgG (antibody) and their enzyme conjugated antibody are cheap, available and can be applied for the authentication of any species in meat (not only pig) by using the same principle either by the developed ELISA or by immunosensor. Seen these informations, the present work is based on an immuno-enzymatic techniques (ELISA and immunosensor) using a simple extraction of IgG directly from muscle for meat authentication.

Due to its cheap cost than its meat counterpart, pork is often added in food products and has been identified as a potential adulterant in raw meat. For the detection of animal species in food products, food control laboratories have to be able to differentiate species used in raw materials (Aida, Man, Wong, Raha, & Son, 2005). Analytical methods commonly used for halal analysis or pork adulteration are based on proteins or DNA analysis, such as electrophoresis (Hernandez-Chavez, Gonzalez-Cordova, Rodriguez-Ramirez, & Vallejo-Cordoba, 2011), real-time polymerase chain reaction (RT-PCR) (Al-Kahtani, Ismail, & Ahmed, 2017), chromatography (Yan et al., 2016), PCR amplification of mitochondrial DNA (Karabasanavar, Singh, Kumar, & Shebannavar, 2014) and single strand conformational polymorphism (SSCP) analysis (Aali, Moradi-Shahrbabak, Moradi-Sharbabak, Sadeghi, & Kohram, 2016). However, these methods are not convenient for routine sample analysis because they are relatively costly, time consuming, laboriously demanding and complex to perform.

However, immunotechniques are characterized by their simplicity of sample preparation, absence of the need for complex equipment and

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qualified personnel and high productivity of serial testing. As well, for food authentication, electrochemical immunosensors are an alternative detection tool and are highly feasible for on-site usage; therefore, there is only one previously reported immunosensor for meat authentication (Lim and Ahmed, 2016). In a very recent work, an immune-strip test was developed for the detection of pork adulteration based on anti-swine IgG polyclonal antibody (Kuswandi, Gani, & Ahmad, 2017).

A high-affinity anti-pig IgG polyclonal antibody was evaluated to develop a user-friendly, rapid and sensitive ELISA (direct and competitive ELISA) for pork adulteration in beef meat samples. Moreover, for the first time, in the proposed study, a sensitive electrochemical immunosensor (direct and competitive immunosensor) based on a detection of pig-IgG was achieved via electropolymerized polypyrrole (PPy) modified graphite paste electrodes (PPy/GPE). The capture of pig-IgG was performed using peroxidase conjugated anti-pig IgG polyclonal antibody (anti-pig IgG-HRP) and the detection was monitored by chronoamperometry.

2. Experimental methods

2.1. Apparatus

ELISA measurements were performed using an automated ELISA plate reader (Biotek) by measuring the absorbance at 450 nm and the data were analyzed using Gen5 software. Chronoamperometric measurements, electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were carried out by an electroanalytical instrument (Palmsens BV Houten, Netherlands) in connection with a PC controlled by PSTrace software. Three electrodes system was employed, consisting of stainless steel as an auxiliary electrode, a reference electrode Ag/AgCl (saturated with KCl) and graphite paste electrode (GPE) as a working electrode. The electrodes were connected to the software and were immersed inside an electrochemical cell containing 3 mL of the electrolyte.

2.2. Reagents

The anti-pig IgG (whole molecule)-peroxidase antibody produced in rabbit and pure IgG from porcine serum (used as standard) were purchased from Sigma. Bovine serum albumin (BSA) as blocking agent was obtained from Amresco. Hydrogen peroxide (H_2O_2)/3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for ELISA and pyrrole were purchased from Sigma-Aldrich. All the used reagents were of analytical grade.

2.3. Preparation of simulated pork adulterated beef meat

In order to study the sensitivity of the immune-enzymatic techniques (ELISA/immunosensor), pork adulterated beef meat were prepared by spiking pork to beef meat in concentration range of 1; 5; 10; 25; 50; 100 (%w/w). Seen the difficulty of measuring the meat below 0.01 g, the meat adulteration in the range of 0.1 to 0.001% was performed by mixing well-defined proportions of beef protein extract with pork protein extract.

Meat containing 100% of pork was also made to be used as a positive control, while 100% of beef or no pork (0%) was used as negative control.

2.4. Preparation of simulated pork adulterated beef meatballs

Meatballs were prepared by emulsifying fine ground meat (beef and or pork) and mixing it with salt and certain spices, and finally shaping it into balls to make uncooked meatballs. It was then cooked in boiling water for 20 min. Afterword, the meatball was cooled and stored in refrigerator for further used. In order to study the sensitivity of the immunosensor, pork adulterated beef meatballs were prepared by spiking pork to beef meatballs in a concentration range of 1; 10; 20; 40; 80% (%w/w). Meatball containing 100% of pork was also made to be used as positive control, while the beef meatball sample containing 100% beef or 0% pork was included as pure or an unadulterated negative control.

2.5. Specificity test

Pork (Variant: *Sus Scrofa Domesticus*), beef (cross between *Brune Atlas* and *Charolaise*), lamb (*Sardi*), chicken (*Chair Ross*) and turkey (*Larger white*) extracts solution were prepared separately. The pork was used as comparison with other meats in order to show the specificity of the proposed immunosensor toward pork.

2.6. Preparation of meat extracts

Two types of meat samples, which are beef and pork meat were used. Beef meat was utilized as control in this study. The raw meat samples were purchased from a supermarket in Casablanca, Morocco. Each kind of meat samples (beef and pork) were freed from fat and connective tissue, then chopped and blended separately in a blender (Moulinex Bocshe) and a portion of 10 g was weighted. In order to avoid contamination among kind of prepared meat, every time the blender and other equipment used were thoroughly cleaned. The meat samples were stored at −20 °C until used for the protein extraction in order to minimize any deteriorative changes to the samples.

The extraction of protein from meat was performed as following: 2 g of meat samples was mixed with 15 mL of the extraction buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES) with 0.6 M sodium chloride (NaCl)) and incubated at 75 °C for 30 min, a rapid stirring followed by centrifugation for 10 min (10.000 rpm) was carried out. Then, the supernatant was recovered and filtered three times with coffee filters (Scheme 1).

The cold extraction was performed by mixing 1 g of meat samples with the extraction buffer without temperature incubation and the proteins extracted were kept in ice throughout the extraction process.

2.7. Protein purification

After filtration of the protein extracts, 10 mL of ammonium sulfate 100% of saturation (6.27 g in 10 mL of H_2O) was added, then the mixture was centrifuged for 10 min at 10.000 rpm. The precipitate was then solubilized in 3 mL of 0.154 M NaCl, which correspond to the physiological saline concentration in the muscle. The final protein extracts were stored at −20 °C until use (Scheme 1). The concentration of extracted and purified proteins was determined based on their absorbance at 280 nm by spectrophotometer (HACH LANGE, DR 2800, Germany).

2.8. Direct ELISA procedure

The pig-IgG extracted from meat were diluted in phosphate buffered saline (PBS) (0.1 M, pH 7.4) and were coated (100 µL/well) and incubated overnight at 4 °C. Then, the 96 well plate was washed with PBS then blocked using BSA (5%) and incubated at room temperature for 1 h. Subsequently, diluted HRP-conjugated anti-pig IgG (1:1000) was added (100 µL/well) and the plate was incubated at 4 °C for 1 h followed by a washing step. As a final step, H_2O_2/TMB (200 µL/well) liquid substrate system for ELISA (1/20 diluted in citrate buffer pH 5.0) (see optimization of different concentration of the TMB/H_2O_2 in supplementary material s.m.1) was added to each well and was incubated 15 min in the dark. Sulfuric acid 0.1 M (50 µL/well) was used to stop the enzymatic reaction and the absorbance was measured at 450 nm using ELISA plate reader (Biotek) (see data in brief. Scheme 1).