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Development of an enzyme-linked immunosorbent assay for the detection of gentamycin residues in animal-derived foods

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed using a polyclonal antibody with high affinity and specificity for detecting gentamycin in animal-derived foods. The half-maximum inhibition concentration (IC_{50}) and limit of detection (LOD, calculated as IC_{15}) of the ELISA for gentamycin in phosphate buffer were 0.3 and 0.03 ng/ml, respectively. The assay showed low cross-reactivity with other aminoglycoside antibiotics except sisomycin (52.5%), which indicated that the assay had high specificity. The trichloroacetic acid solution was selected as extraction buffer for eight animal-derived foods to effectively eliminate the matrix effect and extract gentamycin. The recoveries for gentamycin from eight fortified food samples, at three concentrations of 8, 20, and 50 μg/kg, were ranged from 69% to 118% and the coefficients of variation were less than 12%. The LODs of this assay for gentamycin in eight chosen animal-derived foods were 3 μg/kg. The whole detecting process could be finished within 1.5 h. The ELISA allows for a rapid, sensitive, specific, accurate, and low-cost determination of gentamycin residues in animal-derived foods.

1. Introduction

Gentamycin is an aminoglycoside antimicrobial agent produced by fermentation of *Micromonospora purpurea* or *Micromonospora echinospora* (Prins, Buller, Kuijper, Tange, & Speelman, 1993). Its mechanism of action is probably analogous to that of streptomycin: interaction with the ribosome to induce inappropriate amino acid incorporation into a protein during its synthesis (Hahn & Sarre, 1969). However, gentamycin is not a single molecule but a complex of three major and several minor components (Isoherranen & Soback, 2000), which make it difficult to be further separation. Therefore, gentamycin is marketed as a mixture.

Gentamycin is toxic in man, manifested as loss of hearing or of vestibular function (ototoxicity) and impairment of renal function (nephrotoxicity) (Wersall, Lundquist, & Bjorkroth, 1969). For this reason, in many countries maximum residue limit (MRL) of gentamycin have been defined. European Union (EU) established MRL as 50 μg/kg for meat and fat, 100 μg/kg for milk, 200 μg/kg for liver, 750 μg/kg for kidney (EC. No. 2377/90). Up to date, several methods such as chromatography (Al-Amound, Clark, & Chrystyn, 2002; Isoherranen & Soback, 1999; Niessen, 1998), microbiological growth-inhibition assay (Lantz, Lawrie, Witebsky, & MacLowry, 1980; Rosner & Aviv, 1980) and radioimmunoassay (RIA) (Mahon, Ezer, & Wilson, 1973) for the detection of gentamycin have been developed. But due to the requirement of expensive instruments, long time and many manipulations in these methods, their application has been limited. Among the immunoassays for gentamycin, some dealt with a few complex samples, but with poor sensitivity (Haasnoot et al., 1999; Jin, Jang, Han, & Lee, 2005, 2006; Jin, Jang, Lee, & Han, 2006). Others reported higher sensitivity but only applied in swine tissues (Chen, Shang, Li, Wu, & Xiao, 2008). Due to the widespread use of gentamycin in the treatment of animal diseases as antibiotic, resulting in more and more prominent hazards in food, there is an urgent need to develop a rapid detection method for gentamycin with high sensitivity in complex food samples. This article is aimed to overcome the shortcomings, establish a simple and rapid detection method with high sensitivity, which may be applied in detecting gentamycin residues in food products from different animal species.

2. Materials and methods

2.1. Reagents and chemicals

Gentamycin sulfate, sisomycin sulfate, kanamycin sulfate, tobramycin sulfate, amikacin sulfate, neomycin B sulfate, streptomycin

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sulfate, ovalbumin (OVA), keyhole limpet hemocyanin (KLH), 3,3′,5,5′-tetramethylbenzidine (TMB), hydrogen peroxide 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC), glutaraldehyde (GDA), horseradish peroxidase (HRP), Freund's complete and incomplete adjutants were purchased from Sigma–Aldrich (St. Louis, MO, USA). Dimethyl sulphoxide (DMSO) and bovine serum albumin (BSA) were obtained from Merck (Darmstadt, Germany). Protein A-Sepharose 4B was obtained from Amersham Biosciences (Uppsala, Sweden).

2.2. Materials and instruments

Polystyrene 96-well microwell plates were obtained from Nunc (Rockilde, Denmark), and a microplate washer was purchased from Bio-Rad (Hercules, USA). Immunoassay absorbance was measured with a Multiskan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland) and operated in the dual-wavelength mode (450 nm as test wavelength and 650 nm as reference wavelength).

2.3. Solutions

Phosphate-buffered saline (PBS, pH 7.4) was consisted of 10 mmol/l sodium phosphate and 0.137 mol/l NaCl. The coating buffer (CB) was sodium carbonate buffer (50 mmol/l, pH 9.6), and the PBST was PBS containing 0.4 mmol/l Tween-20. TMB substrate solution was prepared by adding 4.5 mg of TMB in 450 μl of DMSO to 14.6 ml of phosphate citrate buffer (0.1 mol/l citric acid + 0.2 mol/l Na_2HPO_4; pH 4.3) containing 3.25 μl of a 1.29 mol/l H_2O_2 solution, and the sulfuric acid (1.25 mol/l) was used as terminating solution.

2.4. Preparation of protein and enzyme conjugates

The gentamycin protein or enzyme conjugate (KLH for immunogen, OVA for coating antigen, HRP for enzyme tracer) was prepared by two methods in our experiment, using EDC (Mahon et al., 1973) and GDA (Chen, Wang et al., 2008) as linker, respectively. 10 mg of protein (enzyme), 30 mg of gentamycin were dissolved in 1 ml potassium phosphate buffer (50 mmol/l, pH 9.3). Then 400 mg of EDC or 25 μl of GDA (50 g in 100 ml) freshly prepared in 0.5 ml of double distilled water was added dropwise into the above mixture. Under stirring, the reaction mixture was kept at room temperature for 2 h and at 4 °C overnight. Then the resulting solution was dialyzed against PBS for 3 days.

2.5. Antibody production

Antibodies were produced as described by Wang et al (Wang, Allan, Skerritt, & Kennedy, 1998). The specificities of antibodies were tested by an indirect competitive ELISA: the microplate wells were coated with 100 μl per well of the gentamycin–OVA diluted in coating buffer by incubation overnight at room temperature. The plate was washed three times with PBST and the unbound active sites were blocked with 200 μl of BSA/PBS (1 g in 100 ml) per well for 1 h at room temperature. After washing, a solution of 50 μl per well of standard and 50 μl per well of antiserum in PBS were added and incubated at room temperature for 1 h. After the plate was washed again, an aliquot of 100 μl per well of goat anti-rabbit IgG–HRP in PBS was added and incubated at 37 °C for 0.5 h. The plate was washed again followed by addition of 100 μl of TMB substrate solution into each well. The reaction was stopped with 1.25 mol/l sulfuric acid solution (50 μl per well) after an incubation of 30 min. The absorbance values were read in dual-wavelength mode (450 nm for test and 650 nm for reference).

The antiserum with highest specificity will be selected for further experiment. The antiserum was purified by protein A-Sepharose 4B affinity chromatography.

2.6. Direct competitive ELISA

The microplate wells were coated with purified antibodies at 0.5 μg per well in 100 μl of coating buffer by incubation at 37 °C for 2 h. The plate was washed three times with PBST and blocked with 200 μl of BSA/PBS (1 g in 100 ml) per well by incubation at 37 °C for 0.5 h. After washing, a solution of 50 μl per well of standards or analytes and 50 μl per well of gentamycin–HRP in PBS were added and the mixtures were incubated for 1 h at room temperature. The plate was washed again followed by addition of 100 μl TMB substrate solution into each well. The reaction was stopped after an incubation of 30 min and the absorbance values were read in dual-wavelength mode (450 nm for test and 650 nm for reference).

2.7. Determination of cross-reactivity

The specificity of the antibody was evaluated by the determination of the cross-reactivity with other aminoglycoside antimicrobial agents, such as sisomycin, kanamycin, tobramycin, amikacin, neomycin B and streptomycin. The cross-reactivity (CR) values were calculated as follows:

$$CR(\%) = IC_{50}(\text{gentamycin})/IC_{50}(\text{compound}) \times 100$$

2.8. Extraction of samples

Eight varieties of animal-derived food samples, including pork, chicken, beef, pig kidney and liver, egg, fish, and milk, were purchased from the local supermarkets. Until recovery experiments, all chosen samples were analyzed by commercial gentamycin ELISA kit (Euro-Diagnostica, Holland) and these samples were certified as free of gentamycin. For recovery study, all samples were fortified with gentamycin to give the final concentration at 8, 20, and 50 μg/kg.

The extraction and dilution procedures of samples are described as follows:

Tissue: Tissue samples were stored at −20 °C until analysis. A 1.0 g aliquot of muscle, liver or kidney sample was transferred into a 15 ml plastic centrifuge tube and 2 ml of trichloroacetic acid (TCA) solution (0.46 mol/l) was added. The sample was thoroughly vortexed for 2 min and centrifuged at 5000 × g for 10 min at 4 °C, the supernatant was separated and diluted 50-folds with PBS and then used for analysis.

Milk: 10 ml of milk sample was centrifuged at 5000 × g for 10 min at 4 °C to remove the fat. 2 ml of the liquid layer was transferred into a plastic centrifuge tube and 240 μl of TCA solution (0.46 mol/l) was added. The sample was thoroughly vortexed and centrifuged at 5000 × g for 5 min at 4 °C, the supernatant was separated and diluted 100-folds with PBS and then used for analysis.

Egg: Yolk and egg white were mixed adequately and stored at −20 °C, then defreezed before using. 1.0 g of sample and 2 ml of TCA solution (0.46 mol/l) were thoroughly mixed for 2 min and centrifuged at 5000 × g for 10 min at 4 °C. The supernatant was diluted 20-folds with PBS and then used for analysis.