Biosensors and Bioelectronics 51 (2014) 426–430

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

An indirect competitive assay-based aptasensor for detection of oxytetracycline in milk

Chong-Han Kim [a], Lyun-Pyo Lee [a], Jeong-Ran Min [a], Myung-Woon Lim [b], Sang-Hee Jeong [a,*]

[a] Department of Bio Applied Toxicology, Hoseo Toxicology Research Center, Hoseo University, Asan 336-795, South Korea
[b] Joongkyeom Co., Ltd., Ansan 425-836, South Korea

ARTICLE INFO

Article history:
Received 28 May 2013
Received in revised form
3 August 2013
Accepted 5 August 2013
Available online 13 August 2013

Keywords:
Oxytetracycline
Aptamer
SELEX
Single-stranded DNA
Indirect competitive assay

ABSTRACT

Oxytetracycline (OTC) is a common antibacterial agent used for the control of animal diseases. OTC abuse can seriously affect human health; therefore, we developed a biosensor using single-stranded DNA (ssDNA) aptamers for the detection of OTC. The binding probe aptamers for OTC were selected by a Systematic Evolution of Ligands by the exponential enrichment (SELEX) process and identified by the enzyme-linked aptamer assay (ELAA). Among the selected 5 aptamers, aptamer OTC3 showed the strongest affinity (K_d=4.7 nM) and highest specificity for OTC compared to structurally similar antibiotics, tetracycline and chlortetracycline. OTC was detected using indirect competitive ELAA. The limit of detection and quantitation with aptamer OTC3 were 12.3 and 49.8 μg/L, respectively, in milk and showed recovery rates of more than 90% in OTC-spiked milk. This biosensor method with high sensitivity and specificity based on indirect competitive ELAA can be applied to OTC detection in food products on-site because of the simplicity of detection.

1. Introduction

Oxytetracycline (OTC) is a member of the broad-spectrum tetracycline (TC) group of antibiotics. TCs are widely used to prevent bacterial infections in livestock and increase their growth rate. Abuse of TCs in farm animals can cause accumulation of antibiotics in food products, including meat, milk and chicken eggs (Cinquina et al., 2003; De Wasch et al., 1998; Furusawa, 1999). Ultimately, this accumulation is likely to have serious implications for human health. Antibiotic residues in milk can trigger the emergence of antibiotic-resistant bacteria (Kitazono et al., 2012) therefore, several countries have set maximum residue limits (MRLs) for many food products (Casella and Picerno, 2009), and extensive efforts have been made to develop a sensing system for the enhanced detection of antibiotics in contaminated food products (Mehta et al., 2011; Niazi et al., 2008a; Song et al., 2012).

Traditionally, chromatography methods, including high-performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC–ESI-MS/MS), have been used for the detection of TCs in food products (Lv et al., 2012; Boscher et al., 2010). Although these methods provide simultaneous and accurate detection of TCs, they demand expensive equipment, tedious sample extraction procedures, and expert technical skill. Moreover, immunochemical methods using antibody have been used owing to simplicity and cost-effectiveness and detection with high sensitivity and specificity (Pastor-Navarro et al., 2007), but the use of an antibody as a probe for detection has drawbacks in terms of stability and production.

Aptamers are short single-stranded oligonucleotides with a three-dimensional structure that show high affinity binding and high-specificity target recognition (Jenison et al., 1994; Shangguan et al., 2007). Aptamers have a number of advantages over antibodies because they are small and can be raised against any type of target, including toxic compounds or poor immunogenic targets (Bruno et al., 2009), in a reproducibly synthesized and stabilized manner (Ahmad et al., 2011; Juskowiak, 2011). In addition, a variety of derivatives such as labeled molecules can be conveniently attached at the 3′ or 5′ end of an aptamer without affecting the target-binding site (Huang et al., 2010; Pultar et al., 2009). In recent years, many biosensors using ssDNA aptamers selected by the Systematic Evolution of Ligands using the EXponential enrichment (SELEX) process have been reported for various small molecular targets (Ding et al., 2009; Reinemann et al., 2009; White et al. 2001). Aptamers for several antibiotics belonging to the TC class have been used for the development of biosensors for the detection of TCs in many food products from animals. Electrochemical aptasensor showed a lower limit of detection (LOD, 1 ng/mL) and short detection time (Zhang et al., 2010). However, this sensing system required a special instrument for signal detection, and the immobilization procedure of the aptamers on an electrode is time

* Corresponding author. Tel.: +82 41 540 9675; fax: +82 41 540 9829.
E-mail address: jeongsh@hoseo.edu (S.-H. Jeong).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.08.003

consuming. Colorimetry has been proposed as a simple technique for the detection of signaling with the naked eye on-site. Despite the convenience, specificity, and sensitivity, detection of OTC in real food samples has not yet been applied. In addition, the observed LOD (5 μM) by naked eye was not significantly higher than the LOD (25 nM) by UV/vis spectrophotometer analysis for OTC detection (Kim et al., 2010; Kim et al., 2011).

In this study, we performed an indirect competitive enzyme-linked aptamer assay using a biotin-labeled aptamer for the detection of OTC in milk for the first time. This proposed method offers good sensitivity (LOD=27 nM in milk) similar to colorimetric aptasensor with high specificity and stability, and does not involve complicated sample extraction steps.

2. Materials and methods

2.1. In vitro selection of aptamers for OTC

A random ssDNA library containing 40 random nucleotides, flanked by invariant primer annealing sites (5′-CGTACG-GAATTCGCTAGC-N40-GGATCCGAGCTCCACGTG-3′), was used for the initial pool. Three different primers were used for PCR amplification: aptF, 5′-CGTACGGAATTCGCTAGC-3′; aptR, 5′-CACG-TGGAGCTCGGATCC-3′; and aptRBioT (equivalent to aptR with 5′ Biotin). Prior to use, 10 μM of the random DNA library pool was dissolved in 100 μL of binding buffer (20 mM Tris–HCl, 50 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, pH 7.2) and denatured at 95 °C for 10 min, quickly cooled at 4 °C for 5 min, and then placed at RT for 5 min.

Selection of aptamers for detecting OTC was performed using a SELEX method. First, the OTC–BSA conjugate (OTC–BSA conjugate for aptamer selection was purchased from Glory Science Co., TX, USA) or BSA was adsorbed onto the wells of microtiter plates. The coating step was performed in 50 mM carbonate buffer, pH 9.6. The plates were coated with 100 μL/well of 5 μg/mL of OTC–BSA conjugate or BSA solution and incubated overnight at 4 °C. The incubated plates were covered with 200 μL/well of 1% (w/v) BSA solution prepared in the binding buffer for 60 min at 37 °C, and unbound BSA was removed. The denatured ssDNA library pool was then added to the BSA-coated wells to eliminate the candidates capable of nonspecific binding of BSA. The unbound ssDNAs were collected and added to the OTC–BSA coated wells for 60 min at 37 °C. After incubation, the unbound and weakly bound ssDNAs were removed and washed 3 times with PBS containing 0.01% Tween-20 (PBST). The bound ssDNAs to OTC were incubated in 100 uL of 20 mM NaOH at RT for 10 min with mild shaking, and the ssDNAs were eluted. The process was repeated 2 times to elute the bound ssDNAs. The eluted oligonucleotides were precipitated by ethanol precipitation and the ssDNAs were dissolved in a final volume of 10 μL of EB buffer (10 mM Tris–HCl, pH 8.5). To monitor the progression of SELEX, the amount of eluted ssDNAs from OTC–BSA-coated wells was measured by Nanodrop device. The eluted ssDNA fraction was amplified by PCR (1 cycle at 95 °C for 5 min; 35 cycles at 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s; followed by 1 cycle at 72 °C for 5 min), using a biotinylated reverse primer (aptRBioT) that allowed the subsequent purification of the aptamer strand by alkali denaturation on Dynabeads streptavidin M-280 (Wochner et al., 2007). The amount of separated ssDNAs was measured using a Nanodrop device, and the separated ssDNAs were used for the next rounds for further enrichment of aptamers. The selection procedure was repeated until the twelfth round. After the last round, the eluted ssDNAs were amplified with unmodified primers aptF and aptR, and cloned into the TOPO PCRII plasmid vector (Invitrogen). Aptamer inserts of 12 candidates were then sequenced.

2.2. Binding assays of aptamers for OTC

Sequenced OTC-specific aptamers were synthesized as biotinylated aptamers and tested for their individual characteristics for binding to OTC. These aptamers were also verified for their specific binding affinity using OTC–BSA-conjugate or BSA-coated plates. The random oligonucleotide (Oligo) library was used as the negative control. First, biotinylated aptamers were heated at 95 °C for 10 min, cooled at 4 °C for 5 min, and placed at RT for 10 min. Each aptamer (5 nM) was then incubated for 60 min at 37 °C in BSA-coated or OTC–BSA coated wells of microtiter plates blocked by 1% (w/v) OVA in the binding buffer for 60 min at 37 °C. After the binding reaction, a streptavidin–HRP (0.5 ng/mL) solution was added and allowed to react (30 min, 37 °C). In the final step, 100 μL of TMB solution was added to measure the absorbance after 30 min at 450 nm (after quenching with 100 μL/well of 1 N H_2SO_4). Each step was performed with constant shaking and protection from light. The wells were thoroughly rinsed 3 times with PBST between each step. The assays were performed in triplicate.

The K_d of the aptamers OTC3, OTC6, OTC9 and OTC12 were determined. Binding reactions were conducted as described above, but with increasing amounts of biotinylated aptamers (1–100 nM). The amount of OTC-bound aptamers was determined by absorbance measurement at 450 nm and converted into the corresponding percentage of binding. The value was determined as follows: $(A/A_{100})\% = (A/A_{100}) \times 100$, where A is the absorbance value of the amount of aptamer with a concentration ranging from 1 to 100 nM and A_{100} is the absorbance value of 20 nM of aptamer OTC3. K_d was calculated by nonlinear regression analysis.

2.3. Indirect competitive enzyme-linked aptamer assay (ic-ELAA)

The indirect competitive OTC binding assay was performed as previously described. Competition was allowed to proceed by adding nonlabeled OTC and biotinylated aptamers. Solutions were prepared in the binding buffer, and the competition time was 60 min at 37 °C. After the competitive reaction, a streptavidin–HRP solution was added and allowed to react. Finally, 100 μL of TMB solution was added, and the absorbance was measured at 450 nm. The assays were performed 5 times. The absorbance values were converted into their corresponding test inhibition values (A/A_0) as follows: $(A/A_0)\% = (A/A_0) \times 100$, where A is the absorbance value for the competitive assay and A_0 is the absorbance value for the noncompetitive assay.

2.4. Specificity of the OTC aptamer for TCs

Analysis of the specificity of OTC aptamers for TCs was performed in the same way as the ic-ELAA method. TC and chlortetracycline (CTC), which are structurally similar to OTC, were used as cross-reactants, and the presence of cross-reactivity was calculated by determining the inhibition values of the competitive assay.

2.5. Pretreatment for the analysis of milk samples

Milk samples were purchased from local supermarkets and stored at 4 °C before use. Milk contains protein, fat, carbohydrate and cations such as Ca^{2+} and Mg^{2+}, which can form chelation complexes with OTC. To remove these components, milk samples (5 mL) were mixed with 5 mL of McIlvaine buffer containing 20 mM EDTA (pH 5.0) and 0.5% (v/v) trifluoroacetic acid to denature milk proteins. The milk samples were then defatted and deproteinized by centrifugation at 4 °C for 20 min at 8000 rpm. Then, 1 M NaOH was added dropwise to the supernatant to adjust the pH to 7.0. The supernatant fluid was filtered