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Development of an indirect competitive assay-based aptasensor for highly sensitive detection of tetracycline residue in honey

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ABSTRACT

Tetracycline (TC) is widely used for prevention and control of animal diseases for its broad spectrum antimicrobial activity and low cost, but its abuse can seriously affect human health and may result in trade loss. Thus there is an imperative need to develop high-performing analytical technique for TC detection. In this study, we developed a biosensor based on an indirect competitive enzyme-linked aptamer assay (*ic*-ELAA). A 76mer single-stranded DNA (ssDNA) aptamer, selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX), was applied for the recognition and detection of TC in honey. The limit of detection was 9.6×10^{-3} ng/mL with a linear working range from 0.01 to 100 ng/mL toward TC in honey, and a mean recovery rate of 93.23% in TC-spiked honey was obtained. This aptasensor can be applied to detect TC residue in food with high sensitivity and simplicity, and it is prospective to develop useful ELAA Kits for TC determination in food.

1. Introduction

Antibiotic residue in foods of animal origin is one of the typical food safety issues and is deemed as an important health hazard owing to abuse and increasing antimicrobial resistance (Butaye et al., 2001). Tetracycline (TC) is a member of the broad-spectrum tetracycline group of antibiotics (tetracyclines, TCs) and can reduce affinity for prokaryotic tRNA by strong binding on the 30S ribosomal subunit (Epe et al., 1987; Spahn and Prescott, 1996). It has been widely used as veterinary drug and as feed additive, thus remains in finished food products including meat, milk, honey (Cinquina et al., 2003; Furusawa, 1999; Wasch et al., 1998). For honey, TC is often used for the treatment of bacterial brood diseases in apiculture, such as American foulbrood (*Bacillus larvae*) and European foulbrood (*Streptococcus pluton*) (Martel et al., 2006). However, there is no harmonious regulatory standard of residual TC in honey across the world. Some countries have set maximum residue limit (MRL) for TC in honey, while others have not because TCs are illegal for use with bees at any level and they do not tolerate any residue level (Li et al., 2008). For instance, the MRL for TC has been set at 300 μg/kg in Korea (Jeon and Rhee Paeng, 2008). A laboratory proposed 20 μg/kg was accepted as the recommended concentration limit but not official MRL for the screening of TC in honey in European Union (Discussion Paper Residue Expert Meeting, 2007). In China, the MRL for TC in honey was formerly set at 0.05 mg/kg (GB 14963-2003) and replaced by new regulation now (GB 14963-2011). Moreover, China is the leading producer and exporter of honey in the world. TC residue is a problem that should not be negligible as it is not only a threat to public health but also a hurdle to the international trade of apiarian products. Thus it is of great significance to develop efficient determination methods for detection of TC in honey.

In recent years, the determination of TC has been widely studied using officially accredited chromatographic methods, including high-performance liquid chromatography (HPLC) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Boscher et al., 2010; Khong et al., 2005; Lv et al., 2012; Martel et al., 2006; Nakazawa et al., 1999; Oka et al., 1994; Pagliuca et al., 2002; Samanidou et al., 2005; Viñas et al., 2004; Wang et al., 2003). They can provide simultaneous and precise results of detection, but require expensive equipments, tedious sample extraction procedures and professional technical skills. Complementary antibody-based methods for rapid detection of TC in food samples have been reported, such as enzyme-linked immuno-sorbent assay (ELISA) or gold immune-chromatographic assay (GICA) with simplicity and high-throughput screen ability. However, it left much to be desired in terms of antibody production, preservation, ethical problems with the use of animals, and the non-specific polyclonal or unsteady

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monoclonal binding properties for *in situ* or real-time analysis (Dong et al., 2013).

Novel aptamer-based biosensor (aptasensor) has been emerged as a powerful tool which can meet the requirements of simplicity, specificity, and sensitivity for the detection of diverse substances at trace levels (Park and Paeng, 2011). Aptamers are short single-stranded oligonucleotides of DNA or RNA showing high affinity binding and high-specificity target recognition. Aptamers are also named "chemical antibody" (Song et al., 2008) due to their artificial process using Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). Aptamers appear to be superior alternatives to antibodies or other biological recognition elements because they have the following advantages: (a) they can be selected *in vitro* in a stable way for various type of targets with uniform activity and not involved with immunogenicity (Jayasena, 1999; Kim et al., 2014; Nimjee et al., 2005; Tombelli et al., 2007); (b) they fold and bind upon the target molecules (Hermann and Patel, 2000; Song et al., 2008) and can be structurally modulated (Dong et al., 2013) or modified at 3′ or 5′ end (Huang et al., 2010; Pultar et al., 2009); (c) they demonstrate advantages respect to other "non-natural" receptor, such as oligopeptides, which cannot be amplified during their selection procedure (Tombelli et al., 2005). Studies on aptasensors utilizing different signal transducers, such as colorimetric (Liu and Lu, 2006; Medley et al., 2008; Stojanovic and Landry, 2002), optical (Lee and Walt, 2000; Li and Ho, 2008; McCauley et al., 2003), mass-dependant (Lee et al., 2008; Liss et al., 2002; Medley et al., 2008; Minunni et al., 2004), and electrochemical (Kim et al., 2008; Willner and Zayats, 2007) have been reported in the past few years.

Both DNA and RNA aptamer have been reported to bind specifically with TC (Berens et al., 2001; Müller et al., 2006; Niazi et al., 2008). Several studies have been reported to detect TC in food using aptamers, most of which are electrochemical aptamer-based biosensors to detect TC residue in milk (Kim et al., 2010; Zhang et al., 2010; Zhou et al., 2012), and few studies (Jeong and Rhee Paeng, 2012) are performed on microtiter plate platform which can achieve high throughput detection.

In this study, we developed an indirect competitive enzyme-linked aptamer assay (*ic*-ELAA) based on a 76mer-ssDNA (Kd = 63.6 nM) for the determination of TC in honey, and to the authors' knowledge, this study is the first report that uses validated ELAA for the determination of TC in honey. Furthermore, a biotin–streptavidin mediated system was introduced to improve target detect ability. The assay offers excellent sensitivity (the limit of detection, LOD = 9.6×10^{-3} ng/mL) with a wide linear range (0.01–100 ng/mL) and does not require complicated sample extraction steps.

2. Materials and methods

2.1. Reagents

A single-strand 76mer DNA aptamer was custom synthesized with 3′-end biotinylated modification by Sangon Biotech Co., Ltd. (Shanghai, China), and has the following sequence (Fig. 1): 5′-CGTACGGAATTCGCTAGCCCCCCGGCAGGCCACGGCTTGGG TTGGT CCCACTGCGCGTGGATCCGAGCTCCACGTG-3′-biotin (Mw. (23747.43), mp (83.77 °C)). TC–BSA was obtained from NanKai Biotech Co., Ltd. (Hangzhou, China). Tetracycline standard and Hammerstein bovine casein were purchased from Sigma-Aldrich (St. Louis, MO, USA) and 2-amino-2-(hydroxymethyl)-1, 3-propanediol (Tris) was purchased from Sigma-Aldrich (Shanghai, China). Bovine serum albumin (BSA), horseradish peroxidase labeled Streptavidin (SA–HRP) and 3,3′,5,5′-tetramethylbenzidine dihydrochloride (TMB) were purchased from

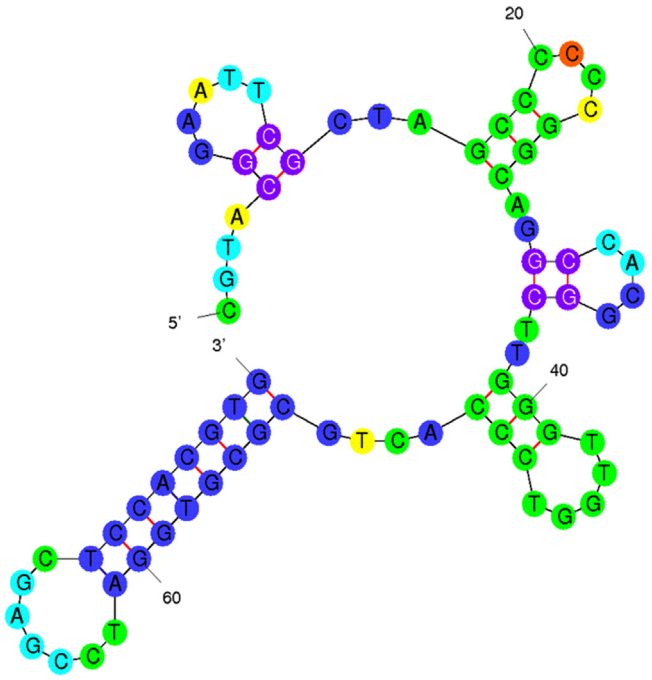

Fig. 1. Secondary structure of the 76mer ssDNA aptamer. The structure was predicted by m-fold program according to the free minimization algorithm. Color annotation means the probability.

KPL (Gaithersburg, MD, USA). All chemicals were of analytical grade and ultrapure water was prepared by Thermo Scientific Barnstead GenPure Water Purification System (Thermo Electron LED GmbH, Stockland 3, D-56412 Niederelbert). All buffers were filtered using 0.22 μm membrane filter before use.

2.2. Instrumentation

ELAA was performed in 8 well Flat-Bottom Immuno Plate (Nunc, Denmark). Absorbance was measured using *ELx800* absorbance microplate reader (BioTek Instruments, Inc., Winooki, Vermount, USA) at both 450 nm and 630 nm. The plates were washed using *ELx50* microplate strip washer (BioTek Instruments, Inc., Winooki, Vermount, USA). The absorbance of honey at 280 nm was obtained using Nanodrop2000C (Thermo Fisher Scientific Nanodrop2000C, Wilmington, Delaware, USA). Centrifugation was performed in Sigma refrigerated centrifuge 3K15 (Sigma Laborzentrifugen GmbH, An der Unteren Söse 50, D-37520 Osterode, Germany).

2.3. Optimization of assay conditions

Prior to the competition assay, the optimization was carried out to achieve an appropriate absorbance of negative control (non-competitive assay) around 1.0 in order to match with the sensitivity of the microplate reader and get an inhibition curve with relatively larger slope. The absorbance of the negative control was compared while varying concentrations of binder (aptamer) and competitor (TC–BSA). The TC–BSA conjugate (initial molar ratio is 3:1) was prepared in a concentration of 0.5–10 μg/mL (ppm) and the aptamer was diluted to 1–20 nM. Moreover, in light of the alteration of the recognition element with respect to ELISA, it was necessary to characterize the effects of coating buffer, blocking agent, binding buffer and concentration of SA–HRP. Thereupon five types of coating buffer were compared, referring to those usually used in the ELISA. They were 50 mM bicarbonate buffer (CB, pH 9.6), 10 mM Tris–HCl buffer (Tris, pH 8.0), 10 mM phosphate buffer saline (PBS, pH 7.4), 100 mM phosphate buffer (PB, pH 7.2) and