LWT - Food Science and Technology 64 (2015) 1138–1142

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Identification and characteristics of aptamers against inactivated *Vibrio alginolyticus*

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form
22 June 2015
Accepted 10 July 2015
Available online 13 July 2015

Keywords:
Detection
Affinity constant (Kd)
Secondary structure

ABSTRACT

Vibrio alginolyticus is an opportunistic pathogen that can infect many aquatic animals and cause economical losses to aquaculture and the food industry. Therefore, sensitive and rapid detection of *V. alginolyticus* is urgently needed. In the present paper, three aptamers were proved to have good specificity towards inactivated *V. alginolyticus*, and they could distinguish inactivated *V. alginolyticus* from inactivated *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Edwardsiella tarda*, *Aeromonas hydrophila* and *Escherichia coli*. Incubating heat- and formaldehyde-treated cells with aptamers #7, #8, and #23, washes with binding buffer, heat-induced release of bound aptamers, and application of the polymerase chain reaction to amplify the aptamers allowed the detection of 10, 10 and 10^3 cells/ml, respectively. Aptamer #8 had good affinity with both live and inactivated *V. alginolyticus*. The combination of aptamers #8 and #7, or aptamers #8 and #23, could distinguish live *V. alginolyticus* from inactivated *V. alginolyticus* based on their different affinities with the microorganism. The putative secondary structures were determined for these aptamers using software RNAstructure 5.3 and the affinity constants (Kds) 28.39 ± 11.46, 12.82 ± 6.00 and 46.89 ± 15.87 nM, were calculated respectively by employing their saturation curves. The results demonstrated that an aptamer-based method can be used for the detection of *V. alginolyticus*.

1. Introduction

Vibrio alginolyticus is widely distributed around oceans and estuaries, and it is one of the most serious opportunistic pathogens among marine *Vibrios* since it is abundant, highly pathogenic and involved in worldwide epidemics (Austin, 2010; Gonzalez-Escalona, Blackstone, & DePaola, 2006; Zhenyu et al., 2013). The pathogen can infect many kinds of aquatic animals such as fish, shrimp and mollusk, which causes huge economic losses to aquaculture and the food industry. It is also a human pathogen that can cause otitis, food poisoning and wound infection (Austin, 2010; Reina Prieto & Hervas Palazon, 1993; Scharer, Savioz, Cernela, Saegesser, & Stephan, 2011; Spark, Fried, Perry, & Watkins, 1979). Therefore, rapid and sensitive detection for *V. alginolyticus* is necessary in aquaculture and food industry to control the disease caused by the microorganism.

Traditional detection of the pathogen mainly relies on time-consuming cultivation of the microorganism (Lee, Han, Maeng, Cho, & Lee, 2012; Paolucci, Landini, & Sambri, 2010). Detection based on 16S rDNA is not feasible because of the high sequence similarity among the ribosomal genes from different *Vibrio* species (Jing-jing et al., 2011; Sawabe, Kita-Tsukamoto, & Thompson, 2007). Therefore, a sensitive, efficient and accurate detection method is required for the pathogen *V. alginolyticus*.

Aptamers are single strand oligonucleotides that can bind to their targets with high affinity and specificity based on their specific three-dimensional structures (Ellington & Szostak, 1990; Tuerk & Gold, 1990). They are selected from a randomized oligonucleotide library by an in vitro process called the systematic evolution of ligands by exponential enrichment (SELEX) method (Ellington & Szostak, 1990; Tuerk & Gold, 1990). Compared to antibodies, aptamers have many advantages such as high affinity, small size, easy chemical synthesis and modification, and lack of immunogenicity (Stoltenburg, Reinemann, & Strehlitz, 2007). Aptamers have been synthesized for the detection of microorganisms, enzymes,

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http://dx.doi.org/10.1016/j.lwt.2015.07.021
0023-6438/

toxins, as well as the diagnosis and therapy of some diseases (Stoltenburg et al., 2007). Since it is potentially useful to work with samples in which the pathogen has been inactivated and is no longer a safety concern, aptamers were developed that were able to bind inactivated *V. alginolyticus*. The affinity constants (Kds) and secondary structures of these aptamers were determined.

2. Materials and methods

2.1. Microorganisms

V. alginolyticus, *Edwardsiella tarda*, *Aeromonas hydrophila*, *Vibrio harveyi*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Escherichia coli DH5a* were provided and identified by the Laboratory of Pathogenic Microorganisms of Jimei University. *E. coli DH5*a was inoculated in Luria–Bertani (LB) medium, cultured in a 100 rpm shaker at 30 °C for 6–8 h and stored at 4 °C. The other six bacteria were inoculated in Tryptic Soy Broth (TSB) medium, cultured in a 100 rpm shaker at 30 °C for 6–8 h and stored at 4 °C. Bacteria were inactivated by adding a solution containing 6% formaldehyde and 0.9% NaCl, heating in a 62.5 °C water bath for 1 h, washing three times with 0.9% NaCl, and suspending in 2 × binding buffer (0.1 M NaCl, 5 mM KCl, 50 mM Tris–HCl, 1 mM $MgCl_2$, pH7.4), and stored at 4 °C (Spotts Whitney et al., 2003). Bacteria were counted and the concentration was calculated according to a linear fitting equation based on the bacteria counts and optical density.

2.2. Aptamers and primers

The sequences of the tested aptamers (#7, #8, #23) and two PCR primers (P1 and P2) are shown in Table 1. All the aptamers and primers were synthesized and labeled by Sangon Biotech Company (Shanghai, China).

2.3. Application of aptamers in detection of V. alginolyticus

The aptamers were diluted to 0.5 μM with 2 × binding buffer, denatured at 95 °C for 5 min and cooled in an ice bath for 10 min. One hundred microliter of the aptamer solution was mixed with 100 μl live bacteria suspension in their culture medium or inactivated bacteria suspension in 2 × binding buffer at different concentrations (10, 10^2, 10^3 or 10^4 cells/ml) on a rotary shaker at 30 °C for 30 min to allow formation of the binding complex. The mixture was centrifuged at 6000 × *g* for 5 min to isolate the aptamer–bacteria complexes, and the unbound aptamer in the supernatant was discarded. After six washes with 500 μl 1 × binding buffer, the bacterial complexes were suspended in 100 μl 1 × binding buffer, heated at 95 °C for 5 min and pelleted by centrifugation at 15,000 × *g* for 10 min. The supernatants containing the binding aptamers were collected and used as template for PCR (primer ratio P1:P2 = 1:1). The 25 μl PCR system contained 2 μl of 10 μM primer P1, 2 μl of 10 μM primer P2, 2 μl template, 2 μl of 10 × PCR buffer, 0.4 μl of 10 mM dNTP, 1U of Taq DNA polymerase. The PCR was performed by using the following thermocycling parameters: 94 °C for 4 min for denaturation followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 20 s. A final extension step at 72 °C for 7 min followed the last cycle. After amplification, the PCR products were analyzed by agarose gel electrophoresis. In negative control, bacteria suspension was replaced with distilled deionized water and carried out all the steps needed for the detection of bacteria. For the positive control, the bacteria suspension was replaced with an inactivated or live *V. alginolyticus* suspensions 10, 10^2, 10^3 cells/ml.

2.4. Affinity analysis of aptamer

The aptamers were labeled with digoxin by asymmetric PCR with primer P2 and a 5′-digoxin-modified primer P1 (primer ratio P1: P2 = 25: 1). The PCR mixture (25 μl) contained 2 μl of 10 × PCR buffer, 0.4 μl of 10 mM dNTP, 1U of Taq DNA polymerase, 2 μl of 10 μM 5′-Digoxin-modified primer P1, 2 μl of 0.4 μM primer P2, and 10 pmol of the aptamer. The thermocycling parameters were as follows: 94 °C for 4 min for denaturation followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 20 s. A final extension step at 72 °C for 7 min followed the last cycle. The concentration of ssDNA in the PCR products was determined with a K5500 micro-spectrophotometer (Beijing Kaiao Technology Development Co., Ltd, China). Ten pmol ssDNA PCR product was diluted to 100 μl with 2 × binding buffer. After denaturated at 95 °C for 5 min and cooling in an ice bath for 10 min, the diluted PCR products were mixed with 100 μl excess inactivated *V. alginolyticus* (4 × 10^8 cells/ml) on a rotary shaker at 30 °C for 30 min to allow formation of the binding complex. The mixture was centrifuged for 5 min at 10,000 × *g* for isolation of the ssDNA-bacteria complexes, and the unbound ssDNA in the supernatant was discarded. After resuspension in 500 μl 1 × binding buffer, the bacterial complexes were peletted by centrifuging at 6000 × *g* for 5 min. Subsequently, a 100 μl aliquot of horse reddish peroxidase (HRP) – conjugated antibody against digoxin (1:1000 diluted by TBS buffer composed of 0.05 mol/L Tris–HCl and 0.9% NaCl) was added to the complexes and incubated for 10 min. The reaction products were centrifuged at 6000 × *g* for 5 min and the supernatant containing unbound ssDNA was discarded. The bacterial precipitate was washed three times by resuspension in 500 μl 1 × binding buffer pelleting by centrifuging at 6000 × *g* for 5 min to remove weakly bound aptamers. The bacterial complexes were suspended in 400 μl ddH_2O, and incubated with 200 μl freshly prepared substrate (1 mg/ml TMB or tetramethylbenzidine: substrate buffer: 30% v/v H_2O_2 = 100:900:1) in the dark for 10 min. Finally, 200 μl of 2 mol/L H_2SO_4 was added to stop the color reaction. The absorbance of the final solution was measured at 450 nm using a BioTek microplate reader. This measurement was the affinity of the aptamer against the bacterium (Bruno & Kiel, 1999; Hao et al., 2011).

2.5. Determination of affinity constant (Kd) of aptamer

The ssDNA aptamers were synthesized and labeled with digoxin

Table 1
Sequences of the tested aptamers and primers.[a]

Name	Sequence
#7	5′-TCAGTCGCTTCGCCGTCTCCTTCGGGGGCGCGGTGAGGGGCTGCACAAGAGGGAGGCACAAGAGGGAGACCCCAGAGGG-3′
#8	5′-TCAGTCGCTTCGCCGTCTCCTTCAGCCGGGGTGGTCAGTAGGAGCAGCACAAGAGGGAGCACAAGAGGGAGACCCCAGAGGG-3′
#23	5′-TCAGTCGCTTCGCCGTCTCCTTCGTAGGAGGTAGTCGGAGAGGCGAATGAGAGGGGAAGCACAAGAGGGAGCACAAGAGGGAGACCCCAGAGGG-3′
P1	5′-TCAGTCGCTTCGCCGTCTCCTTC-3′
P2	5′-CCCTCTGGGGTCTCCCTCTTGTGC-3′

[a] Primer sequences in aptamers are shown underlined.