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Electrochemical DNA sensor for simultaneous detection of genes encoding two functional enzymes involved in lignin degradation

Zhen Li[a,b], Guangming Zeng[a,b,*], Lin Tang[a,b], Yi Zhang[a,b], Yuanping Li[a,b], Ya Pang[a,b], Jie Luo[a,b], Yuanyuan Liu[a,b]

[a] College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China
[b] Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, PR China

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ABSTRACT

An electrochemical DNA sensor for simultaneous detection of functional genes encoding manganese peroxidase (MnP) and cellobiose dehydrogenase (CDH) on a gold electrode was developed. After two thiolated capture probes assembled on the electrode surface, the electrode was exposed to a monolayer of 6-mercapto-1-hexanol (MCH) solution to prevent nonspecific adsorption of target DNA and detection probes. Horseradish peroxidase–streptavidin (HRP–SA) conjugate and laccase–streptavidin (LAC–SA) conjugate were applied for enzyme-amplified amperometric measurement. The two target genes were simultaneously quantified in the same system. The DNA conformation and surface coverage on electrode were characterized by impedance spectroscopy and cyclic voltammetry. The amperometric current responses to HRP and LAC-catalyzed reactions were linearly related to the common logarithm of two target nucleic acids concentrations, ranging from 1×10^{-11} M to 4×10^{-8} M and 1×10^{-10} M to 4×10^{-8} M. The correlation coefficients were 0.9884 and 0.9881, and the detection limits were 6.2×10^{-12} M and 3.0×10^{-11} M, respectively. The effectiveness of this DNA sensor was confirmed by simultaneous detection of two gene fragments extracted from *Phanerochaete chrysosporium* using polymerase chain reaction (PCR) and restriction endonuclease digestion. The DNA biosensor exhibited good selectivity, precision, stability and reproducibility.

1. Introduction

Biodegradation resistance of wood and other lignified materials is directly related to the presence of lignin [1,2]. Lignin is the most abundant natural aromatic polymers in nature and is extremely recalcitrant in an obligate aerobic oxidative process, carried out appreciably only by the white-rot fungi during secondary metabolism [3,4]. The white-rot basidiomycete *Phanerochaete chrysosporium* has become the model system for studying the physiology and genetics of lignin degradation [5]. Manganese peroxidase (MnP) and cellobiose dehydrogenase (CDH), the two key enzymes produced by *P. chrysosporium*, may mutually effect in an extracellular pathway in fungal lignin mineralization [6]. MnP is an extracellular heme-containing enzyme which can oxidize phenolic lignin model compounds and non-phenolic aromatics via radical intermediates [7,8]. Production and optimization of MnP were investigated by employing different agro-industrial wastes and using response surface methodology [9,10]. CDH is an extracellular hemoflavoprotein which has been proposed to prevent the polymerization reactions and increase the rate of depolymerization in depolymerizing lignocellulose [11,12]. Amperometric detection with mediated electron transfer and high pressure liquid chromatography (HPLC) were used to reveal the kinetic model and effect of CDH on lignin model compounds [13,14]. Over the past decade, MnP and CDH encoding genes have been cloned and characterized from a large number of basidiomycete fungi using polymerase chain reaction and molecular screening assays [15–20].

Recently, electrochemical DNA biosensors have received particular attention due to their fast response, high sensitivity and low cost in environmental and microbial analysis [21–25]. The combination of PCR amplification and DNA sensor was also utilized to improve the detection sensitivity [26,27]. Our group has developed an electrochemical DNA sensor based on the sandwich hybridization recognition of target sequence of lignin peroxidase genes [28], and the simultaneous determination of lignin peroxidase and manganese peroxidase activities using artificial neural networks was also studied [29]. Several papers have proposed that CDH displayed a synergism with MnP during lignin biodegradation and also discussed the relationship between the two enzymes [30,31]. In order to understand the synergistic effects of CDH and

* Corresponding author at: College of Environmental Science and Engineering, Hunan University, Changsha 410082, PR China. Tel.: +86 731 88822754; fax: +86 731 88823701.
E-mail addresses: happylizhen@yeah.net (Z. Li), zgming@hnu.cn (G. Zeng).

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Table 1
Sequences of oligonucleotides used in biosensor.

Oligonucleotide	Sequence (5′–3′)
Capture probe of MnP (M_1)	HS-$(CH_2)_6$-CTGATGGTGTCGTGTTTCT
Detection probe of MnP (M_2)	GATGCCGTTGTTGGCGGAGAA-biotin
Target oligonucleotide of MnP (M_3)	TTCTCCGCCAACAACGGCATCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGAAACACGACACCATCAG
Two-base-mismatched oligonucleotide of MnP (M_4)	TTCTCCTCCAACAACGGCATCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAGAAACACCACACCATCAG
Capture probe of CDH (C_1)	HS-$(CH_2)_6$-TGTCAAAGTGGCAGTTCCCGT
Detection probe of CDH (C_2)	CAGCGAGTGGAGGAAACAA-biotin
Target oligonucleotide of CDH (C_3)	TTGTTTCCTCCACTCGCTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACGGGAACTGCCACTTTGACA
Two-base-mismatched oligonucleotide of CDH (C_4)	TTGTTTCCTCCACTCACTGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTACGGGAACTGCCACTTTGATA
Sense primer of MnP (M_5)	CATCCGTCTGACCTTCC
Anti-sense primer of MnP (M_6)	AGCCTGTGCCCTTGAGC
Sense primer of CDH (C_5)	ATTGTTTCCTCCACTCG
Anti-sense primer of CDH (C_6)	CCGCCATTGTTCCACTC

MnP on the recalcitrant aromatic compounds degradation better, establishing an effective and sensitive determination protocol for simultaneously monitoring genes encoding the two enzymes from ligninolytic fungi would be of considerable value [32,33].

In this work, an electrochemical DNA biosensor based on two-enzyme labels was developed for simultaneously detecting the hybridization of MnP and CDH gene fragments from *P. chrysosporium*. The consensus oligonucleotide probes and primers were self-designed and synthesized after Clustal alignment of gene sequences in Gene Bank. Two thiolated capture probes and two biotinylated detection probes were hybridized with target sequences respectively on a gold electrode, and the signals were amplified by horseradish peroxidase (HRP) and laccase (LAC), resulting in the low detection limit and good selectivity. The restriction digestion product of MnP genes and PCR product of CDH genes from *P. chrysosporium* were applied to the DNA detection and the results were consistent with the values from electrophoresis and UV spectrometry. The electrochemical DNA biosensor based upon the functional genes of biodegradation enzymes was convinced to hold a potential for further application in the analysis of microbial community functional diversity [34]. This DNA detection technique in the same system should be a platform for simultaneous determination and identification of multiple species of pathogenic microorganisms and functional genes for environmental pollutant biodegradation.

2. Experimental

2.1. Materials

The oligonucleotide primers and target-specific probes were self-designed by Primer Premier 5.0, which were synthesized by Sangon (Shanghai, China). The two capture probes (M_1 and C_1) were modified with $(CH_2)_6$-SH at 5′ end and another two detection probes (M_2 and C_2) were biotinylated at 3′ end to characterize the sensor performance. Target oligonucleotides (M_3 and C_3) were complementary sequences to both capture probes and detection probes. Two-base-mismatched sequences (M_4 and C_4) were applied to test the selectivity of the DNA biosensor. Two pairs of sense primer and anti-sense primer were used in polymerase chain reaction (PCR) amplifications of *P. chrysosporium* MnP and CDH genes. The sequences of oligonucleotides are shown in Table 1. Horseradish peroxidase–streptavidin conjugate (HRP–SA) and dialysis membranes (molecular weight cut off (MWCO) of 14 Da and 25 Da) were purchased from Dingguo Biotechnology Co., Ltd. (Beijing, China). Laccase (EC 1.10.3.2, 23.3 U mg^{-1}) was from Fluka. Streptavidin (SA), tris (hydroxymethyl) aminomethane (Tris) and 6-mercapto-1-hexanol (MCH) were from Sigma–Aldrich. λDNA/HindIII, Marker I, Marker II, restriction endonuclease XbaI, 2× Taq PCR Master Mix, bromophenol blue buffer (6×), TIANquick Midi Purification Kit and TIANgel Midi Purification Kit were provided by Tiangen Biotech Co., Ltd. (Beijing, China). SYBR Green I was from Bio-Vision, Inc. (Xiamen, China). All chemicals used were of analytical grade or better quality, and all solutions were prepared in deionized water of 18 MΩ purified from a Milli-Q purification system. A sodium chloride–sodium citrate buffer (SSC, 0.3 M NaCl and 0.03 M sodium citrate, pH 8.00) was prepared as the hybridization solution. Phosphate buffer saline (PBS, 67 mM KH_2PO_4 and 67 mM Na_2HPO_4), Tris–HCl buffer (0.1 M Tris adjusted to pH 8.00 with 0.1 M HCl) and Tris–EDTA buffer (TE, 10 mM Tris–HCl and 1 mM EDTA, pH 8.00) were used in this work.

2.2. Apparatus

Electrochemical measurements were carried out on CHI660B electrochemistry system (Chenhua Instrument, Shanghai, China) with a three-electrode system consisting of a gold working electrode with a diameter of 2 mm, a saturated calomel electrode (SCE) and a Pt foil electrode, which were used as the reference electrode and auxiliary electrode, respectively. The PCR reaction was carried out in a Bio-Rad MyCycler (Bio-Rad Laboratories, USA) and the Gel electrophoresis analysis was in a DYY-7C electrophoresis system (Liuyi Instrument, Beijing, China). Gel images were captured on a Gel Doc 2000 imaging system (Bio-Rad Laboratories, USA). An Eppendorf BioPhotometer was used to determine the concentration of gel electrophoresis-purified DNA fragment. A UV-2250UV-vis spectrophotometer (Shimadzu, Japan), a CS501-SP thermostat (Huida Instrument, Chongqing, China), a Model 85-2 magnetic stirring apparatus (Lida Instrument, Shanghai, China) and a Branson200S ultrasonic wave washing machine (Xuyang Instrument, Hebei, China) were used in the assay. The solution pH was measured with a model pHSJ-3 digital acidimeter (Shanghai Leici Factory, China). All the work was done at room temperature (25 °C) unless otherwise mentioned.

2.3. Preparation of laccase–streptavidin conjugate

The synthesis of laccase–streptavidin conjugate (LAC–SA) was achieved in a typical procedure. Five milligrams laccase was dissolved in 1 mL of deionized water and 0.2 mL 0.1 M $NaIO_4$ solution was added under stirring, keeping light-resistant for 20 min at room temperature. The mixture solution was dialyzed with 1 mM sodium acetate buffer (pH 4.40) for 12 h at 4 °C and the pH was adjusted to 9.00 with 0.2 M carbonate buffer (pH 9.50). Then 1 mL 0.02 M carbonate buffer (pH 9.00) containing 2.0 mg streptavidin was added and kept stirring gently under light-resistant condition for 2 h at room temperature. Afterwards, the solution was mixed with 0.1 mL 4.0 mg mL^{-1} $NaBH_4$ and continuously reacted for 2 h at 4 °C, and the mixture was dialyzed with 67 mM PBS (pH 7.38) for 12 h at 4 °C. Furthermore, isometric saturated ammonium sulphate solution was added drop by drop under stirring and was centrifugated with 9000 rpm for 15 min after reacting for 2 h at 4 °C. The pre-