Biosensors and Bioelectronics 71 (2015) 103–107

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

A novel strategy to analyze L-tryptophan through allosteric Trp repressor based on rolling circle amplification

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ARTICLE INFO

Article history:
Received 22 January 2015
Received in revised form
25 March 2015
Accepted 5 April 2015
Available online 8 April 2015

Keywords:
Tryptophan detection
Rolling circle amplification
Repressor
Allosteric effect

ABSTRACT

Rolling circle amplification (RCA) has been considered as a powerful tool for nucleic acids detection. Here, a novel repressor-RCA-based method for L-tryptophan (L-Trp) detection was developed. This method utilizes the specific interaction between the RCA circular template and the Trp repressor protein (TrpR) involved in *trp* operon of *Escherichia coli* (*E. coli*). In the absence of L-Trp, the TrpR protein could not bind to the RCA template, and the RCA process can be continued. When L-Trp is present, the activated TrpR will bind to the operon sequence on the RCA template and inhibit the RCA reaction. Thus, the concentration of L-Trp is correlated directly with the fluorescent RCA signals. We succeeded in detecting L-Trp in a single step in simple homogeneous reaction system. The detection limit was estimated to be 0.77 μM (S/N=3) with good linearity. The method can unambiguously distinguish L-Trp from other 19 standard amino acids and L-Trp analogs. This strategy is also promising for detecting many small molecules such as other amino acids and carbohydrates.

1. Introduction

Amino acid (AA) assay plays a significant role in protein sequencing, food nutrition analysis (Rezazadeh et al., 2013), microbial fermentation, biological sample examination (Bertels et al., 2012; Lian et al., 2012), medical diagnosis (Iizuka et al., 2013). L-tryptophan (L-Trp) is an essential amino acid in protein synthesis and also a precursor of many biologically active substances. It has been found that the unbalance of L-Trp and its metabolic products has closely correlated with human diseases (Lesniak et al., 2013). The ratio of L-Trp and L-kynurenine in serum is considered to be a marker of some disorders (Huang et al., 2013). High level of L-Trp in gastric juice could indicate the gastric cancer progression (Deng et al., 2012).

The significance of the L-Trp analysis has inspired the development of various analytical methods. The commonly used methods for L-Trp analysis are based on spectroscopy (fluorescence, ultraviolet, mass spectrum) with the help of separation techniques (HPLC, gas-chromatography, electrophoresis) (Kaluzna-Czaplinska et al., 2010; Sa et al., 2012; Zinellu et al., 2012). These methods demonstrate relative high sensitivity of even several nM level and a wide range of sample suitability. However, the expensive instruments and laborious operation procedure have limited their applications.

Much effort has been devoted to find a simple convenient L-Trp assay. For the susceptibility of L-Trp to oxidation, many electrochemical biosensors have been proposed. The sensitivity varies from several nM to several μM (Deng et al., 2013; Li et al., 2013; Moradi et al., 2013; Ozcan and Sahin, 2012). However, these methods bear intrinsic drawbacks of interference from other reductants present in detection systems. The pH values of sample solution could also interfere with the result accuracy. Moreover, the quality of the electrochemical signals is strongly dependent on the proper modification of electrodes, and the long-time stability of electrode is unsatisfied. To achieve better sensitivity and specificity, many modified electrodes had to be developed.

Direct sensing of L-Trp using chemiluminescent (CL) and colorimetric methods has been reported frequently. A simple colorimetric assay for analyzing L-Trp and Trp residues in proteins, although being convenient, has detection limit about 5.1 μM (Huang et al., 2011). Simple CL and fluorescence sensors for Trp assay show high sensitivity, however, the amino acid selectivity remains to be improved (He et al., 2012; Lin et al., 2008). Thus, there is an urgent need for L-Trp analysis methods which can offer good specificity, sensitivity, operation convenience and test cost.

Rolling circle amplification (RCA) is an isothermal reaction catalyzed by strand-displacing DNA polymerases such as phi29 DNA polymerase. Using a circular single-stranded DNA (ssDNA) as

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http://dx.doi.org/10.1016/j.bios.2015.04.017
0956-5663/

the template, phi29 DNA polymerase can extend the primer along the circular template round and round to synthesize a long ssDNA product. For RCA offers isothermal, high efficient and cost-effective advantages, it has been widely used in molecular detections such as nucleic acids detection (Bi et al., 2013; Long et al., 2013) and protein detection (Cheng et al., 2010; Xue et al., 2012). RCA-based assay for enzyme catalytic activity has also been developed (Zhao et al., 2014). Whether RCA can be employed to probe other small molecules is also highly desired (Yin et al., 2009). However, only a few small molecules such as Pb^{2+}, NAD and ATP have achieved RCA detecting (Cho et al., 2005; Ma et al., 2011; Peng et al., 2014; Tang et al., 2013; Xue et al., 2013; Yi et al., 2014; Zhao et al., 2012). To our knowledge, amino acid has seldom been reported in this way.

In this paper, we presented a novel method for L-Trp detection based on RCA principle. The attractive feature is that it utilizes the allosteric protein of Trp repressor (TrpR) to recognize L-Trp specifically, and uses the activated TrpR binding with the circular template of RCA to regulate the RCA reaction. Using this convenient one-step method, we succeeded in detecting L-Trp with an extraordinary high capability of distinguishing from other standard 19 amino acids and L-Trp analogs. We also tested its feasibility to determine the L-Trp abundance in real culture medium samples.

2. Experimental

2.1. Chemicals and oligonucleotides

Oligonucleotides at HPLC grade were purchased from Sangon Biotech Co. Ltd. (Shanghai, China) without further purification. Their concentrations were determined by the absorption coefficiency of each sample. Their sequences were listed in Table S1, where the oligonucleotide for forming the circular template had a phosphoric acid group at the 5′-terminal. Phi29 DNA polymerase was purchased from NEB (Ipswith, MA). SYBR Green II was from Invitrogen (Waltham, MA). T4 DNA Ligase was purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). *Pfu* DNA polymerase, DNA Gel Extraction Kit and Plasmid Miniprep Kit were purchased from Tiangen Biotech (Beijing, China). Restriction endonucleases EcoRI and HindIII were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). Ni-NTA column was from GE Healthcare (Buckinghamshire, UK). Plasmid pET28a and *Escherichia coli* (*E. coli*) strain DH5α and BL21 were kept in our lab.

2.2. Cloning, expression and purification of TrpR

The *trpR* gene was cloned by PCR from *E. coli* strain DH5α. The upstream and downstream primers contained EcoRI and HindIII recognition sites, respectively (Table S1). The 345-bp PCR product and the pET28a plasmid were both cleaved by EcoRI and HindIII at 37 °C for 2 h. After gel extraction purification, the digested *trpR* gene fragment and the pET28a plasmid were ligated by T4 DNA ligase at 16 °C overnight and transformed into competent cell DH5α. The transformed DH5α was cultured in Luria-Bertani (LB) plate containing kanamycin (30 μg/ml), and the recombinant plasmids were selected by EcoRI and HindIII double digestion after plasmid Miniprep and were further confirmed by subsequent DNA sequencing.

The recombinant plasmid pET28a-trpR was transformed into BL21 competent cells, which then were cultured in LB containing kanamycin. When the absorbance OD_{600} of the cell culture reached 0.6, 5 mM isopropyl β-*D*-1-Thiogalactopyranoside (IPTG) was added to induce the *trpR* gene expression. After continuous incubation at 37 °C for 4 h, the cells were collected by centrifugation. The BL21 cells were re-suspended in lysis buffer and treated by ultrasonication. The Cell lysates were centrifuged at 4 °C. The supernatant was purified by Ni-NTA column following the GE product manual. Briefly, after flow-through of the supernatant, the column was washed with 10 volumns of washing buffer and then eluted with 500 mM imidazole. The eluent was dialyzed at 4 °C and the protein solution was prepared in 20 mM PBS buffer pH 8.0 and stored at −20 °C. The protein purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Circularization of oligonucleotide

For circularization, the 5′-end of the template oligonucleotide (CT-trpO) was phosphorylated. The 5′-end and 3′-end of the template oligonucleotide (1 μM) were then hybridized with 2 μM ligation oligonucleotide (trpO) in a head-to-tail fashion, and were covalently linked by T4 DNA ligase (175 U) at 16 °C for 60 min. The ligation buffer contained 50 mM Tris pH 8.0, 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM ATP in 10 μl reaction system. The ligation product was used directly for the following RCA analysis.

2.4. TrpR binding and RCA detections

The 0.5 pmol circular template was mixed with 2.5 μM TrpR in 100 μl RCA reaction solution which contained 50 mM Tris, 10 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 4 mM DTT (pH 7.5), SYBR Green II (1:10000) and phi29 DNA polymerase (3 U). For the sensitivity study, L-Trp at different concentrations were also added in the reaction solution. Fluorescence signals were recorded on Microplate Reader (Infinite M200, Tecan, USA) with excitation wavelength at 480 nm and emission wavelength at 518 nm. The fluorescence emission was monitored for over ~20 min at 37 °C. The inhibited RCA rate (IRR) and relative inhibited RCA rate (RIRR) were calculated by the formulas, respectively: $IRR = RR_c - RR_{aa}$, $RIRR = (RR_c - RR_{aa})/(RR_c - RR_{trp})$. RR_c: RCA rate without L-Trp; RR_{aa}: RCA rate with amino acid; RR_{trp}: RCA rate with L-Trp.

3. Results and discussion

3.1. Concept proof of the proposed assay

It has been well known that the *trp* operon is an elaborate machine in *E. coli* genome for the bacteria to regulate the tryptophan metabolism in response to the L-Trp abundance in environment (Youderian and Arvidson, 1994). The key element for the regulation is the allosteric protein TrpR (Haran et al., 1992). When the tryptophan abundance is more than needed, the excess L-Trp will bind to the TrpR protein. Due to the allosteric effect of this repressor, the activated TrpR then attaches the operator site of the *trp* operon with a high binding affinity (Arvidson et al., 1986). As a result, the RNA polymerase could not initiate the transcription at the promoter region, leading to the inhibition of gene expression involved in tryptophan metabolism (Jeeves et al., 1999). (Schemes 1A and B).

Based on this gene expression regulation scheme, we proposed a novel repressor-RCA-based strategy to analyze L-Trp abundance. A circular template and a primer constitute a duplex fragment containing the TrpR binding site. Thus, in the presence of L-Trp, the activated TrpR binds to this recognition site with a high affinity and is expected to prevent phi29 DNA polymerase from initiating RCA reaction. Therefore, the higher the L-Trp concentration is, the lower the RCA efficiency is (Schemes 1C and D). Additionally, a recent study has proved that a protein binding to an aptamer designed in circular template can efficiently arrest the RCA process because protein-binding DNA aptamer can form highly stable