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An ultrasensitive fluorescence method suitable for quantitative analysis of mung bean nuclease and inhibitor screening *in vitro* and *vivo*

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ABSTRACT

Mung bean nuclease is a single stranded specific DNA and RNA endonuclease purified from mung bean sprouts. It yields 5′-phosphate terminated mono- and oligonucleotides. The activity level of this nuclease can act as a marker to monitor the developmental process of mung bean sprouts. In order to facilitate the activity and physiological analysis of this nuclease, we have developed a biosensing assay system based on the mung bean nuclease-induced single-stranded DNA scission and the affinity difference of graphene oxide for single-stranded DNA containing different numbers of bases. This end-point measurement method can detect mung bean nuclease in a range of 2×10^{-4} to 4×10^{-2} with a detection limit of 1×10^{-4} unit/mL. In addition, we demonstrate the utility of the assay for screening chemical antibiotics and metal ions, resulting in the identification of several inhibitors of this enzyme *in vitro*. Furthermore, we firstly report that inhibiting mung bean nuclease by gentamycin sulfate and kanamycin *in vivo* can suppress mung bean sprouts growth. In summary, this method provides an alternative tool for the biochemical analysis for mung bean nuclease and indicates the feasibility of high-throughput screening specific inhibitors of this nuclease *in vitro* and *in vivo*.

1. Introduction

Mung bean nuclease (MBN) is one of enzymes that can cleave the phosphodiester bonds between the nucleotide subunits of nucleic acids(Sung et al., 1962; Kowalski et al., 1976). Many evidences have indicated that cleavage reactions of single-stranded DNA (RNA) catalyzed by this nuclease play key roles in a variety of fields ranging from transcript mapping studies, flushing staggered ends and separation of cDNA strand after synthesis with reverse transcriptase and DNA polymerase, as well as in many biological processes involving replication, recombination and tumor therapy (Murray, 1986; Castro et al., 2004; Soucek et al., 2006). Thus, to obtain further insight into the cellular and physiological roles of this nuclease, novel tools such as potent, selective and cell-permeable inhibitors of mung bean nuclease are still desirable. Similarly, such molecules would be valuable as chemical probes to complement the use of RNAi-based tools, as they would inhibit the enzymatic activity rather than interfere with potential structural roles of nucleases. However, most current assays available for the biochemical analysis of this nuclease are time consuming, and less suitable for quantitative analysis and screening (Vernick et al., 1988; Grafi et al., 1991; Castro et al., 2003; Ma et al., 2014). For example, gel-based assays based on oligo-nucleotides labeled with fluorescent or radioactive moieties are widely used but difficult to quantify and laborious. Meanwhile, quantitative assays based on colorimetry are insensitive and require high protein and substrate concentration (Cheng et al., 2006). Recently developed quantitative assays based on size-exclusion chromatography also have limited sensitivity, require relatively large reaction volumes and are not suitable for high-throughput screening (He and Yan, 2012).

Graphene oxide (GO), a single-atom thick and two-dimensional nanomaterial, has attracted great attention due to its remarkable electronic, mechanical and thermal properties (Dikin et al., 2007). This kind of nanomaterial can directly interact with single-stranded DNA by π–π stacking interactions between nucleotide bases and GO. At the same time, it is an excellent energy acceptor of the fluorescence resonance energy transfer which makes the fluorescence detection a promising application of GO in biomolecules sensing technology (Zhu et al., 2010; He et al., 2013). Recently, Lu and coworkers designed a Y-shaped DNA probe for multiplex

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nucleases detection using GO as a platform (Lu et al., 2011). Subsequently, Lee and coworkers developed an exonuclease detection method based on the preferential binding of single-stranded DNA over double-stranded DNA to GO (Lee et al., 2011). However, both of them were only designed for nucleases whose substrates were dsDNA molecules. Recently, a detailed investigation on the affinity difference of GO for ssDNA containing different numbers of bases in the length proved that short ssDNA had weaker affinity to GO than long ssDNA (Leung et al., 2010; He et al., 2013). Based on these findings, Zheng and He developed a nuclease detection method by using single-stranded DNA as substrate (Zheng et al., 2012; He et al., 2014). The two methods based on GO have shown great advantages on the single-stranded nuclease analysis due to the simplicity, rapidity and low cost comparing with existed reports. Thus, developing GO-based biosensors for other nucleases sensitive assay, whose substrates are ssDNA molecules, are also required and possible. Herein, a simple, rapid and ultra-high sensitive GO-based sensing platform is constructed for fluorescence detection of mung bean nuclease, an important and specific nuclease of mung bean sprouts. By using the difference in affinity of GO for ssDNA containing different numbers of bases in length, mung bean nuclease, which mainly digests ssDNA, was taken to further confirm the "proof-of-principle" verification of this method. The assay described here was based on the end-point measurement and also suitable for 96-and 384-well microplate formats(Maryati et al., 2014). To broad the use range of the assay, we screened the impact of antibiotics and metal ions on this nuclease, which has been confirmed as inhibitors of other nucleases (Campbell and Jackson, 1980; Woegerbauer et al., 2000; Beernink et al., 2001; Liu et al., 2007). These inhibitors may be useful tools for the biochemical and biofunctional analysis of mung bean nuclease.

2. Material and methods

2.1. Chemicals and materials

The fluorophore-labeled ssDNAs (P1-P4, Table S1) were synthesized from Takara Biotechnology Co., Ltd. (Takara, China). Mung bean nuclease was purchased from the same company. GO was purchased from Sinocarbon Materials Technology Co., Ltd. (China). The reactive buffer used in this experiment consisted of 30 mM NaAc, pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 0.35 mM DTT, 2% glycerol. Milli-Q purified water was used to prepare all the solutions.

2.2. Apparatus

Fluorescent emission spectra were performed on F-2500 Fluorescence Spectrophotometer (Hitachi, Japan). The sample cell was a 700-μL quartz curvet. The fluorescence intensity (FI) was monitored by exciting the sample at 521 nm and measuring the emission at 578 nm. Both of the slits for excitation and emission were set at 10 nm. The fitting of the experimental data was accomplished using the software Sigmaplot 8.0.

2.3. Optimization of the reaction time between TMARA-labeled ssDNA and GO

GO was sonicated in Milli-Q purified water for 2 h to get a homogeneous black solution and stored at 4 °C for use. The working solution containing ssDNA was obtained by diluting the stock solution to a concentration of 100 nM using 20 mM Tris–HCl buffer. To optimize the reaction time between fluorescence-labeled ssDNA and GO, 1 μL of the ssDNA stock solution (10 μM), and 0.75 μL GO solution (2 mg/mL) as prepared were mixed. The mixed solution was diluted with Tris–HCl buffer to 100 μL. The above prepared solution was incubated for 0, 1, 5, 10, 15, 20, 25, and 30 min at room temperature. Finally, the fluorescence intensity of the sample was measured at 578 nm with excitation at 521 nm.

2.4. Optimization of the reaction time between fluorophore-labeled substrates and mung bean nuclease

To optimize the reaction time between fluorophore-labeled substrates and mung bean nuclease, 1 μL of the substrate stock solution (10 μM), and 4 units mung bean nuclease solution were mixed in the reactive buffer. The above prepared solution was incubated for 0, 5, 10, 15, 20, 25, 30, 35 and 40 min at 37 °C. Then, 1.5 μg GO was added and the mixed solution was diluted with Tris–HCl buffer to 100 μL. The above prepared solution was incubated for 10 min at room temperature. Finally, the fluorescence intensity of the sample was measured at 578 nm with excitation at 521 nm.

2.5. Performance of mung bean nuclease detection in a Tris–HCl buffer

For mung bean nuclease assay, 1 μL of the ssDNA stock solution (10 μM), and appropriate concentrations of mung bean nuclease solution were mixed. The above-prepared solution was incubated for 30 min at 37 °C. Then, 1.5 μg GO was added to the solution, the mixed solution was diluted with Tris–HCl buffer to 100 μL. The above-prepared solution was incubated for 10 min at room temperature. Finally, the fluorescence intensity of the incubated solution was measured at 578 nm with excitation at 521 nm.

2.6. Antibiotics screening

For the inhibition assay, a total volume of 100 μL standard solutions that contained 100 nM P3 and various amounts of antibiotics were initially incubated at 37 °C for 10 min Next, 1 μL mung bean nuclease (4 Units) was added and the sample incubated for 30 min Then, 1.5 μg GO was added and incubated for 15 min Finally, the fluorescence intensity of the incubated solution was measured at 578 nm with excitation at 521 nm. Impacts of antibiotics on the mung bean nuclease were evaluated from the maximum fluorescence intensity at 578 nm.

2.7. Mung bean nuclease assay in cell-free extracts of sprouts

Mung bean seeds were sterilized in a 5% sodium hypochlorite solution for 10 min, rinsed thoroughly with deionized water several times, and subsequently placed in the wet cotton at a controlled temperature of 25 °C in the dark. After 24 h, the seeds were checked for the germination, and seeds that had sprouted were used in the test. The inhibiting tests were conducted in a Petri dish test unit (18 mm). Each test unit contained 30 mL of culture water with a specific concentration of antibiotics. The test units were placed in an incubator at a controlled temperature of 25 °C in the dark. The exposure period was 48 h. Each antibiotics concentration was prepared in three replicates. After an incubation period of 72 h, plants were collected and seedling growth was measured. The length of each seedling was measured with a ruler. The whole sprouts were grinded with 5 mL cold PBS, centrifuged and resuspended in the 0.5 mL ice-cold cell lysis buffer (Cell Signaling) on ice for 5 min Cells were pulse-sonicated on ice 5 times for 5 s each. Then, centrifuged extracts at 15,000*g* for 20 min at 4 °C and supernatants were collected. Concentration of cell-free extracts was assayed by measuring absorbance at 595 nm using Coomassie blue protein reagent (Pierce, Rockford, USA).