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A fluorescence turn-on method for real-time monitoring of protease activity based on the electron transfer between a fluorophore labeled oligonucleotide and cytochrome *c*

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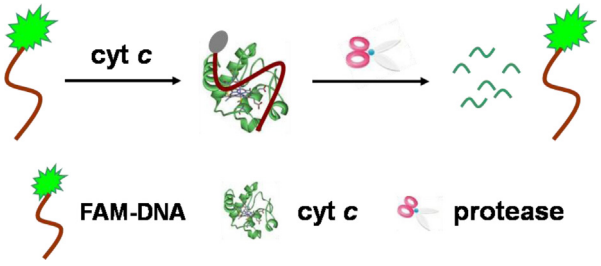
HIGHLIGHTS

- Rapid detection of protease activity with fairly good sensitivity and selectivity.
- A turn-on fluorescence assay reduces the likelihood of false positive signals.
- Cytochrome *c* efficiently quench the fluorescence of the FAM single stranded DNA.
- The enzymatic reaction could be monitored in real-time.
- The assay could be used for the screening of potential protease inhibitors.

GRAPHICAL ABSTRACT



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ABSTRACT

A new continuous fluorescence turn-on assay for protease activity and inhibitor screening has been developed. A fluorophore labeled single stranded DNA (FAM-DNA) and cytochrome *c* (cyt *c*) were employed. The fluorescence of the FAM-DNA was efficiently quenched when binding to cyt *c*, through the electron transfer between the FAM fluorophore and the heme cofactor of cyt *c*. In the presence of a protease, such as trypsin, cyt *c* was digested into small peptide fragments. The FAM-DNA was released, which resulted in the recovery of the FAM fluorescence. The rate of the cyt *c* digestion could be reduced via the addition of an inhibitor. As a result, reduced degree of the fluorescence recovery was obtained. The limit of detection of our assay is 1 nM trypsin and the IC_{50} values are 3.23 μg mL^{-1} and 0.303 μg mL^{-1} for the inhibitor from egg white and the inhibitor from soybean, respectively. Our method could be used for the sensing of protease activity for various biochemical applications, and for the screening of protease inhibitors as drugs for the treatment of various related diseases.

1. Introduction

Proteases, as one of the most important groups of digestive enzymes, exist naturally in all organisms. They are involved in various physiological processes, such as digestion, fertilization, growth, differentiation, cell signaling, cell migration, immunological defense, and apoptosis [1–4]. The abnormal proteolysis plays important roles in a number of diseases, such as emphysema, stroke, viral infection, cancer, Alzheimer's disease, inflammation, and arthritis [3–7]. Trypsin is an important serine protease found in the digestive system of many vertebrates. It can catalyze the hydrolysis of peptide and ester bonds containing lysine and arginine residues at the C-terminus. Abnormal trypsin activity is associated with a number of diseases such as pancreatic insufficiency

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[8]. Therefore, there is an increasing interest in the sensing of protease activity and the corresponding inhibitors for the potential therapeutic intervention of a variety of disease states [9–13].

There are a variety of techniques developed for protease activity assay, including enzyme linked immunosorbent assay (ELISA) [14,15], gel electrophoresis [16,17], mass spectrometry [18,19], and a number of electrochemical [20,21], colorimetric [22,23], and fluorometric methods [24–27]. Among them, fluorescence-based enzymatic assay has drawn more attentions due to its rapid response and easy operation. It is therefore of great value to develop a simple, rapid, sensitive and high-throughput routine assay to detect protease activity.

Cytochrome *c* (cyt *c*) is a heme containing protein which contains 104 amino acid residues in most mammals [28]. It is an electron transfer protein and loosely associated with the inner membrane of the mitochondrion. Its heme moiety could act as an efficient fluorescence quencher. The isoelectric point (p*I*) of cyt *c* is 10.0–10.5 [29].

Herein we report a simple, convenient, and sensitive fluorescence turn on method for protease activity assay. Cyt *c* and a fluorophore labeled single-stranded DNA (FAM-DNA) were selected. FAM-DNA (a polyanion) could bind to the positively charged cyt *c* at pH 8.2 through electrostatic and hydrophobic interactions. The fluorescence of the FAM-DNA was efficiently quenched by the metal-containing heme in cyt *c*. In the presence of a protease, cyt *c* was digested, and the FAM-DNA was released. Fluorescence recovery of the FAM-DNA was observed. A new and continuous fluorescence assay for the sensing of protease activity was therefore established. Our assay is sensitive, selective, and fairly straightforward. It has a good potential to be used for the detection of protease activity in various biochemical applications, and for the screening of protease inhibitors as potential drugs.

2. Experimental

2.1. Materials

Oligonucleotide (FAM-DNA: 5′-FAM-TTT TTT TTT TTT TTT TTT TT-3′), protease K, and the trypsin inhibitor from soybean (Type I-S) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Trypsin and the inhibitor from egg white (Type II-O, partially purified ovomucoid) were obtained from Sigma–Aldrich (St Louis, MO, USA). Cyt *c*, chymotrypsin, horseradish peroxidase (HRP), bovine serum albumin (BSA) were purchased from Bio Basic Inc. (BBI, Markham, Canada). Hemoglobin and carboxypeptidase were purchased from Worthington Biochemical Corporation (Lakewood, USA). Other reagents were all of analytical grade and used without further purification. Water was doubly distilled and purified by a Milli-Q system (Millipore, Billerica, MA, USA).

2.2. Instrumentation

Fluorescence measurements were performed on a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA). Sample solutions were excited at 490 nm and fluorescence emission spectra were recorded with slits for excitation and emission both of 2 nm. UV–vis absorption spectra were obtained with a Cary 50 Bio spectrophotometer (Varian Inc., CA, USA).

2.3. Assay Procedures

Trypsin of different concentrations was added to 5 mM Tris–HCl buffer solution (pH 8.2) containing 250 nM cyt *c* (the substrate) and 100 nM FAM-DNA. The final concentrations of trypsin were 0, 2, 10, 50, 100, 200 and 300 nM, respectively. Assay solution temperature was kept at 37 °C. The emission intensity changes at 515 nm were monitored in real time.

2.4. Trypsin Inhibitor Screening

Different amounts of the trypsin inhibitors were added to the FAM-DNA (100 nM) and cyt *c* (250 nM) sample solution in 5 mM Tris–HCl (pH 8.2) buffer. 300 nM trypsin was then added. The fluorescence intensity changes at 515 nm were recorded with the assay temperature kept at 37 °C.

The inhibition efficiency (IE) is defined as: [30]

$$\mathrm{IE} = \left[F_{(\text{no inhibitor})} - F_{(\text{inhibitor})}\right] / \left[F_{\text{no inhibitor}} - F_0\right] \quad (1)$$

where $F_{(\text{no inhibitor})}$ and $F_{(\text{inhibitor})}$ are the fluorescence intensities at 515 nm after 10 min of the trypsin enzymatic reaction in the absence or presence of an inhibitor. F_0 is the fluorescence intensity of the blank sample.

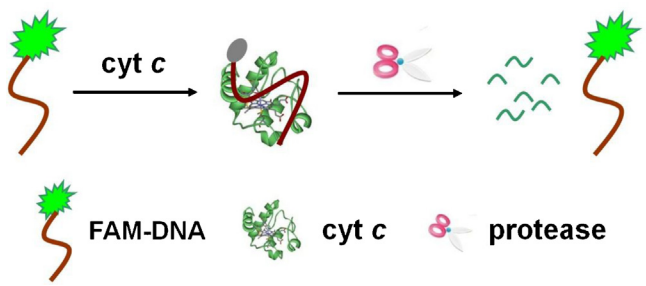
3. Results and discussion

3.1. Assay strategy

Scheme 1 depicts the principle of the FAM-DNA/cyt *c* based fluorescence turn-on protease assay. FAM-DNA is a fluorophore labeled single-stranded DNA, and it is a polyanion. Cyt *c* is positively charged when the assay solution pH is lower than its p*I* value. FAM-DNA could bind to the positively charged cyt *c* via electrostatic and hydrophobic interactions. The fluorescence of FAM-DNA was effectively quenched through electron transfer from the FAM fluorophore to the metal-containing heme in cyt *c*. In the presence of a protease, cyt *c* was hydrolyzed into small peptide fragments, including the heme-containing peptide fragment. The FAM-DNA was released. Increased fluorescence intensity (decreased degree of fluorescence quenching) was therefore detected. The fluorescence intensity changes could be directly related to the amount of trypsin added to the assay solution. A simple fluorescence turn-on assay for protease detection was therefore established.

3.2. The interactions between FAM-DNA and cyt c

The interactions between FAM-DNA and cyt *c* were investigated (Fig. 1). 100 nM FAM-DNA displayed strong fluorescence in 5 mM Tris–HCl (pH 8.2) aqueous buffer solution. However, with the addition of increasing amounts of cyt *c*, the fluorescence of FAM-DNA gradually decreased, because of the electron transfer between FAM-DNA and cyt *c*. When 250 nM cyt *c* was added, a quenching efficiency of 93.7% was obtained (Fig. 1B). Further increase of the cyt *c* concentration caused no obvious decrease of the fluorescence signal of FAM-DNA. 250 nM cyt *c* was therefore used for the following protease detection.



Scheme 1. Schematic illustration of the principle of the protease activity assay.