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Label–free, turn–on fluorescent sensor for trypsin activity assay and inhibitor screening

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ABSTRACT

The development of new detection methods for proteases activity assay is important in clinical diagnostics and drug development. In this work, a simple, label–free, and turn–on fluorescent sensor was fabricated for trypsin, a protease produced in the pancreas. Cytochrome c, a natural substance of trypsin, could be selectively cleaved by trypsin into heme–peptide fragment. The produced heme–peptide fragment exhibited an intensive catalytic role on the H_2O_2–mediated the oxidation of thiamine to form strong fluorescent thiochrome. The fluorescence intensity was closely dependent on the amount of trypsin presented. The procedure allowed the measurement of trypsin over the range of 0.5–20.0 μg/mL with a detection limit of 0.125 μg/mL. The sensor showed better precision with a relative standard deviation of 1.6% for the measurement of 1.0 μg/mL trypsin solution (n=11). This sensing system was applied to screen the inhibitor of trypsin, the IC_{50} values were calculated to be 12.71 ng/mL for the trypsin inhibitor from soybean and 2.0 μg/mL for benzamidine hydrochloride, respectively, demonstrating its potential application in drug development and related diseases treatment.

1. Introduction

Many efforts have been devoted to developing new detection methods and new detection techniques toward biological macromolecules, e.g. protein and nucleic acid, as the result of increasing attention paid to human health, and diagnosis and treatment of disease [1]. Protease, also known as proteolytic enzyme, exists widely in natural organisms and plays important roles in catalyzing the hydrolytic cleavage of specific peptide bonds in the target proteins into smaller fragments [2]. Proteases are involved in the control of a variety of physiological and pathological processes [3,4]. On the other hand, proteases are also implicated in many physiological diseases. The abnormal level of protease would lead to a number of diseases such as inflammation, cancer, Alzheimer's [5,6]. More and more studies have been paid to develop simple and sensitive methods toward the detection of protease activity and the related inhibitor screening [7–12], which is significance to the disease diagnosis and treatment as well as to the drug research and development. Among the reported techniques, fluorescence–based assays are more attractive due to its high sensitivity, easy operation and rapid response. The probes of labeled specific peptide sequence were often designed and used as the enzyme substrates in most of fluorescent–based methods [10–12]. However, chemical modification of peptide substrate may depress the affinity of the probe to the target protease and thus reduce the detection sensitivity. In addition, the synthesis and modification of specific peptide substrate are more tedious and expensive. Hence, it is highly desirable to develop simple and sensitive fluorescent method for the assay of protease activity by employing a natural substrate in a label–free mode.

Trypsin is an important serine protease produced by the pancreas. It promotes the pancreatic proenzymes into the active forms and controls pancreatic exocrine function. A deficiency in the trypsin level can cause pancreatic diseases such as meconium ileus and hereditary pancreatitis [13]. The development of simple and convenient method for trypsin activity assay and its inhibitor screening has therapeutic implications for these pancreatic diseases. Cytochrome c is a highly water–soluble hemeprotein widely found in plants, animals, and unicellular organisms [14]. As an electron transfer protein, cytochrome c can act as an efficient fluorescence quencher through the electron transfer between the fluorophore and the heme moiety of cytochrome c [15]. Cytochrome c could be selectively cleaved by trypsin into smaller fragments on the C–terminal side of arginine and lysine residues [16]. The isoelectric point (pI) of the heme–peptide fragment formed (7.0) was significant different with that of cytochrome c (pI 10.2) [17]. This difference renders the scientists the opportunity to develop fluorescent method for trypsin detection by using

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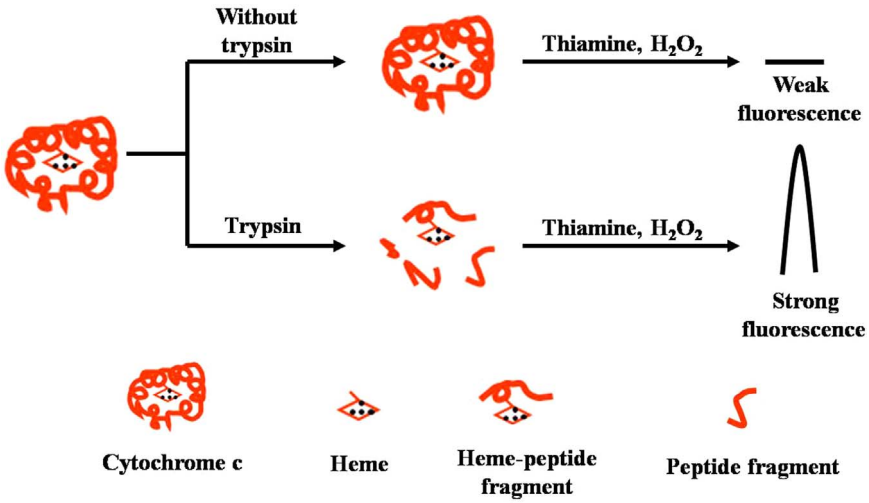

Scheme 1. A schematic diagram for the fluorescent sensing of trypsin.

cytochrome c as the natural substrate, in which cytochrome c acts as a fluorescent quencher [18–22]. In the presence of trypsin, cytochrome c could be degraded into the heme–peptide fragment in the form of Cys–Ala–Gln–Cys–His–Thr–Val–Glu–Lys–heme at pH 8.9 [23,24]. Zhang et al. [25] and our previous work [26] indicated that the formed heme–peptide fragment had peroxidase–like activity and could catalyze the oxidation of organic molecule, such as luminol and 3,3′,5,5′–tetramethylbenzidine, in the presence of hydrogen peroxide (H_2O_2). These properties have been explored to develop chemiluminescent and colorimetric sensor for the detection of trypsin.

The main aim of the present work is to develop a fluorescent sensor for trypsin assay by using peroxidase–like activity of heme–peptide fragment. Scheme 1 depicts the schematic diagram of this strategy for fluorescent sensing of trypsin. Thiamine, a fluorescent substrate of peroxidase [27], was employed as the model substrate of heme–peptide fragment. As expected, the heme–peptide fragment exhibited an intensively catalytic role on the H_2O_2–mediated oxidation of thiamine to produce strong fluorescent thiochrome. This fluorescent sensor has several advantages. Firstly, cytochrome c was used as a natural enzyme substrate. Thus, no specific peptide chain is synthesized, making the operation more simple and convenient. Secondly, no label is required on the substrate, which would not only reduce the cost, but also improve the sensitivity. This sensing system has been successfully applied to trypsin inhibitor screening, demonstrating its potential application in drug development.

2. Experimental

2.1. Apparatus

Fluorescence measurement was carried out on a F–2700 spectrofluorometer (Hitachi, Japan) with slit widths of both 5 nm for excitation and emission, respectively. The hydrolysis of cytochrome c by trypsin was incubated at 37 °C in the DF–101S constant temperature heating magnetic stirrer (Zhengzhou Kefeng Instrument Equipment Co. Ltd., China). The pH of the buffer solution was measured with a PB–10 pH meter (Sartorius Scientific Instrument Co. Ltd., Germany).

2.2. Chemicals

All chemicals were of analytical grade, and ultrapure water was used throughout the experiments. Trypsin (from pig pancreas), cytochrome c (from the horse's heart), trypsin inhibitor from the soybean, benzamidine hydrochloride hydrate, lysozyme, thrombin and pepsin were purchased from Sigma–Aldrich (MO, USA). Bovine serum albumin (BSA) and hemoglobin was purchased from Solarbio technology Co., Ltd. (Beijing, China). Thiamine was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). H_2O_2 (30%, v/v) and $CaCl_2$ were purchased from Xilong Chemical Co., Ltd. (Guangdong, China). Tris (hydroxymethyl) aminomethane was purchased from Huamei Biological Engineering Co., Ltd. (Henan, China). Trypsin stock solution (10.0 mg/mL, containing 1.0 mmol/L $CaCl_2$) and cytochrome c solution (0.25 mmol/L) were prepared in 50 mmol/L tris–HCl buffer (pH 9.0). Thiamine solution (10.0 mmol/L) and H_2O_2 solution (10.0 mol/L) were prepared with water.

2.3. Procedure for trypsin detection

Typically, cytochrome c solution (20 μL, 250 μmol/L) was incubated with various concentrations of trypsin in 4.9 mL of 50 mmol/L tris–HCl buffer solution (containing 1.0 mmol/L $CaCl_2$, pH 9.0) at 37 °C for 75 min. After that, thiamine solution (50 μL, 10.0 mmol/L) and H_2O_2 solution (50 μL, 10.0 mmol/L) were added. The fluorescence spectrum of the resultant solution was scanned immediately with fixing the excitation wavelength at 373 nm. The fluorescence intensity at 432 nm was employed to optimization and quantization.

2.4. Procedure for trypsin inhibitor screening

For trypsin inhibitor screening, trypsin (0.5 μL, 10.0 mg/mL), cytochrome c (20 μL, 250 μmol/L), and various concentrations of inhibitor were mixed in 4.9 mL of 50 mmol/L tris–HCl buffer solution (containing 1.0 mmol/L $CaCl_2$, pH 9.0). After incubation at 37 °C for 75 min, thiamine solution (50 μL, 10.0 mmol/L) and H_2O_2 solution (50 μL, 10.0 mol/L) were added. The fluorescence spectra of the resultant solutions were scanned immediately with fixing excitation wavelength at 373 nm. The inhibitory efficiency was calculated according to the formula $(F-F_i)/(F-F_b)$, where F_b was the fluorescence intensity of blank solution, F and F_i were the fluorescence intensity in the absence of and presence of the inhibitor.