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A sensitive and label-free trypsin colorimetric sensor with cytochrome c as a substrate

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ABSTRACT

The development of simple and sensitive methods for protease sensing plays important roles in clinical diagnostics and drug development. Here a simple, rapid, label-free, and sensitive trypsin colorimetric sensor was developed by employing cytochrome c (cyt c) as an enzyme substrate and 3,3′,5,5′-tetramethylbenzidine (TMB) as a chromogenic reagent. It was found that cyt c hardly catalyzes H_2O_2-mediated TMB oxidation to produce a blue solution. But the hydrolysate of cyt c by trypsin displays an intense catalytic effect on the aforementioned reaction, resulting in the formation of a blue solution immediately. The detection process allows visually perceiving as low as 50 ng/mL trypsin with the naked eyes. With the aid of a spectrophotometer, the absorbance at 652 nm was proportional to the concentration of trypsin in the range from 5.0 ng/mL to 2.0 μg/mL with a detection limit of 4.5 ng/mL. The sensor showed better precision with relative standard deviation of 2.5% and 1.7% for eleven repetitive measurements of 50.0 ng/mL and 1.0 μg/mL trypsin solution, respectively. The procedure has been successfully applied to the determination of trypsin in human urines and for inhibitor screening, demonstrating its potential application in clinic diagnosis and drug development.

1. Introduction

Many efforts have been attracted to the development of new analytical methods toward detecting biological macromolecules, such as protein and nucleic acid, as the result of increasing attention paid to human health in the purpose of diagnosis and treatment of disease. Proteases, also known as proteolytic enzymes, are a unique class of enzymes playing important roles in a variety of physiological and pathological processes, such as protein digestion, blood clotting and disease development (Neurath, 1999; Turk, 2006). As such a protease, trypsin (EC 3.4.21.4) is produced by the pancreas and belongs to a kind of serine protease (Rawlings and Barrett, 1994; Hirota et al., 2006). It is widely found in the digestive system of many vertebrates and specific for cleaving peptides mainly at the C-terminal side of arginine or lysine residues (Olsen et al., 2004). Trypsin plays a critical role in regulating pancreatic exocrine function (Rawlings and Barrett, 1994; Hirota et al., 2006). The level of trypsin in biological fluids can serve as a reliable and specific diagnostic biomarker for pancreatic function and its pathological changes (Noone et al., 2001; Whitcomb, 1999). The biological fluids of acute pancreatitis and pancreas transplant patients had higher level of trypsin than that of healthy person's (Artigas et al., 1981; See and Smith, 1991). Therefore, the development of simple, sensitive, and convenient methods for the detection of trypsin is important for the diagnosis and therapeutics of these diseases, and for screening of protease inhibitors.

A variety of techniques have been reported for the determination of trypsin in biological fluids and for screening of protease inhibitors, including gel electrophoresis (Lefkowitz et al., 2010), liquid chromatography (Bures et al., 2004), enzyme-linked immunosorbent assay (Seia et al., 2014), fluorescence (Ou et al., 2015; Wu et al., 2014; Fan et al., 2012), chemiluminescence (Zhang et al., 2013; Zhang et al., 2014), and electrochemical methods (Stoytcheva et al., 2012; Park and Yang, 2014; Dong et al., 2015). Although the above-mentioned techniques are routine methods for the detection of trypsin and possess their respective advantages, most of which are laborious, requiring specific instrumentation, or need to design probes with suitable structure to interact with trypsin to produce a detectable signal.

Colorimetric method has received extensively attention owing to its cost-effective, instrumentation-free, and a color change can be easily observed by the naked eyes. A few colorimetric methods have also been suggested for the determination of trypsin (Xue et al., 2011; Miao et al., 2013; Ding et al., 2014; Lou et al., 2010; Wang et al., 2015). Most reported colorimetric methods are based on trypsin regulating the dispersed and aggregated states of

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nanoparticles upon substrate hydrolysis (Xue et al., 2011; Miao et al., 2013; Ding et al., 2014). Such kind of the method had high sensitivity by taking advantage of high molar absorptivity of the Plasmon band of metal colloids, however, they usually need carefully design/synthesize a substrate (peptide chain)-functionalized metal nanoparticles. Lou et al. reported a colorimetric method for the detection of trypsin by using a Rhodamine B derivative and Cu^{2+} as the chromogenic reagents and bovine serum albumin (BSA) as the substrate (Lou et al., 2010). The detectability of this method (2.5 μg/mL) was not very satisfactory. Wang et al. found that BSA-stabilized gold nanoclusters (BSA-stabilized Au NCs) could catalyze the oxidation of peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) under visible light irradiation without adding hydrogen peroxide (H_2O_2) (Wang et al., 2015). Through digestion of the protein template of BSA-stabilized Au NCs, a colorimetric method with a detection limit of 0.6 μg/mL was achieved for trypsin. It is still highly desirable to develop simple, rapid and sensitive colorimetric methods for the detection of trypsin.

Cytochrome c (cyt c) is a well-known heme-containing electron transfer protein that plays significant roles in the mitochondrial respiratory chain (Fan et al., 2002). Cyt c could be digested by trypsin into heme-peptide fragment, which has significantly lower isoelectric point (pI 7.0) than that of cyt c (pI 10.2) (Bushey and Jorgenson, 1990; Wahl et al., 1994). This feature made cyt c have been widely used as a natural substrate to develop fluorescence method for the assay of trypsin activity, in which cyt c acted as a quencher (Wang et al., 2010; Liao et al., 2013). Recently, Zhang et al. (2014) reported that the hydrolysate of cyt c by trypsin, the heme-peptide fragment, had higher catalytic activity on luminol-H_2O_2 chemiluminescence reaction than that of cyt c. By using these facts, they developed a sensitive chemiluminescence method for the determination of trypsin (Zhang et al., 2014).

In this work, we attempt to develop a simple colorimetric method for the detection of trypsin by employing cyt c as an enzyme substrate and TMB as a chromogenic agent. TMB is colorless in reduction state and can be oxidized by H_2O_2 into oxidation state, a blue charge-transfer complex, in the presence of a catalyst, such as horseradish peroxidase (HRP) (Josephy et al., 1982) and free heme (Huy et al., 2005). Scheme 1 shows the schematic diagram of the colorimetric sensing of trypsin. Cyt c hardly catalyzed the oxidization of TMB in the presence of H_2O_2 and the color of the solution was almost colorless. In the presence of trypsin, cyt c was hydrolyzed and released heme-peptide fragment, which exhibited an intensive catalytic role on H_2O_2-mediated TMB oxidization. The solution changed into blue quickly. The resultant color change could be perceived with the naked eye or monitored the absorbance change at 652 nm. The method was rapid, cost-efficient, sensitive, and could be used to the determination of trypsin in biological samples and for inhibitor screening.

2. Experimental

2.1. Chemicals

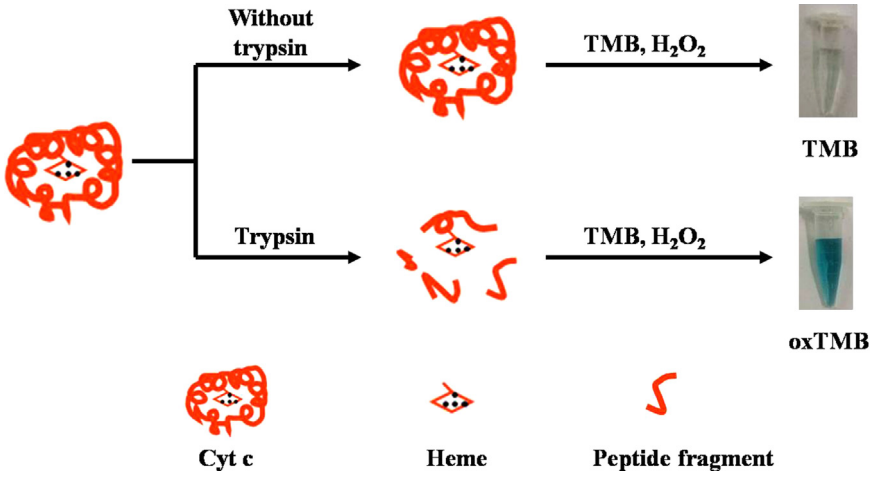
All chemicals were of analytical grade; water was produced from Mole gene 1810b ultrapure water system (Chongqing Mole, China). Trypsin (from pig pancreas), cytochrome c (cyt c, from horse's heart), trypsin inhibitor (from soybean), lysozyme, thrombin, S1 nuclease, pepsin, and chymotrypsin were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Solarbio Technology Co., Ltd (Beijing, China). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Xintai Reagent Co., Ltd (Shandong, China). Hydrogen peroxide (H_2O_2) (30%, v/v) and $CaCl_2$ were purchased from Xilong Chemical Co., Ltd. (Guangdong, China). Tris(hydroxymethyl)aminomethane was purchased from Sin-American Biotechnology Co., Ltd. (Henan, China).

2.2. Apparatus

Absorption spectra were taken on a TU-1901 double beam ultraviolet-visible spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China). The pH of the solution was measured with a PB-10pH meter (Sartorius Scientific Instrument Co., Ltd., Germany). The hydrolysis of cyt c was incubated in a DF-101S constant temperature heating magnetic stirrer (Zhengzhou Kefeng Instrument Equipment Co., Ltd., China).

2.3. Color development and trypsin assay

In a typical experiment, trypsin solutions with various concentrations were firstly mixed with 0.5 μmol/L cyt c solution in 50 mmol/L Tris buffer (containing 1.0 mmol/L $CaCl_2$ as the activator) at pH 9.0. After incubation at 37 °C for 60 min, TMB (0.5 mmol/L) and H_2O_2 (10.0 mmol/L) were rapidly added into the above solution to color development. The absorption spectrum of the mixture was measured with 1 cm cuvette within 5 min. The absorbance at 652 nm was used to quantify trypsin.



Scheme 1. Schematic diagram for the colorimetric sensing of trypsin.