A label-free real time fluorometric assay for protease and inhibitor screening with a released heme†

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A label free continuous assay for protease activity and inhibitor screening has been developed. A protease (trypsin) could digest hemoglobin. Free heme molecules were released. Strong π–π stacking and hydrophobic interactions with the perylene probe resulted in efficient quenching of the probe's monomer fluorescence.

Proteases are one of the most abundant classes of enzymes that can substantially speed up the hydrolysis of proteins. They occur naturally in all living organisms and play key roles in a variety of cellular processes (such as cell signaling and apoptosis).Ill-regulated or undesired protease activity is associated with a number of widespread human diseases including cancer, arthritis, viral infection, and Alzheimer’s disease. They are often the therapeutic targets selected by the pharmaceutical industry. And inhibitor drugs are currently employed for the treatment of many diseases such as HIV infection and diabetes. Therefore, the development of assay methods for efficient detection of protease activity and the screening of potential inhibitors is of great importance.

A variety of techniques have been developed to analyze protease activity, such as gel electrophoresis, mass spectrometry, enzyme linked immunoabsorbent assay (ELISA), and a number of colorimetric, electrochemical, and fluorometric methods. Among them, fluorometric methods are often employed due to the high sensitivity and easy operation. Protease activity fluorometric assays based on quantum dots, conjugated polyelectrolytes, fluorescence resonance energy transfer (FRET), and aggregation-induced emission (AIE) have been reported in recent years. However, certain limitations still exist. For example, the complexity of conjugated polymer synthesis, fluorophore labeling, and the toxicity of some of the quantum dots limit their applications.

Herein we report a novel label free assay for the sensing of protease activity and inhibitor screening. Trypsin was selected as the model protease, and the heme containing bovine hemoglobin was selected as the substrate. When hemoglobin was digested by trypsin, heme was released. The negatively charged heme molecules bound strongly to the positively charged perylene probe (probe 1), which caused efficient quenching of the probe’s fluorescence. The changes in probe’s emission intensity could be monitored in real time and directly related to the amount of trypsin added in the assay solution. The assay is label free, highly sensitive, selective, fairly simple, and inexpensive.

Trypsin was selected as the model protease. It is an important serine protease produced by the pancreatic acinar cells, and plays a key role in food digestion, and the activation of other enzymes of the pancreatic juice such as chymotrypsinogen and carboxypeptidase. Trypsin preferably cleaves peptide bonds on the C-terminal side of lysine and arginine amino acid residues. Its level is closely related to a number of pancreatic diseases.

Hemoglobin is a metalloprotein and has four subunits. Each subunit is composed of a polypeptide chain and a heme group. The iron atom is coordinated by protoporphyrin IX and an imidazole nitrogen atom of a histidine residue. The major function of hemoglobin is oxygen transportation.

Scheme 1 outlines the assay strategy. (1) Hemoglobin was digested when mixed with trypsin in the assay solution. Heme molecules were released. (2) Heme contains two negatively charged carboxylic acid functional groups, probe 1 contains four positive charges, and both molecules possess large planar aromatic structures. Heme and probe 1 would form an aggregation complex through strong electrostatic and π–π stacking interactions.

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Probe 1’s fluorescence was efficiently quenched. (3) The changes in emission intensity could be directly related to the amount of trypsin added in the assay solution. (4) In the presence of a trypsin inhibitor, the activity of trypsin would be reduced, less amount of hemoglobin was digested, and increased probe 1 monomer fluorescence would be observed, the emission restoration could be used to evaluate the performance of the inhibitor.

Probe 1 emits strong green fluorescence in a buffer solution with a maximum emission of 488 nm. Fig. 1 shows that with the increase in the enzymatic reaction time, the fluorescence intensity of probe 1 gradually decreased, indicating that the substrate bovine hemoglobin was gradually degraded by trypsin, and more heme molecules were released into the assay solution. Probe 1 bound to the free heme molecules and resulted in gradual increase in quenching of probe 1 monomer fluorescence.

Since the free Fe(II)-heme could be easily oxidized to Fe(III)-heme in the air (less than 1 s),13 it seems likely that the heme–probe 1 aggregation complex contained mostly the Fe(III)-heme molecules (Fig. S1, ESI†). Control experiments were conducted to verify that the observed probe 1 fluorescence quenching was indeed a result of its binding to the Fe(III)-heme molecules. Fig. S2 (ESI†) shows that with the increase in the solution Fe(III)-heme concentration, probe 1 fluorescence was gradually quenched. In contrast, when probe 1 was replaced by fluorescein, a commonly used fluorescence dye, very little fluorescence quenching was observed (Fig. S3, ESI†). In addition, a negatively charged perylene probe was also selected (probe 2, Fig. S1, ESI†). Fig. S4 (ESI†) shows that with probe 2 concentration kept at 5 μM, an increase in the solution Fe(III)-heme concentration from 0 to 10 μM caused rather small quenching of the probe 2 fluorescence. Since probe 2 contains two negative charges, and Fe(III)-heme also contains two negative charges, electrostatic repulsive interactions to a large degree prevented binding of probe 2 to Fe(III)-heme. The results clearly show that the observed fluorescence quenching was a result of binding of probe 1 to the released heme molecules. The degree of fluorescence quenching depended on the concentration of the free heme, and the extent of the enzymatic digestion reaction.

The probe 1 emission intensity changes at 488 nm were monitored in real time (Fig. 2). The results show that the emission intensity remained mostly unchanged with no trypsin added. And with the increase in the assay solution trypsin concentration, more dramatic probe 1 emission intensity changes were observed. The gradually increased degree of probe 1 fluorescence quenching with the prolonged incubation time clearly suggests that the substrate hemoglobin was gradually digested by trypsin, and increased concentrations of heme molecules were released into the assay solution. At a trypsin concentration of 2 μM, a saturation point was reached after about 50 min of enzymatic digestion. The results indicate that at such a high concentration of trypsin, after 50 min of reaction, a sufficient amount of heme molecules was released, and complete quenching of the probe’s fluorescence was obtained. The kinetic parameters \( K_M \) and \( \nu_{max} \) were determined to be 43.36 μM and 1.18 μM min\(^{-1}\), respectively (Fig. S10–S12, ESI†). The quenching efficiency after 15 min of the enzymatic digestion reaction was in direct proportion to trypsin concentration in the range of 0–100 μM mL\(^{-1}\) [Fig. S6(a), ESI†]. The initial reaction rate (\( \nu_0 \)) also displays a linear relationship with trypsin concentration in the range of 0–2000 μM mL\(^{-1}\) [Fig. S6(b), ESI†]. The limit of detection of our assay is estimated to be 2 μM mL\(^{-1}\), which is among the best trypsin assay methods reported to date.14 In addition, a number of other proteases were also tested (Fig. S7, ESI†). The results clearly show that our assay method could also be used to analyze the activity of other proteases.

In order to test the specificity of the assay, control experiments were conducted. A number of commonly used enzymes such as S1 nuclease, alkaline phosphatase (ALP), lysozyme, and a mixture of these enzymes were tested. Fig. 3 shows that under the same experimental conditions, none of these enzymes produced noticeable probe 1 emission intensity changes. The results clearly suggest that the assay is highly specific for the protease. We also tested a positively charged protein (lysozyme) and bovine albumin (a common protein found in many biological samples). The results show that they did not significantly interfere with our assay (Fig. S8 and S9, ESI†).

An enzyme inhibitor can bind to a specific enzyme and reduce its catalytic activity considerably. Many enzyme inhibitors are
used nowadays as drugs to treat a variety of diseases.2
A library screening approach has often been used in many
cases to find specific enzyme inhibitors. The development of
a novel inhibitor screening method with low cost and high
efficiency is therefore of critical importance.

Three known trypsin inhibitors were tested. They were the
inhibitor from soybean, the inhibitor from egg white, and
the inhibitor from bovine hemoglobin. The rate of enzymatic hydrolysis
of bovine hemoglobin would decrease in the presence of these
benzamidine hydrochloride. The rate of enzymatic hydrolysis
inhibitor from soybean, the inhibitor from egg white, and
of bovine hemoglobin would decrease in the presence of these

Heme molecules were released. Electrostatic and π–π stacking
interactions among heme and probe 1 molecules resulted in
efficient quenching of the probe’s monomer fluorescence. The
decrease in the probe’s emission intensity was directed related to
the amount of trypsin added to the assay solution. A limit of
detection of 2 mU mL⁻¹ was achieved. And the IC₅₀ values of
three trypsin inhibitors were determined. Our assay is simple,
sensitive, selective, and inexpensive. It could be used for protease
activity detection in related biological/biochemical applications,
and the screening of protease inhibitors as potential drugs.

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