Electronic Supplementary Information

A Label-Free Real Time Fluorometric Assay for Protease and Inhibitor Screening with a Released Heme

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EXPERIMENTAL SECTION

Materials

Trypsin and thrombin were obtained from Beijing Dingguo Biotechnology Co., Ltd. (Beijing, China). Bovine hemoglobin (BHb) was obtained from Worthington Biochemical Corporation (Lakewood, NJ, USA). Lysozyme and bovine serum albumin (BSA) were purchased from Bio Basic Inc. (Markham, Ontario, Canada). Alkaline phosphatase (ALP) was from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Fe(III)-heme (hematin) and the trypsin inhibitor from soybean (Type I-S) were obtained from Sangon Biotechnology Co., Ltd. (Shanghai, P. R. China). The inhibitor from egg white (Type II-O, partially purified ovomucoid) was from Sigma. and benzamidine hydrochloride was from Aladdin Chemistry Co., Ltd. (shanghai, P. R. China). Details of the synthesis of the positively charged perylene probe 1 and the negatively charged perylene probe 2 will be reported elsewhere. The protein solutions were stored at 4 ºC before use. All stock and buffer solutions were prepared using water purified with a Milli-Q A10 filtration system (Millipore, Billerica, MA, USA).

Instrumentation

UV-vis absorption spectra were obtained with a Cary 50 Bio spectrophotometer (Varian Inc., CA, USA). Emission spectra were recorded using a Fluoromax-4 spectrofluorometer (Horiba Jobin Yvon Inc., USA), with an excitation wavelength of 442 nm. Excitation and emission slit widths were 1 nm. Quartz cuvettes with 10-mm path length and 2 mm window width were used for UV-vis and emission

measurements. The buffer pH was measured using a pH meter with a glass/reference electrode, calibrated with standard buffer solutions of pH values of 4, 7, and 10 from Sangon Biotechnology Co., Ltd. (Shanghai, China). Unless specified, all spectra were taken at 37 ºC in a 5 mM Tris-HAc buffer solution (pH 8.2).

Assay procedures

Trypsin (8 μL) of different concentrations was added to 392 μL of 5 mM Tris-HAc buffer solution (pH 8.2) containing 5 μM bovine hemoglobin (the substrate) and 5 μM probe 1. The final concentrations of trypsin were 0, 5, 10, 25, 50, 100, 250, 500, 1000, and 2000 mU/mL, respectively. Assay solution temperature was kept at 37 ºC. The emission intensity changes at 488 nm were monitored in real time with data points taken every 0.1 s.

Kinetic parameters

8 μL trypsin (2 U/mL) was added to 392 μL Tris-HAc buffer solution (5 mM, pH 8.2) containing 5 μM probe 1 and bovine hemoglobin (the substrate) of different concentrations. The final concentrations of the substrate were 1.0, 1.5, 2.5, and 5 μM, respectively. The emission intensity changes at 488 nm were record.

Michaelis – Menten equation has often been used to characterize the enzymatic reaction kinetics. A plot of $1/\upsilon_0$ versus 1/[S], usually called a Lineweaver – Burk plot, yields a straight line, with intercept on the x-axis as $-1/K_M$, and on the y-axis as

$1/\upsilon_{max}$.[S1] The concentrations of hemoglobin (μM) were calculated from the calibration curves in Figure S11, and the initial reaction rates (υ_0, $\mu M \cdot min^{-1}$) were calculated from the changes in emission intensity of the perylene probe 1 in the first 3 minutes of the enzymatic reaction. Figure S12 shows the plot of $1/\upsilon_0$ vs 1/[S], from which the kinetic parameters were obtained.

Trypsin inhibitor screening

Trypsin inhibitor from soybean was first isolated by Kunitz. It is a monomeric protein containing 181 amino acid residues.[S2] Chicken ovomucoid is a glycoprotein, it is comprised of 186 amino acids arranged in three tandem domains.[S3] Benzamidine hydrochloride is a reversible inhibitor of trypsin-like enzymes.

Different amounts of the trypsin inhibitors were mixed with trypsin, and the solutions were incubated at 4 ºC for 30 min. Assay solution containing bovine hemoglobin (5 μM) and probe 1 (5 μM) was incubated at 37 ºC. 8 μL of the pre-incubated trypsin (+ inhibitor) solution was added to 392 μL of the assay solution, and the emission intensity changes were recorded.

Calculation of the IC_{50} value

IC_{50} value is defined as the concentration of the inhibitor required to achieve 50% decrease of the enzyme activity.[S4] It has been widely used to evaluate the inhibition effects of enzyme inhibitors. The IC_{50} values of the selected trypsin inhibitors could

be obtained from the plot of inhibition efficiency (*IE*) versus inhibitor concentration, and the following equation was used to calculate the inhibition efficiency.[S5]

$$IE = \frac{C_t\ (inhibitor) - C_t\ (no\ inhibitor)}{C_0 - C_t\ (no\ inhibitor)}$$

"C_0" is the initial concentration of bovine hemoglobin, "C_t *(inhibitor)*" and "C_t *(no inhibitor)*" are the concentrations of hemoglobin after 15 min trypsin hydrolysis in the presence or absence of the inhibitor, respectively. "C_0", "C_t *(inhibitor)*", and "C_t *(no inhibitor)*" values are obtained from Figure S13 and Figure S11-(d).

RESULTS AND DISCUSSION

Toxicity of the perylene probe

Perylene derivatives have been extensively used as dyes and pigments.[S6] They have also been used for drug delivery,[S7] photodynamic therapy,[S8] and live-cell imaging.[S9] So their toxicity is limited.

Assay optimization

The optimal concentration of bovine hemoglobin added to the assay solution was investigated. Figure S5 shows that at a trypsin concentration of 2 U/mL, with the increase of the solution hemoglobin concentration, the quenching efficiency gradually increased. At a 5 μM hemoglobin concentration, maximum quenching efficiency (99.9%) was obtained. 5 μM hemoglobin concentration was therefore selected as the optimized concentration of the substrate in the current investigation.

Specificity of the assay

S1 nuclease hydrolyzes single-stranded RNA or DNA into mononucleotides. ALP is a phosphomonoesterase that hydrolyzes the 5′-phosphate groups from DNA, RNA, and nucleotides. Lysozyme is a hydrolytic enzyme specific for 1,4-beta-glycosidic linkage between N-acetylmuramic acid and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls.

Comparison of the IC_{50} values with the literature reported values

When the inhibitor from chicken egg white or benzamidine were used as the inhibitor, the inhibition efficiency was comparable to the reported literature values (Table S1). However, when the inhibitor from soybean was used, the inhibition efficiency (IC_{50} value) we obtained was somewhat different from the reported literature value. Literature reports have shown that the IC_{50} value is influenced by many experimental factors such as the source and concentration of the enzyme, the source of the inhibitor, the substrate, etc.[S13] It seems understandable that the inhibition efficiency is somewhat different from the reported literature values.

The label free approach and the turn off mode of detection

For the analysis of complex biological samples, a fluorescence turn on mode is normally preferred. And it has to be emphasized that generally speaking label free assays are more susceptible to experimental factors, and the potential interference compounds in the assay solution. We are currently working on these issues, and we

hope in the near future, better label free assay methods for the convenient, highly sensitive and selective assay of complex biological samples could be developed.

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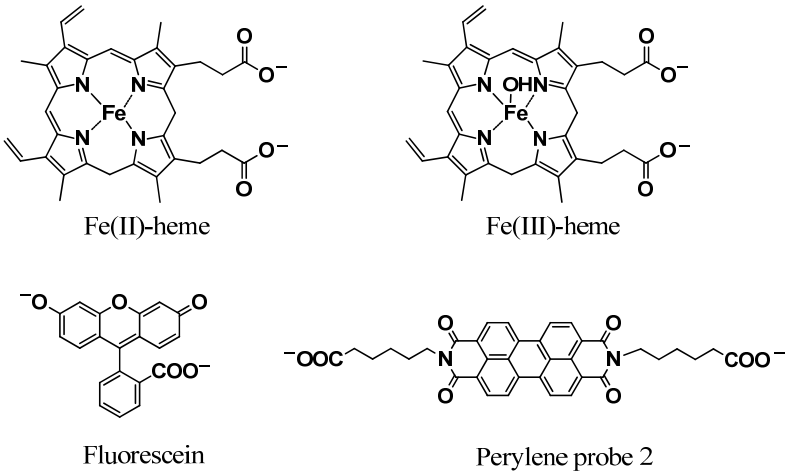

Figure S1. Structures of Fe(II)-heme, Fe(III)-heme, fluorescein, and the negatively charged perylene probe 2.

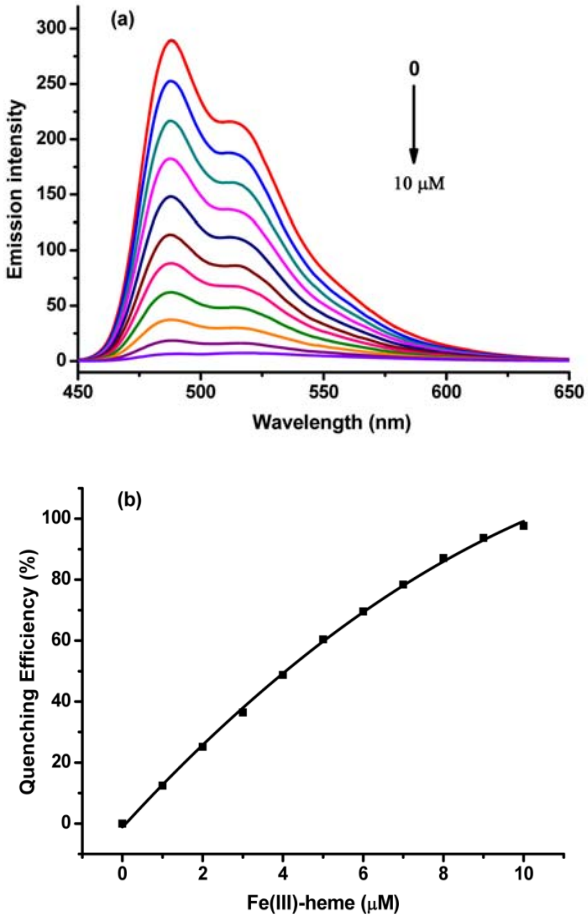

Figure S2. (a) Changes in the emission spectrum of 5 μM probe 1 upon the addition of Fe(III)-heme in different concentrations. **(b)** Quenching efficiency. Quenching experiments were performed by successive addition of Fe(III)-heme to a solution of probe 1 (5 μM) in 5 mM Tris-HAc buffer at pH 8.2 (total sample volume, 2 mL).

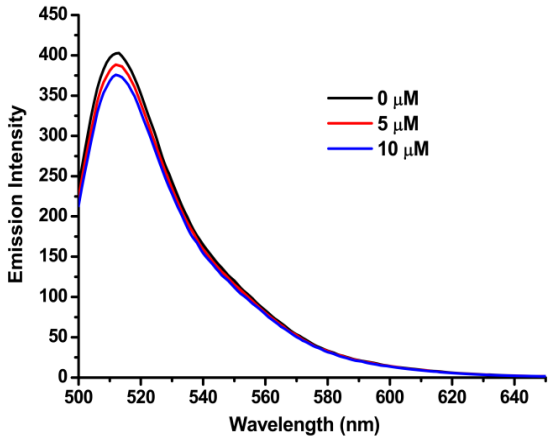

Figure S3. Changes in the emission spectrum of 5 μM fluorescein upon the addition of Fe(III)-heme in different concentrations (0, 5, and 10 μM, respectively).

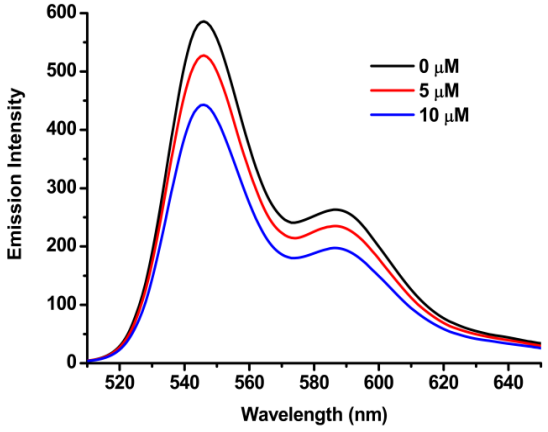

Figure S4. Changes in the emission spectrum of 5 μM perylene probe 2 upon the addition of Fe(III)-heme in different concentrations (0, 5, and 10 μM, respectively).

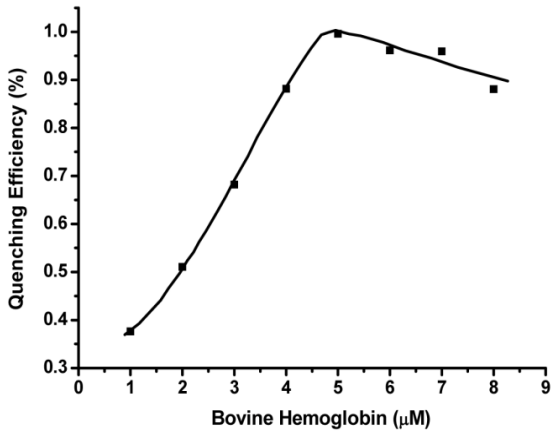

Figure S5. Changes in quenching efficiency upon the addition of bovine hemoglobin in different concentrations. Trypsin, 2 U/mL; reaction time, 90 min.

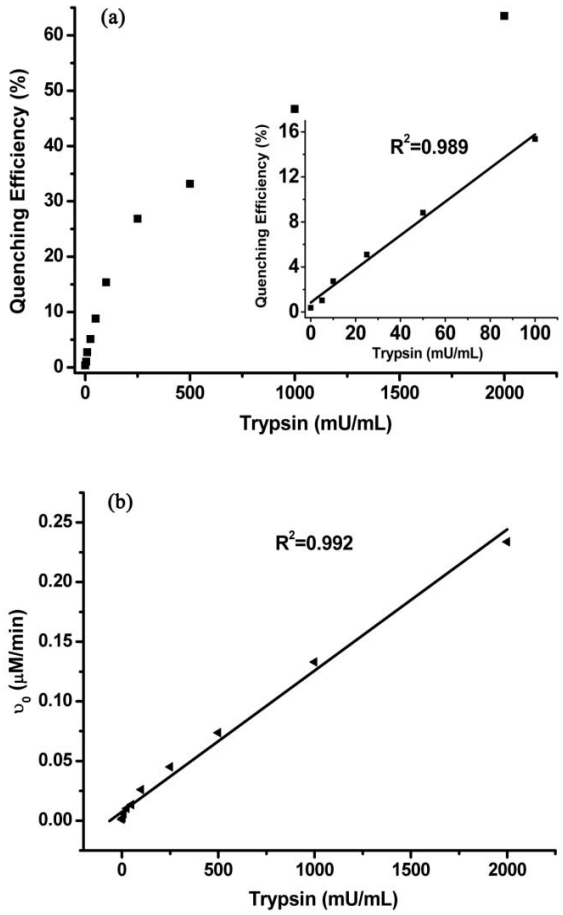

Figure S6. (a) Changes in quenching efficiency against trypsin concentration, reaction time: 15 min. Inset: expanded linear region of the curve. The linear regression equation is: $(F_0\text{-}F)/F = 0.149C + 0.845$ ($R^2 = 0.99$), in which "$(F_0\text{-}F)/F$" is the quenching efficiency and "C" is the concentration of trypsin in mU/mL. **(b)**

Changes in the initial rate of hemoglobin hydrolysis (υ_0) against trypsin concentration. The linear regression equation is: $\upsilon_0 = 1.16 \times 10^{-4}C + 0.0086$ ($R^2 = 0.99$), in which "υ_0" represents the initial reaction rate and "C" represents the concentration of trypsin in mU/mL.

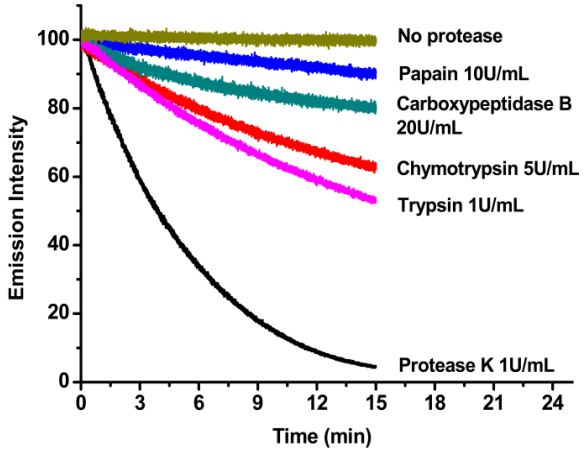

Figure S7. Assay of the enzymatic activities of other proteases. Probe 1 emission intensity changes at 488 nm were monitored as a function of reaction time. Assay conditions: 5 μM hemoglobin, 1 U/mL trypsin, 5 mM Tris-HAc, pH 8.2.

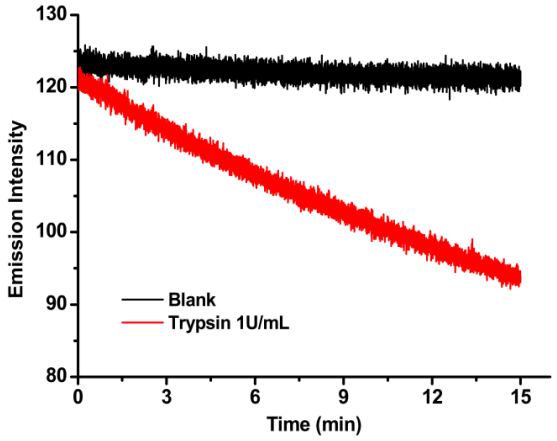

Figure S8. Selectivity of the assay. Probe 1 emission intensity changes at 488 nm were monitored as a function of reaction time. Blank control contained 5 μM hemoglobin, 5 μM lysozyme; Sample solution contained 5 μM hemoglobin, 5 μM lysozyme, and 1 U/mL trypsin. Buffer, 5 mM Tris-HAc, pH 8.2.

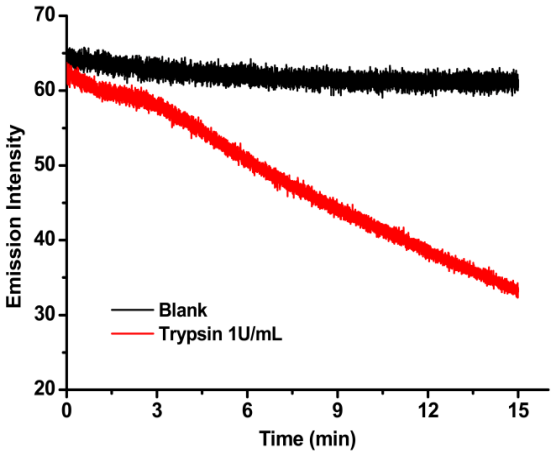

Figure S9. Selectivity of the assay. Probe 1 emission intensity changes at 488 nm were monitored as a function of reaction time. Blank control contained 5 μM hemoglobin, 5 μM bovine albumin; Sample solution contained 5 μM hemoglobin, 5 μM bovine albumin, and 1 U/mL Trypsin. Buffer, 5 mM Tris-HAc, pH 8.2.

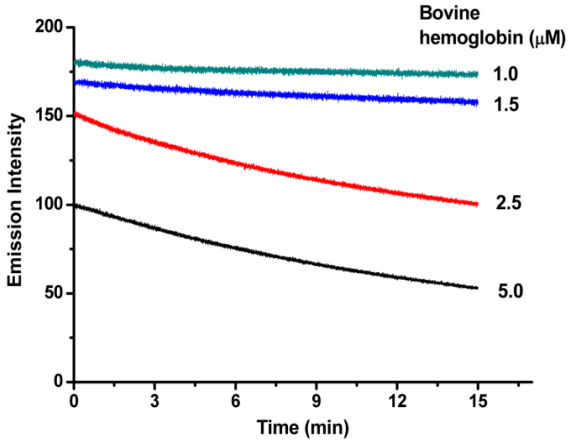

Figure S10. Real time emission intensity changes of probe 1 at 488 nm. Bovine hemoglobin concentrations: 1, 1.5, 2.5, and 5 μM, respectively. Trypsin: 1.0 U/mL.

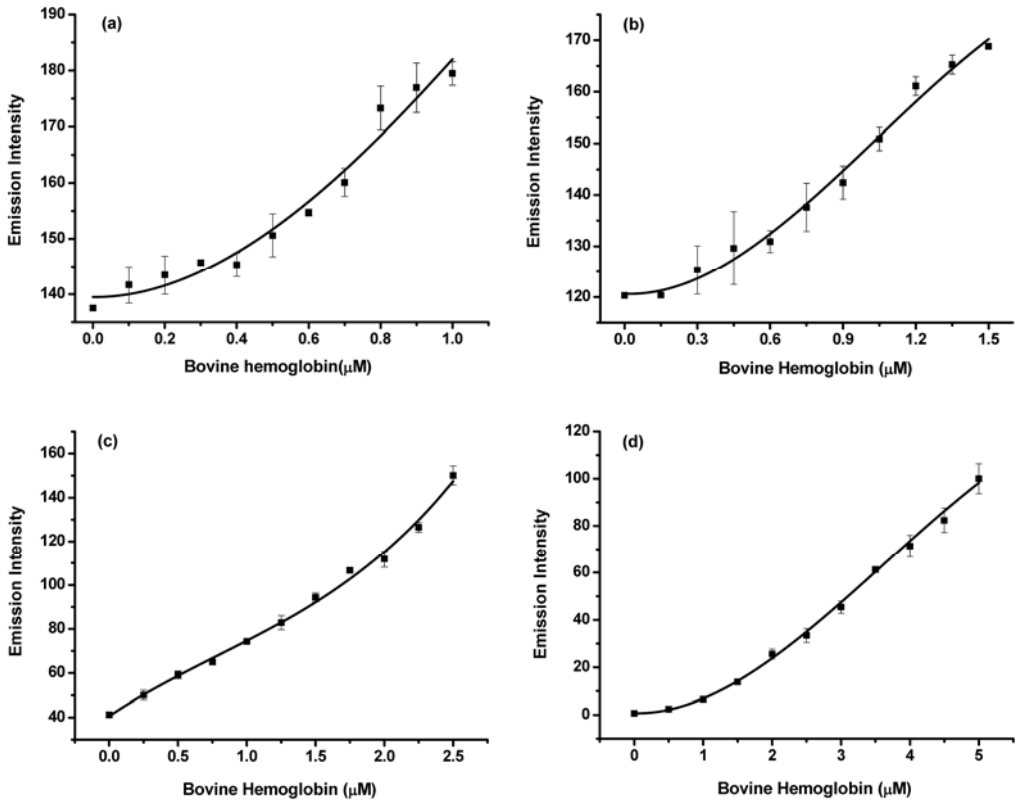

Figure S11. Calibration curves. Figures a-d show the calibration curves with total bovine hemoglobin concentrations (digested + undigested) of 1.0, 1.5, 2.5, and 5 μM, respectively. The values on the x-axis are the concentrations of the undigested bovine hemoglobin. Probe 1 concentration: 5 μM. Data points were obtained by pre-mixing of the known amounts of the digested and undigested hemoglobin with probe 1, and the emission intensities at 488 nm were measured.

The completely digested bovine hemoglobin sample was prepared by the following procedure: 8 μL trypsin (2 U/mL) was added to 372 μL Tris-HAc buffer (5 mM, pH 8.2) solution containing 5 μM bovine hemoglobin (the substrate). The assay mixture was incubated at 37 ºC for 12 hours.

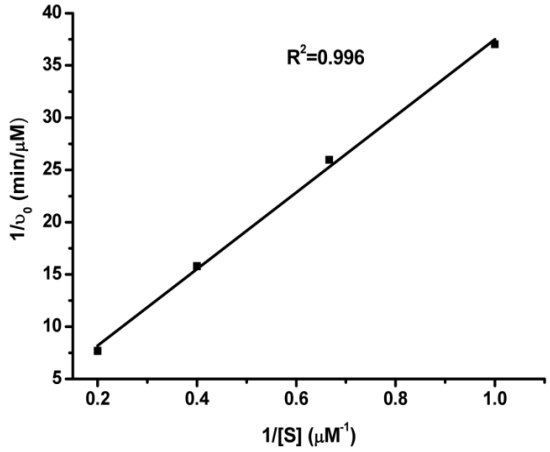

Figure S12. Lineweaver – Burk plot. Bovine hemoglobin hydrolysis was catalyzed by 1.0 U/mL trypsin, in the presence of 5 μM probe 1, and 1.0, 1.5, 2.5, 5 μM bovine hemoglobin.

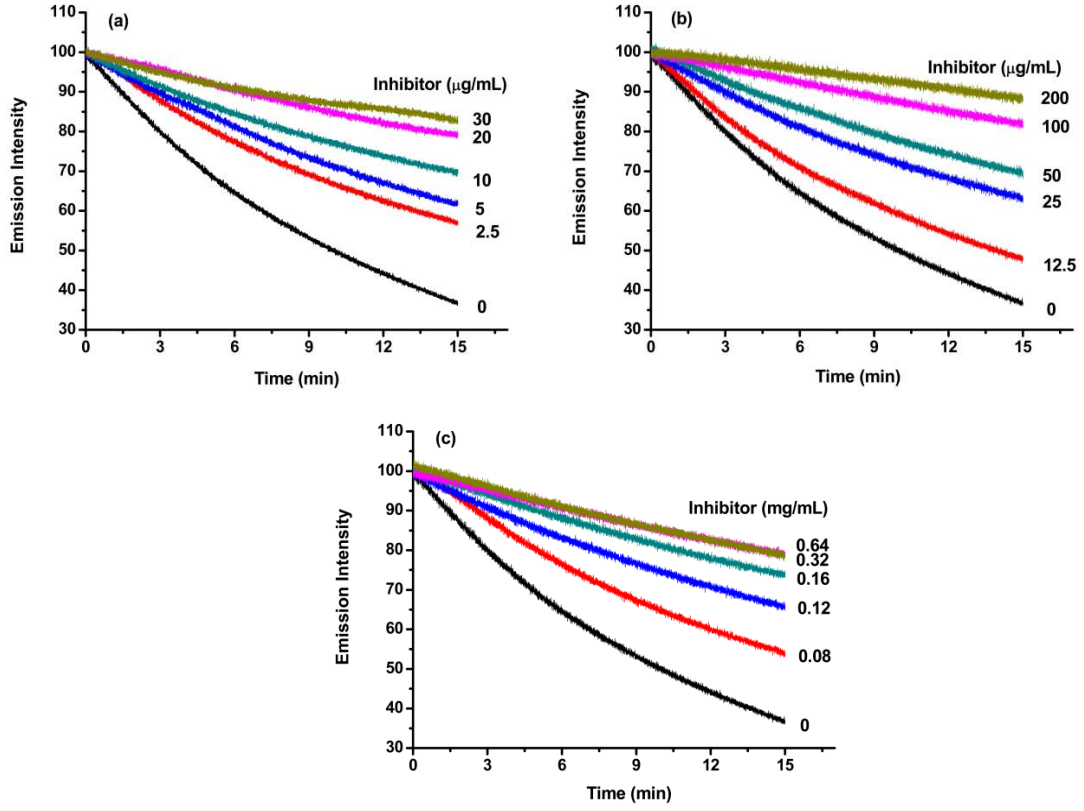

Figure S13. Real time emission intensity changes at 488 nm in the presence of trypsin (2 U/mL), and inhibitors of various concentrations. The inhibitors: (a) from soybean (b) from egg white, and (c) benzamidine hydrochloride.

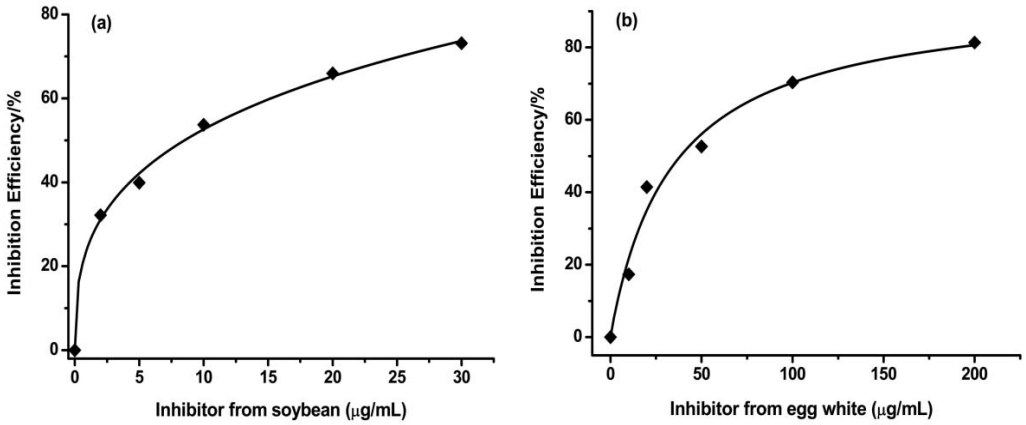

Figure 14. Plot of the inhibition efficiency versus the concentration of inhibitor from (a) soybean, (b) chicken egg white.

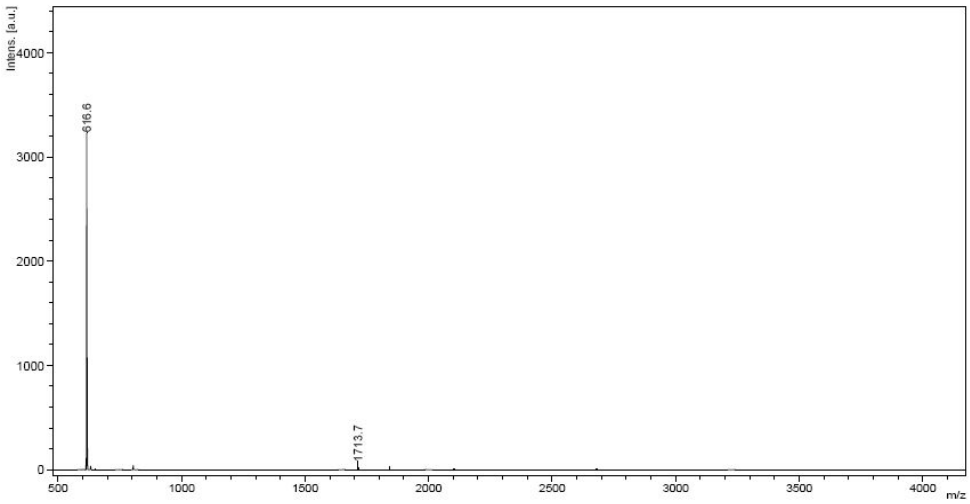

Figure S15. MALDI-TOF mass spectrum. 50 μM bovine hemoglobin sample was digested by 2 U/mL trypsin at 37 ºC in 5 mM Tris-HAc buffer (pH 8.2) for 4 hours. The signal of highest intensity at 616.6 Da is ascribed to free heme.

Table S1. Comparison of the inhibition efficiency with literature methods.

Inhibitor	Our assay		Literature method	
	Substrate	Inhibition efficiency (IC_{50})	Substrate	Inhibition efficiency
From soybean		8.5 μg/mL (0.38 μM)	D-Phe-piperidinyl-Arg-4-nitroanilide[S10]	IC_{50} = 0.009 μM
From chicken egg white	Bovine hemoglobin	38.3 μg/mL	N-α-benzoyl-L-arginine p-nitroanilide[S11]	20 μg/ mL, 13.5% inhibition
Benzamidine hydrochloride		0.126 mg/mL	N-α-benzoyl-DL-arginine-p-nitroanilide[S12]	2 mg/mL, 72% inhibition