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Dual emissive bispyrene peptide probes for highly sensitive measurements of trypsin activity and evaluation of trypsin inhibitors

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ABSTRACT

Peptide substrates were double labeled with pyrenes to prepare fluorescent probes for highly sensitive detection of protease activity and evaluation of protease inhibitors using pyrene monomer/excimer signals. Two proximate pyrene moieties formed excited state dimers in the probes, and these pyrene excimer formations were dissociated by tryptic digestion. The specificity constant of the optimum bispyrene peptide probe was 2.7 times higher than that of the conventional peptide-4-methylcoumarin amide. Moreover, our probe had high sensitivity with an estimated detection limit for trypsin of 4.11 pM. The half maximal inhibitory concentration and dissociation constant of the Bowman–Birk inhibitor were successfully estimated.

1. Introduction

Trypsin (EC 3.4.21.4), which can cleave polypeptide substrates at Arg/Lys-Xaa bonds (where Xaa is any amino acid), plays a key role in protein digestion.[1,2] Trypsin is primarily produced by the pancreas as its inactive precursor, trypsinogen, which is activated to trypsin by enterokinase.[3,4] Once activated, trypsin can activate trypsinogen and many other digestive proenzymes, such as chymotrypsinogen, proelastase, kallikreinogen, procarboxypeptidase, and some prolipases, and also pancreatic and inflammatory cells. Therefore, trypsin plays an essential role in regulating pancreatic exocrine function. Trypsin overexpression or deficiency is directly associated with some pancreatic and other diseases.[5–9] Trypsin is commonly used as a model protease because it is inexpensive and readily available, and a promising biomarker. Therefore, a simple and sensitive method for detection of trypsin in biological samples is essential for early disease diagnosis, therapy, and biological research.[10]

Fluorescent probes are a powerful tool for detecting and monitoring biological compounds because they provide high sensitivity, rapid assays, and versatility toward biologically important analytes.[11–13] Therefore, many fluorescent probes have been developed for biological targets such as enzymes. For measuring protease activity, which is useful for evaluating drug targets or diagnostic and prognostic biomarkers,[14] fluorescent peptide probes such as peptide-4-methylcoumarin amide (MCA)[15–18] and Förster resonance energy transfer (FRET)-based substrates[19–22] are commonly used. Proteolytic cleavage of these probes changes their fluorescent properties, allowing the detection of protease activity. However, the commercially available peptide-MCA and FRET-based substrates are not versatile. The specificity for the *P*1′ amino acid residue, which occupies the S1′ subsite of the protease, is important for substrate recognition and catalytic efficiency. However, the Peptide-MCA substrates have no specificity for *P*1′.[23] In FRET-based assays, the requirement to combine a fluorophore with an appropriate quencher can limit the versatility, and the necessary fluorophores or quenchers are often expensive. Furthermore, the synthesis of FRET-based substrates is usually complicated because at least one pair of orthogonal protecting groups is needed to enable the coupling of the two different dyes required for FRET. Recently, several near infrared (NIR) dyes have been exploited for detecting protease activity.[24,25] However, the preparation of NIR dyes is generally difficult and costly.

Pyrene, a commonly occurring fluorescent molecule, has been exploited for detection and monitoring of protease activity because of its unique fluorescence characteristics: monomeric pyrene shows fluorescence emission (370–420 nm), while an excimer band (450–550 nm) appears when two pyrene molecules are close together (approximately 10 Å apart).[26,27] Usefully, pyrene excimer emission can be easily observed by the naked eye, and causes a large Stokes shift (130 nm).[28,29] Several precisely controlled aggregation and dissociation systems of pyrenyl compounds have been used for protease assays. Xu et al. reported that electrostatic interaction of negatively charged *N*-[4-(1-pyrenyl)butanoyl]-L-tryptophan with positively charged melittin enabled the detection of trypsin activity.[30] Tang et al. also developed a sodium 3-(pyren-1-yloxy)propane-1-sulfonate-protamine complex for detection of trypsin activity.[31] In addition, Wang et al. designed a pyrene-functionalized peptidic inhibitor that contained two pyrene-Lys-Trp-Lys sequences attached via the C-terminus to a lysine amide as a

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branched unit.[32] This cationic peptide bound to the negatively charged surface of β-tryptase, and this non-covalent binding changed the monomer/excimer signals of the pyrene moieties through aggregation-induced emission. In these systems, assembly and disassembly of pyrenyl compounds by intermolecular electrostatic or hydrophobic interactions leads to monomer/excimer emission from pyrene, which enables detection of protease activity. However, the sensitivity cannot be regulated because it depends on the formation and dissolution of the entire assembly of pyrene complexes.[33] Moreover, the kinetic assay is complicated in these systems, and standard Michaelis–Menten kinetics cannot be applied. Intramolecular excimer-forming substrates have been developed as an alternative. Ahn et al. developed two pyrene-labeled peptide substrates that each contained two pyrene moieties incorporated into the side chains of cysteines at the N- and C-termini of the substrate peptide for trypsin.[23] However, these probes showed insufficient kinetic parameters compared with conventional substrates, and could only detect trypsin activity at the nanomolar level.

In this study, fluorescent peptide probes were developed for a simple and sensitive protease assay. We designed bispyrene peptide probes for the detection of trypsin activity by combining the well-known dual emission of pyrene with a protease activity-triggered peptide probe (Fig. 1A). The probes contained two molecules of 1-pyrenebutyric acid (Pba) and one or two substrate peptides for trypsin. Both long and short probes were designed. The long probes (**1–4**) contained two Pba-linked substrate peptides on either side of a core. In the short probe (**5**), one Pba-linked substrate peptide was replaced with Pba. The long probes, which had two cleavage sites, were expected to undergo fast enzymatic reactions in which trypsin facilitated access to the recognition sites in the probes. Hexamethylenediamine was used as the probe core. To study the effect of sterically hindered pyrene moieties, β-alanine was inserted between the Pba and valine residue in **1**, **3**, and **5**. Moreover, one extra amino acid (glycine) was inserted between the arginine residue and the core in **1**, **2**, and **5** to provide a native amino acid in the *P1′* position of the artificial substrate. The two pyrene moieties formed excited state dimers in the probes, and the probes emitted excimer fluorescence (Fig. 1B). After proteolytic cleavage, these pyrene excimers dissociated, and the excimer fluorescence decreased while the monomer fluorescence increased. This change in excimer fluorescence enabled detection of protease activity.

2. Results and discussion

To synthesize the long probes, first, the corresponding protected Pba-containing peptides were synthesized by standard 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis (SPPS). These peptides were then coupled with both edges of hexamethylenediamine, followed by the deprotection of 2,2,4,6,7-pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) or 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) groups (Figs. S1–S8). To synthesize the short probe, *N-tert*-butyloxycarbonyl (Boc)-hexamethylenediamine was coupled with Pba. After the deprotection of the Boc group from the previously obtained compound, the corresponding protected Pba-containing peptide synthesized by Fmoc SPPS was then linked. Finally, the Pbf group was deprotected (Figs. S9–S10).

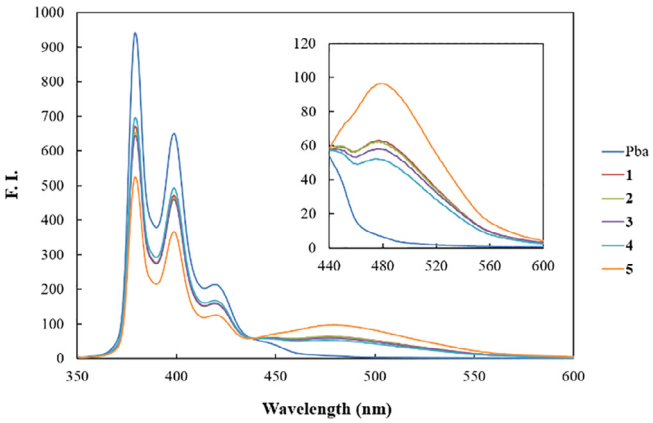

Fig. 2. Monomer and excimer emission of probes **1–5** (2 μM) and Pba (4 μM) in DMSO at room temperature. The excitation wavelength was 344 nm.

Initially, we investigated the intramolecular excimer emission of the probes. Solutions of **1–5** with concentrations of 2 μM were prepared in dimethylsulfoxide (DMSO). A 4 μM Pba solution was also prepared in DMSO for use as a control. The pyrene concentration in all of the solutions was 4 μM. For each sample, a fluorescence spectrum was recorded upon excitation at 344 nm before addition of trypsin. All of the probes exhibited more intense excimer fluorescence than Pba, which indicated that the two pyrene moieties in the probes were spatially proximate (Fig. 2). Probe **5** showed the most intense excimer fluorescence because it had the shortest peptide unit. The excimer fluorescence intensities for **1** and **2** were almost equal, and were slightly higher than that of **3**. Probe **4** had the least intense excimer emission. These results indicated that insertion of β-alanine in the substrate peptides hardly affected the intramolecular excimer formation, whereas the extra glycine suppressed excimer formation. Therefore, the excimer fluorescence intensities of **1–4** were not solely dependent on the length of the peptide moiety. Next, we investigated changes in monomer and excimer emission during tryptic hydrolysis. The final concentration of **1** was adjusted to 10 μM, and the final concentration of trypsin was adjusted to 10 nM with the buffer. The buffer contained PEG6000 as an additional agent, as we had found in a preliminary study that PEG6000

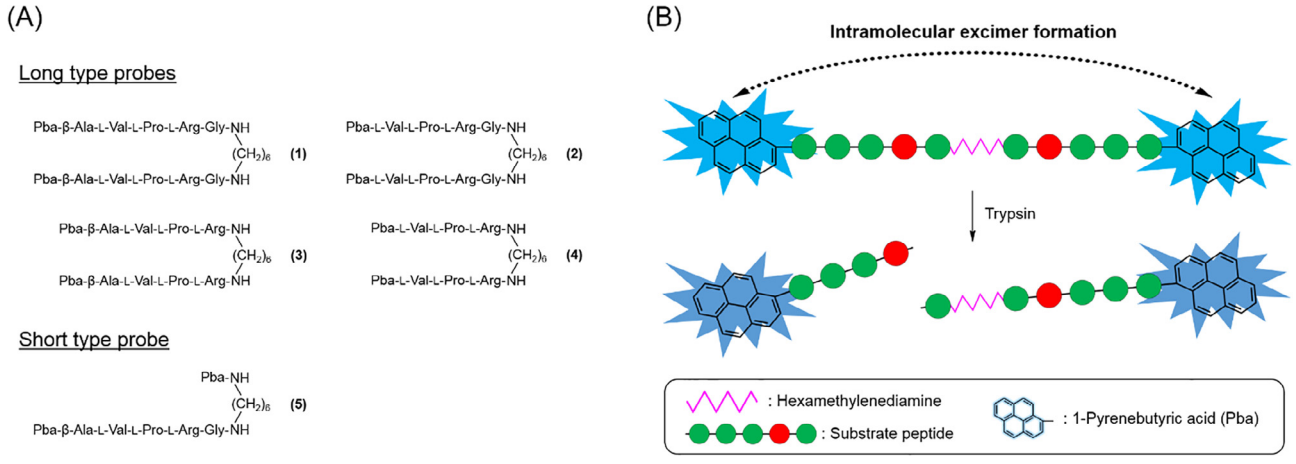

Fig. 1. (A) Design of the bispyrene peptide probes: long probes **1–4** and short probe **5**. (B) Schematic representation of the detection system for **1**.