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Lanthanide-based fluorogenic peptide substrate for the highly sensitive detection of thermolysin

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ABSTRACT

A new fluorogenic, lanthanide-based oligopeptide substrate for the detection of the zinc-dependent endoprotease thermolysin is described. Using time-resolved fluorescence measurement, a highly sensitive assay for thermolysin was developed with a 50 pM detection limit (3.5 fmol).

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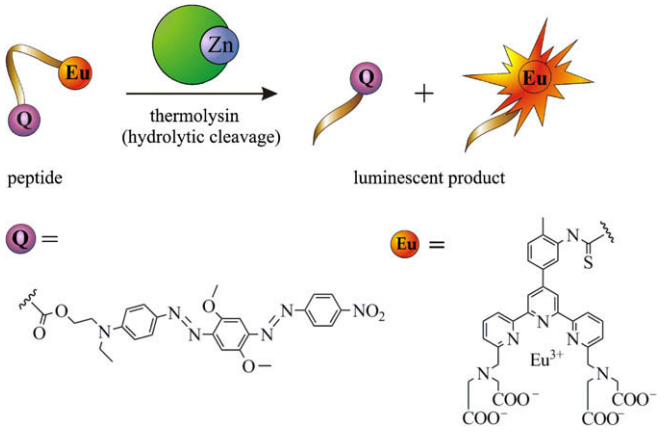
Zinc-containing peptidases are widely distributed in nature and have important roles in many physiological processes. The M4 or thermolysin family comprises numerous zinc-dependent metallopeptidases that hydrolyze peptide bonds. A large number of these enzymes are implicated as virulence factors of the microorganisms that produce them and are therefore potential drug targets.[1] The development of sensitive assays for the detection and screening of protease activity at low concentration is therefore of significant relevance, in particular to the discovery of new protease inhibitors. We became interested in the sensitive detection of zinc-dependent enzymes in a different context, using them as signal-amplifying components in PCR-free nucleic acid detection schemes.[2]

Many synthetic peptides have been coupled with fluorescent dyes to develop sensitive fluorescence resonance energy transfer (FRET) assays for the determination of specific proteases.[3] Proteolytic cleavage of the labeled substrates often leads to large increase of fluorescence. To address the specific binding profile of a protease, extended peptides (typically 10–12 AA) have to be synthesized. More recently, the ensemble of fluorogenic probes has been extended by lanthanide-based compounds suitable for time-resolved measurements.[4] A significant advantage of such probes is their outstanding sensitivity.

Time-resolved fluorescence signals are highly sensitive since practically background-free, scarcely affected by other compounds with short-lived fluorescence that coexist in the sample. Relatively few examples of Eu(III)- and Tb(III)-based protease probes have been reported, and the broad applicability of these substrates has to be demonstrated.

We describe here the first lanthanide-based probe for thermolysin (E.C.3.4.24.27, from *Bacillus thermo-proteolyticus rokko*, type X), a readily available, zinc-dependent bacterial endoprotease (Scheme 1).

In addition, we use for the first time a bis (azo) dye (black hole quencher 2, BHQ-2) as an effective intramolecular quencher of lanthanide fluorescence.



Scheme 1. Cleavage of fluorophore-quencher labeled peptide by thermolysin yields a luminescent signal.

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The fluorescent component of the peptide is the europium(III) complex of a modified terpyridine[5] (referred to as Eu) which was coupled via isothiocyanate functionality to lysine ε-amino group during solid phase synthesis of the peptide sequence. The quencher, commercially available BHQ-2 (referred to as Q), is already conjugated by the supplier via FMOC protected lysine to the peptide resin. A peptide sequence was chosen which presents several feasible adjacent cleavage sites for thermolysin (Gly-Phe, Phe-Ser, Ser-Ala).[6]

As could be observed by MALDI-MS, thermolysin cleaves the substrates at the glycine-phenylalanine bond (data not shown). In order to vary the distance between modified terpyridine and quencher, either Boc-Lys(Fmoc)-OH (K^1) or Fmoc-Lys(Boc)-OH (K^2) were introduced and fully deprotected during cleavage (**1** Eu-$K^1K^2K^2$GFSAK^2K-Q, **2** Eu-$K^1K^2K^2$GFSAK^2K^2K-Q, **3** Eu-$K^1K^1K^1$-GFSAK^1K^1K-Q) (Scheme 2).

The labeled peptides show no luminescence in aqueous solutions (data not shown). Upon addition of thermolysin, the quenching efficiency of BHQ-2 is disrupted due to hydrolysis of the peptide bonds, enabling the terpyridine moiety to act as an effective sensitizer for europium(III) (Scheme 1) generating a luminescent signal.

This can be monitored by the increase in luminescence with the time. Peptides **1–3** showed little difference in their increase in luminescence (data not shown). Therefore, peptide **1** as a representative substrate was used in any further experiments.

In order to demonstrate the sensitivity of the new substrate and find the detection limit, varying amounts of thermolysin were added to a substrate solution and the change of luminescence over the time was recorded (Fig. 1). Up to 50 pM (3.5 fmol) thermolysin yield an 8.4 times higher signal than the background after 18 min.

There are only few commercially available fluorescent substrates for thermolysin.[7] One of them is MOCAc (MOCAc-PLGL(Dpa)AR, 7-methoxycoumarin-4yl) acetyl-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitro-phenyl)-L-2,3-diamino-propionyl]-L-Ala-L-Arg-NH_2) which was compared with peptide **1** in terms of sensitivity (Fig. 2).While reaction rates at 3 μM **1** or MOCAc are quite similar, a much higher signal to noise ratio is observed for **1**, underlining the gain in sensitivity by time-resolved detection of luminescent lanthanide-substrates. At

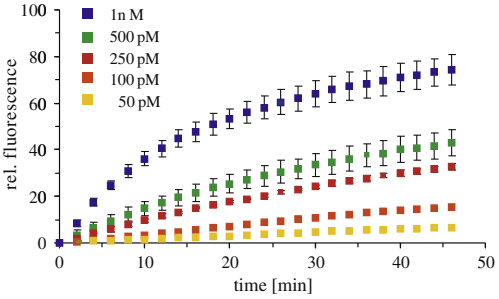
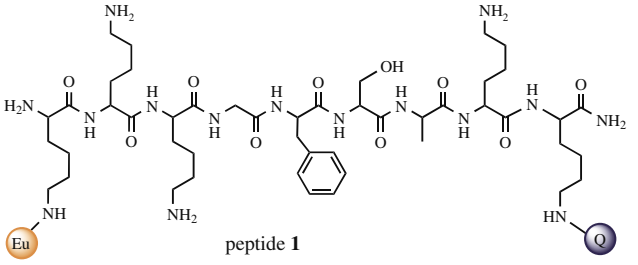
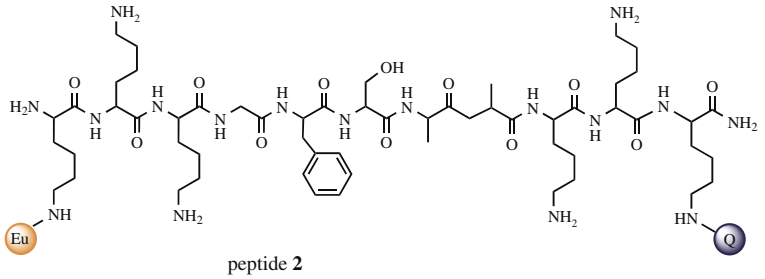
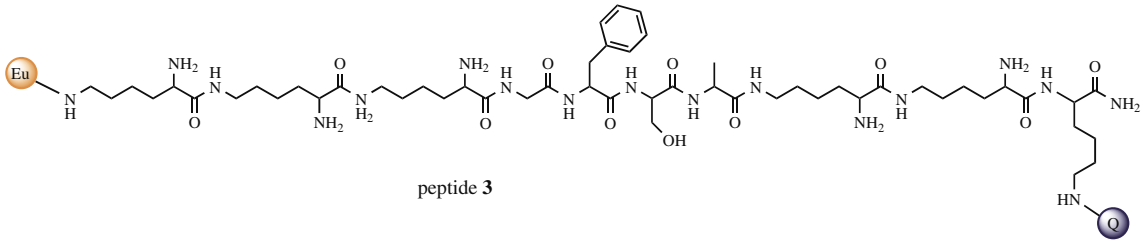


Figure 1. Cleavage of peptide **1** (3 μM) by thermolysin (1 nM–50 pM), monitored as the increase of relative fluorescence over the time (λ_{ex} = 295 nm, λ_{em} = 617 nm, delay time 0.1 ms, gate time 1 ms, total decay time 0.02 s, PMT voltage 800 V), in 2 mM Tris–HCl (pH 7.5) at 23 °C with 40 mM NaCl. All kinetic data represent the means of at least three measurements. Error bars indicate standard variations (omitted for 100 pM and 50 pM).







Scheme 2. Structures of synthesized peptides **1–3**.

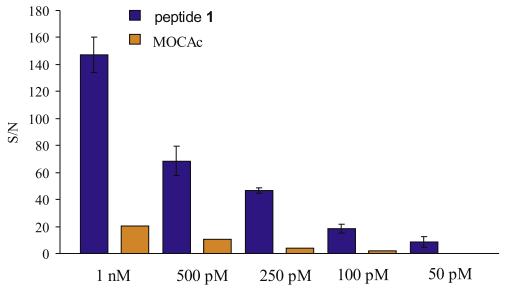


Figure 2. Comparison of the enzyme sensitivity of peptide **1** (3 μM) (λ_{ex} = 295 nm, λ_{em} = 617 nm) and MOCAc-PLGL(Dpa)AR (3 μM) (λ_{ex} = 328 nm, λ_{em} = 388 nm) in 2 mM Tris–HCl (pH 7.5) at 23 °C with 40 mM NaCl, measured at the same PMT voltage of 800 V. The signal to noise ratio (*S/N*) is determined as the ratio between fluorescence intensity in the presence and absence (background) of enzyme after 18 min. Error bars indicate standard variation and represent the means of at least three measurements.

100 pM enzyme the *S/N* ratio for **1** is 18.5 compared with 1.71 for MOCAC. At 50 pM, thermolysin is no longer detectable by MOCAc while the S/N ration of **1** is still 8.4.

Measurements of hydrolysis rates at various substrate concentrations and two different enzyme concentrations revealed that the thermolysin-mediated hydrolysis of peptide **1** obeys a simple Michaelis-Menten relationship (Inset: Fig. 3). Kinetic parameters were determined using double-reciprocal Lineweaver–Burk plots (Fig. 3). The Michaelis constant K_m = 4.49 (±0.6) μM and k_{cat} = 2.60 (±0.08) s^{-1} were determined as mean values of three independent measurements (errors indicate standard variations) with different thermolysin concentrations (Fig. 3 shows the data for 500 pM thermolysin).

It should be noted that under the assay conditions of Fig. 1, hydrolysis of MOCAc by thermolysin is characterized by K_m = 2.0 (±0.5) μM and k_{cat} = 2.36 (±0.3) s^{-1} (data not shown, errors indicate standard variations), that is, under non-saturation conditions, cleavage of MOCAc is somewhat more effective than of peptide **1** due to the lower K_m value. Consequently, the higher sensitivity of substrate **1** can not be attributed to more effective enzymatic cleavage.

In conclusion we have demonstrated that fluorogenic lanthanide-based peptides are suitable for the detection of endopeptidases such as thermolysin. In comparison to a commercially available fluorescent substrate our novel probe shows an about eight times higher sensitivity for thermolysin, and a lower detection limit of 50 pM (3.5 fmol). Potential applications of the lanthanide-based substrate include the rapid detection of low levels of clinically relevant endoproteases in biological matrices. For example, a variety of matrix metalloproteinases are becoming increasingly important as malignant or cardiac serum markers due to their enhanced expression during tumor growth and vascular injury.

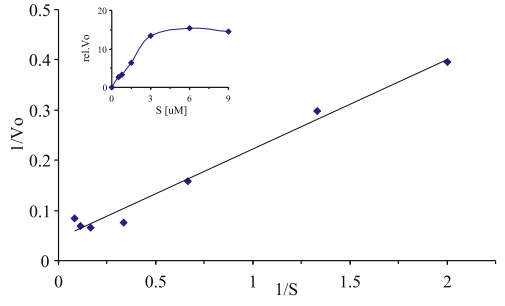


Figure 3. Lineweaver–Burk plot of thermolysin at 500 pM. Inside: enzyme saturation plot. Increase in fluorescence after 18 min (V_0) with varying substrate concentrations of peptide **1** (0.5–12 μM) in 2 mM Tris–HCl (pH 7.5) at 23 °C with 40 mM NaCl (λ_{ex} = 295 nm, λ_{em} = 617 nm, delay time 0.1 ms, PMT voltage 800 V).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.152.

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