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Determining Protease Substrate Selectivity and Inhibition by Label-Free Supramolecular Tandem Enzyme Assays

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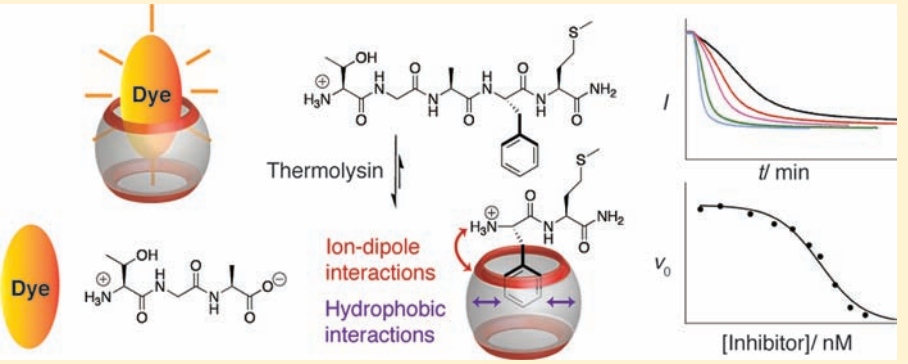
Supporting Information

ABSTRACT:



An analytical method has been developed for the continuous monitoring of protease activity on unlabeled peptides in real time by fluorescence spectroscopy. The assay is enabled by a reporter pair comprising the macrocycle cucurbit[7]uril (CB7) and the fluorescent dye acridine orange (AO). CB7 functions by selectively recognizing N-terminal phenylalanine residues as they are produced during the enzymatic cleavage of enkephalin-type peptides by the metalloendopeptidase thermolysin. The substrate peptides (e.g., Thr-Gly-Ala-Phe-Met-NH_2) bind to CB7 with moderately high affinity ($K \approx 10^4\ M^{-1}$), while their cleavage products (e.g., Phe-Met-NH_2) bind very tightly ($K > 10^6\ M^{-1}$). AO signals the reaction upon its selective displacement from the macrocycle by the high affinity product of proteolysis. The resulting supramolecular tandem enzyme assay effectively measures the kinetics of thermolysin, including the accurate determination of sequence specificity (Ser and Gly instead of Ala), stereospecificity (D-Ala instead of L-Ala), endo- versus exopeptidase activity (indicated by differences in absolute fluorescence response), and sensitivity to terminal charges ($-CONH_2$ vs $-COOH$). The capability of the tandem assay to measure protease inhibition constants was demonstrated on phosphoramidon as a known inhibitor to afford an inhibition constant of (17.8 ± 0.4) nM. This robust and label-free approach to the study of protease activity and inhibition should be transferable to other endo- and exopeptidases that afford products with N-terminal aromatic amino acids.

INTRODUCTION

As the enzymes that catalyze the hydrolytic degradation of proteins, proteases are ubiquitous in living systems and regulate a multitude of cellular processes including the cell cycle, hormone activation, angiogenesis, and apoptosis.[1−5] Aberrations in protease expression or function are therefore implicated in many pathological conditions such as cancer,[6] arthritis,[7] and Alzheimer's disease.[8] In addition, proteases play an essential role in viral replication and in the toxicity of bacteria.[9] Indeed, the potential of proteases as targets for drug development is enormous, as evidenced by the successful development of numerous therapeutics based on protease inhibition.[5]

The characterization of protease activity for the purpose of determining substrate activity and inhibitor potency is unfortunately slow and expensive. The vast majority of assays require labeled substrates,[10−17] which are costly and may not behave the same as their natural counterparts.[18] Label-free protease assays, on the other hand, rely on analytical instruments such as mass spectrometers[18] or employ synthetic/semisynthetic multifunctional pores,[19,20] which are difficult to scale up for high-throughput screening. Hence, the development of rapid and robust assays for protease activity greatly accelerates the characterization of protease targets and the discovery of drug candidates.[17,21]

This paper describes a robust and convenient approach for measuring protease kinetics using optical spectroscopy on label-free substrates and products. Our approach is based on a supramolecular tandem assay,[22−25] which incorporates an essential component of indicator-displacement assays.[26−28] Supramolecular tandem assay is a recently developed technique that

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provides real-time continuous monitoring of enzymatic activity by following a change in the concentration of substrate *or* product as it competitively displaces a fluorescent reporter dye from the cavity of a macrocyclic host. These assays therefore rely on the differential binding of the macrocycle with the fluorescent dye, the enzymatic substrate, and the corresponding product.

Supramolecular tandem assays have been implemented successfully for monitoring enzymatic transformations involving amino acids, biogenic amines, amino aldehydes, and nucleotide phosphates.[22−25] Until now, the technique was limited to substrates and products which, owing to their low molecular weight, could essentially be fully immersed in the macrocyclic host cavity, such that the entire analyte, e.g. arginine or cadaverine, served as recognition motif. Here, the utility of the tandem assay principle is transferred to peptides, which themselves are far too large to be fully included in the macrocyclic cavity. Rather, it is a *residue* of the peptide chain which complexes with differential affinity to the macrocycle in the substrate and the product. For the first time, we demonstrate the quantitative determination of absolute kinetic parameters (k_{cat}/K_M) for protease activity, the application of this analysis to the profiling of enzyme substrates for sequence selectivity, stereospecificity, and endo- vs exopeptidase activity, as well as the quantitative determination of inhibitory constants for protease inhibitors.

RESULTS AND DISCUSSION

Experimental Design. Cucurbit[7]uril (CB7, Figure 1) is a water-soluble macrocycle that has been investigated extensively in biological applications including drug delivery,[29−33] interactions with enzymes,[34,35] plasma membrane protein fishing,[36] and label-free enzyme assays.[22−25] The repeating glycoluril units produce a barrel-shaped container that has a hydrophobic cavity and negatively charged portals.[37] The latter are capable, not only for CB7 but also for its homologues, of binding inorganic cations as well as the cationic sites of organic guests, mostly ammonium groups; nonpolar groups are preferentially immersed in the inner cavity.[38−42] CB7 and its larger homologue CB8 have been shown to bind aromatic amino acids and sequence specifically to peptides and proteins containing phenylalanine (Phe), tryptophan (Trp), or tyrosine (Tyr) at the N-terminal positions.[43−47] Recognition at the N terminus is achieved via the cooperation of hydrophobic inclusion of the aromatic side chain and electrostatic stabilization of the proximal N-terminal ammonium group.

The differential binding of CB7 to an aromatic residue located at the N terminus versus other positions is exploited here in the design of an enzyme assay by choosing a protease (thermolysin) that efficiently hydrolyzes the peptide bond on the amino side of phenylalanine residues and thus generates an N-terminal phenylalanine as its product. The product binds to CB7 more tightly than the starting material and will, therefore, selectively displace a fluorescent indicator from CB7. This allows real-time monitoring of the thermolysin-mediated reaction by the pronounced change in fluorescence intensity. Thermolysin is a thermally resistant (thermophilic) enzyme produced by *Bacillus thermoproteolyticus*. It is selective for bulky, hydrophobic amino acids such as Phe and Leu,[48] and represents the family of thermolysin metalloendopeptidases as relevant therapeutic targets due to their high substrate specificity, their functional role in extracellular transformations of neuroendocrine as well as cardiovascular peptides, and in processes ranging from reproduction to cardiovascular homeostasis.[9,49,50]

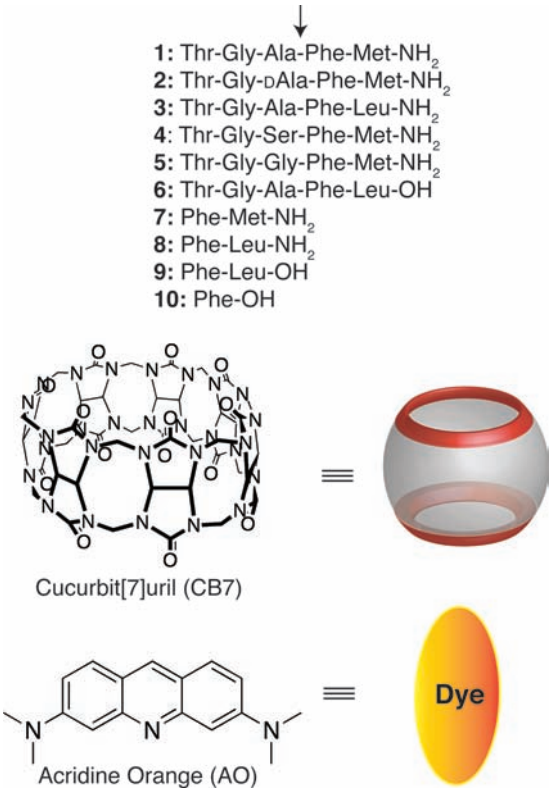
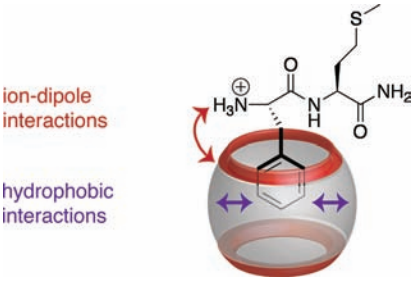


Figure 1. Amino acid sequences of the peptides used in this study; the N termini are unprotected primary amines, the C termini are designated as $-NH_2$ for a primary amide and $-OH$ for a carboxylic acid. The arrow indicates the cleavage site for thermolysin. Also shown are the chemical structures of the macrocycle and fluorescent dye constituting the employed reporter pair.

Scheme 1. CB7 Binds Selectively to N-Terminal Phe Residues Due to Cooperative Hydrophobic and Ion−Dipole Interactions



Enkephalin-based peptides were chosen as substrates to establish proof-of-principle for the protease assay. These neurological pentapeptides of sequence Tyr-Gly-Gly-Phe-Met-OH (natural amino and carboxy termini) or Tyr-Gly-Gly-Phe-Leu-OH are part of the endogenous opioid system involved in pain perception and emotional behavior, and they are implicated in the pathogenesis of Alzheimer's dementia.[51] Thermolysin hydrolyzes these peptides at the Gly-Phe peptide bond, producing Phe-Met-OH and Phe-Leu-OH products that contain an N-terminal Phe and, thus, should bind to CB7 selectively versus the substrates as well as the other peptide product fragments (Scheme 1).[52−54]

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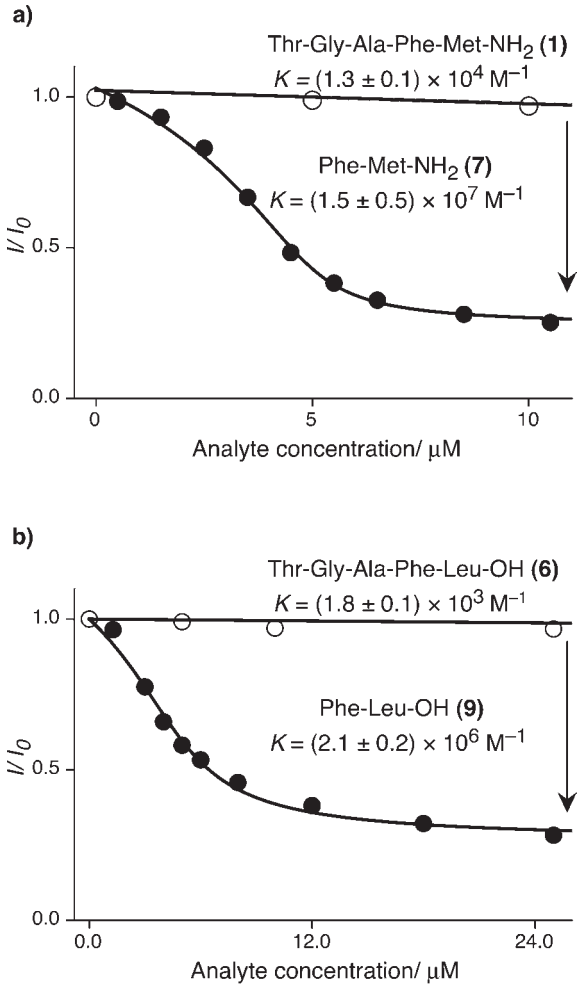

Figure 2. Fluorescence titrations (λ_{exc} = 485 nm, λ_{obs} = 510 nm) of substrates **1** and **6** and their proteolysis products (dipeptides **7** and **9**) by using competitive displacement of AO (0.5 μM) from CB7 (5 μM) in 10 mM ammonium phosphate buffer at pH 7.2, 37 °C. The titrations for the substrates are cut off for clarity, see Supporting Information for more data. I_0 and I are the fluorescence intensities in the absence and presence of competitor, respectively. The arrow indicates the expected fluorescence response in the course of the enzymatic reaction.

The choice of the fluorescent dye is critical to the design of a supramolecular tandem assay. Acridine orange (AO, Figure 1) and CB7 were selected as the "reporter pair" (i.e., the macrocycle and dye pair). AO is a weakly fluorescent dye in aqueous solution, which becomes strongly fluorescent upon encapsulation by CB7.[55] Upon the addition (or enzymatic formation) of a strongly binding analyte to the preformed CB7·AO complex, the fluorescence intensity drops again, leading to a "switch-off" fluorescence response. Important to note, the binding constant of CB7 with AO (2.9×10^5 M^{-1})[24,55] lies *in between* the binding strength of CB7 with the substrate and product of interest. This was demonstrated by simple titration experiments (Figure 2 and Supporting Information), from which the binding constants of peptides **1**−**9** were determined (Table 1). Peptides **1**−**6**, the candidates to potentially act as substrates for thermolysin, have invariably a low binding affinity to CB7, accounted by the presence of only hydrophobic interaction between the amino acid Phe and the host cavity. However, the corresponding proteolytic products (dipeptides **7**−**9**) have 3 orders of magnitude higher affinity for

Table 1. Binding Constants (K) of Peptides 1−9, Phenylalanine 10, and Phosphoramidon 11 with CB7 and Proteolytic Constants (k_{cat}/K_M) for Their Reaction with Thermolysin

entry	peptide sequence	K (10^4 M^{-1})[a]	k_{cat}/K_M (10^4 s^{-1} M^{-1})[b]
1	Thr-Gly-Ala-Phe-Met-NH_2	1.3	14
2	Thr-Gly-D-Ala-Phe-Met-NH_2	2.6	≤0.005[c]
3	Thr-Gly-Ala-Phe-Leu-NH_2	0.35	3.2 [7.0][d]
4	Thr-Gly-Ser-Phe-Met-NH_2	1.9	6.9
5	Thr-Gly-Gly-Phe-Met-NH_2	1.4	1.2
6	Thr-Gly-Ala-Phe-Leu-OH	0.18	2.3
7	Phe-Met-NH_2	1500 ± 500	*e*
8	Phe-Leu-NH_2	2700 ± 1500	*e*
9	Phe-Leu-OH	210	*e*
10	Phe-OH	2.0 [2.5] [f]	*e*
11	Phosphoramidon	0.12	*g*

[a] Determined by competitive fluorescence titrations, *cf.* Figure 2 and Supporting Information; 15% error unless explicitly stated. [b] Determined by supramolecular tandem assay at varying peptide concentrations (5−55 μM, n = 5−6), *cf.* Figure 3; kinetic parameters were determined by nonlinear regression (see Supporting Information); 20% estimated error. [c] Insignificant hydrolysis due to the presence of D-Ala. [d] Value in square brackets refers to exopeptidase activity, see text and Supporting Information. [e] No conversion detected due to Phe N terminus. [f] Value in square brackets in 0.1 M NaCl solution.[56] [g] Phosphoramidon was employed as inhibitor, cf. Figure 5.

CB7 due to the additional electrostatic interaction between the N-terminal ammonium group of Phe with the CB7 carbonyls. This high affinity disappears again for the simple amino acid Phe (**10**), for which the adjacent C-terminal carboxylate group entirely offsets the stabilizing interaction by the ammonium group. Note that the binding affinity of CB7 between the peptides **1** and **2** is slightly different. Nevertheless, it is not surprising due to the diastereomeric differentiation between L- and D-Ala by the achiral host CB7.[56]

When compared to highly selective antibodies, the molecular recognition of peptides by CB7 must be considered as rather unspecific. The synthetic macrocyclic host binds to all pentapeptides **1**-**6** with very similar affinity, and even the binding constant of the amino acid Phe (**10**) falls in the same range (Table 1). CB7 shows also little selectivity toward the dipeptides **7**−**9**. However, it *differentiates* the dipeptides **7**−**9** reliably from the pentapeptides **1**−**6**, and this substrate/product differentiation is sufficient to set up robust enzyme assays. When thermolysin is added to the peptide solution containing an enkephalin-based substrate and the CB7/AO reporter pair (Scheme 2), the enzymatic product, containing an N-terminal phenylalanine residue, should rapidly (relative to the enzymatic transformation itself) and competitively displace the AO dye from the CB7 cavity, thus yielding a decrease in fluorescence intensity that reports the protease activity continuously and in real time. The immediate response is due to the fast rates for the formation and dissociation of the supramolecular assemblies which, as previously discussed, constitutes an advantage of using macrocycles instead of antibodies.[24] As can be further seen from the actual titration plots (Figure 2), even working at relatively low substrate concentrations of 5−20 μM should produce a readily detectable change in fluorescence response upon conversion of a substrate to a product. This working concentration range is exactly desirable in protease assays, including those employed in high-throughput screening for pharmaceutical investigations.[14−17]

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Enzyme Assays. To establish proof of principle for the use of a supramolecular tandem assay to monitor protease activity on unlabeled peptides, we first investigated a series of enkephalin-based peptides (Figure 1) with the sequences Thr-Gly-Ala-Phe-Met-NH_2 (**1**), Thr-Gly-D-Ala-Phe-Leu-NH_2 (**2**), Thr-Gly-Ala-Phe-Leu-NH_2 (**3**), as well as the dipeptide product from proteolysis of enkephalin **1**, Phe-Met-NH_2 (**7**).

As depicted in Figure 3a, peptides **1** and **3** were hydrolyzed rapidly, but peptide **2** showed insignificant hydrolysis. The stark contrast in the rate of cleavage at the Ala-Phe bond for peptides **1** and **2** was due to the substitution of L-Ala by its enantiomer.

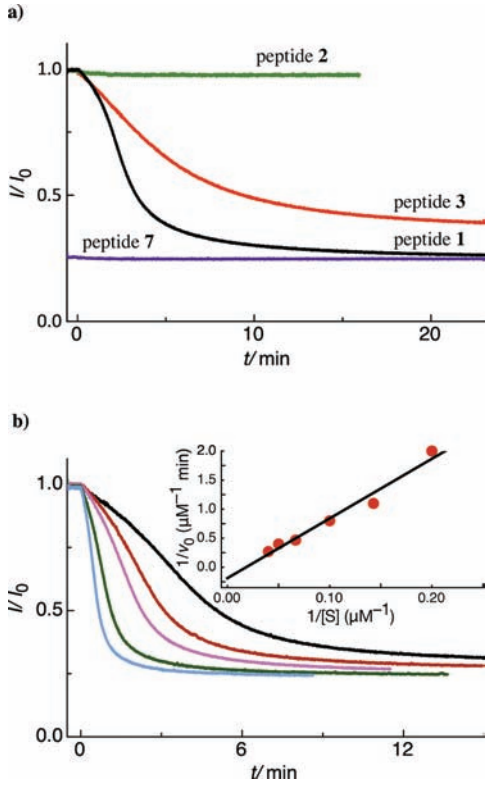


Figure 3. (a) Continuous fluorescence assays (λ_{exc} = 485 nm, λ_{obs} = 510 nm) with the CB7/AO reporter pair (8 μM/ 0.5 μM) upon addition of thermolysin (t = 0 min, 15 nM) to peptides **1**−**3** and **7** (30 μM), at 37 °C. (b) Determination of enzyme kinetic parameters by monitoring of thermolysin (15 nM) activity with varying concentration of enkephalin **1** (5−25 μM) in 10 mM ammonium phosphate buffer, pH 7.2, at 37 °C with the CB7/AO reporter pair (2.5 μM/0.5 μM). I_0 and I are the fluorescence intensities at time t = 0 and time t, respectively.

The tandem assay thus reflects the previously established substrate selectivity of thermolysin, including the remarkable stereospecificity of the P1 position.[48,57−60] As can be also seen from Figure 3a, the dipeptide **7** shows no fluorescence response. This signifies that the peptide bond between Phe and Met in this peptide is not cleaved by thermolysin (exopeptidase activity, see below). If it were cleaved, an increase in fluorescence would have been observed, because the product (free Phe, **10**) would again constitute a weak competitor (see binding constants in Table 1). Again, it was known that substrates lacking a peptide bond N-terminal to Phe (such as **7**) are not digested by thermolysin,[53] such that our result established a negative control experiment.

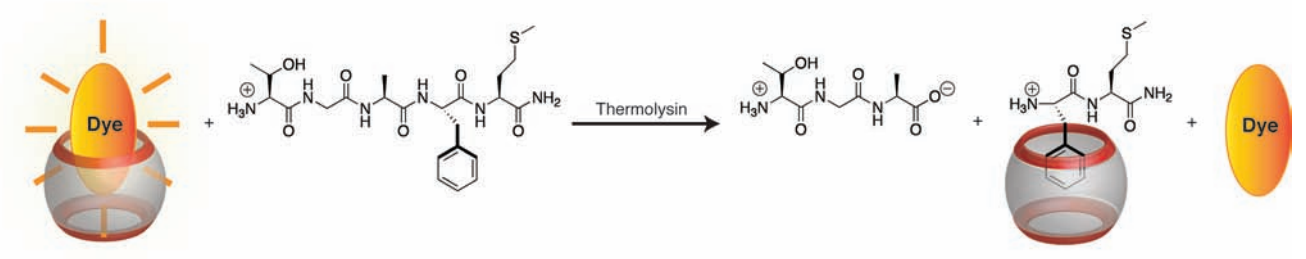
Substrate Selectivity. Given the high sensitivity of the tandem assay observed in the initial experiments, we decided to measure the kinetic behavior of thermolysin for substrates with varying amino acids at the P_1 position. With peptides **1** and **3** as parent compounds, the P_1 mutations of Ala to Ser (peptide **4**) and Ala to Gly (peptide **5**) examined the effects of adding a hydroxyl group to the β carbon, or removing the β carbon, respectively. Peptide **6** is an analogue of **3** with a carboxylate at the C terminus, which was designed to test the effect of C-terminal charge.

The enzyme-kinetic analysis required the determination of initial rates of reaction. For this purpose, the observed fluorescence decay needed to be related to changes in absolute concentration.[25] This relationship was achieved by recording the fluorescence response obtained by addition of a known quantity of an authentic sample of reaction product (see Supporting Information). Analysis of the initial reaction rates at varying substrate concentrations (Figure 3b) yielded the characteristic proteolytic constants (k_{cat}/K_M) for the different peptide sequences (inset of Figure 3b and Table 1).[61]

Note that our tandem assays allow kinetic measurements for unlabeled peptides, while previous assays were carried out with peptides carrying fluorescent labels such as 2-naphthylamide (2NA)[62] or dansyl.[63] The structural differences prevent a direct comparison of the absolute proteolytic constants. Nevertheless, peptides **1** ($k_{cat}/K_M = 14 \times 10^4\ M^{-1}\ s^{-1}$) and **5** ($1.2 \times 10^4\ M^{-1}\ s^{-1}$) showed the same order of magnitude as well as the same trends of substrate selectivity as did the labeled derivatives Glt-Gly-Ala-Phe-2NA ($5.2 \times 10^4\ M^{-1}\ s^{-1}$) and Glt-Gly-Gly-Phe-2NA ($0.15 \times 10^4\ M^{-1}\ s^{-1}$),[62] which was gratifying to observe.

The data in Table 1 show that the identity of the amino acid residue at the P_1 position significantly affects the proteolytic coefficients of thermolysin activity. The values are moderately reduced for the peptides containing glycine and serine at P_1 compared to their parent compound **1** with alanine at the P1 position. The binding of the substrate at P_1 is governed by hydrophobic interactions, which accounts for the fact that, at P_1,

Scheme 2. Product-Selective Fluorescence Switch-Off Tandem Assay Using CB7 and AO as Reporter Pair[a]



[a] It should be noted that the dye, substrate, and product are in rapid dynamic competitive equilibrium for encapsulation within the CB7 macrocycle.

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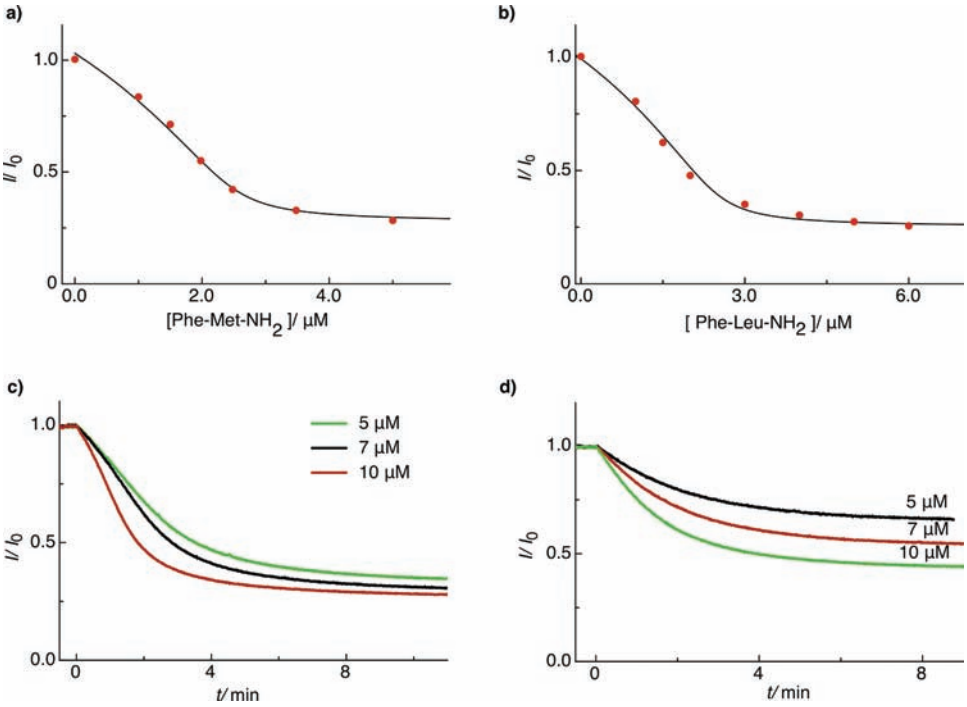

Figure 4. Fluorescence measurements using CB7/AO (2.5 μM CB7 and 0.5 μM AO, λ_{exc} = 485 nm, λ_{obs} = 510 nm) as a reporter pair in 10 mM ammonium phosphate buffer, pH 7.2, at 37 °C. Competitive fluorescence titration plots of (a) Phe-Met-NH_2 (**7**) and (b) Phe-Leu-NH_2 (**8**). Tandem protease assays for thermolysin (15 nM) with substrates (c) Thr-Gly-Ala-Phe-Met-NH_2 (**1**) and (d) Thr-Gly-Ala-Phe-Leu-NH_2 (**3**).

Gly, and Ser are cleaved more slowly than Ala.[62,64,65] The use of a carboxylate group leads to a slight reduction in enzymatic activity, as indicated by the k_{cat}/K_M value, presumably due to the known sensitivity of thermolysin toward adjacent charges (see the following section; note that most model substrates were amidated at the C terminus for the convenience of peptide synthesis).

Exo- and Endopeptidase Behavior. During the determination of the k_{cat}/K_M values for the peptides described above, we stumbled on the unexpected exopeptidase behavior of thermolysin that was specific to the substrate, Thr-Gly-Ala-Phe-Leu-NH_2 (**3**). The *expected* endopeptidase products of the cleavage of substrates Thr-Gly-Ala-Phe-Met-NH_2 (**1**) and Thr-Gly-Ala-Phe-Leu-NH_2 (**3**) are Phe-Met-NH_2 (**7**) and Phe-Leu-NH_2 (**8**), respectively. These products bind tightly to CB7 and are therefore responsible for the change in fluorescence intensity during the tandem assay. Therefore, the final steady-state fluorescence response (i.e., after quantitative enzymatic digestion of substrates **1** and **3**) was expected to be similar to the fluorescence response brought about by the same concentrations of their endopeptidase products **7** and **8**. This similarity was observed for substrate **1** but not for substrate **3**.

In detail, the enzymatic hydrolysis of 5 μM of the substrate Thr-Gly-Ala-Phe-Met-NH_2 (**1**) resulted in complete displacement of the dye from CB7, as observed by the similarity of the steady-state fluorescence response after complete enzymatic conversion and the response of the same concentration of Phe-Met-NH_2 (Figure 4a,c). In the case of the substrate Thr-Gly-Ala-Phe-Leu-NH_2 (**3**), however, the final steady-state fluorescence response upon enzymatic hydrolysis of 5 μM substrate was less than that of its endopeptidase product Phe-Leu-NH_2 (**8**) at the same concentration (see Figure 4b,d). In fact, even a concentration of 10 μM of substrate **3** was insufficient to produce the same fluorescence response as that produced by 5 μM of **8**. We concluded that the expected product was not quantitatively formed. To account for this result, and inspired by previous experimental observations,[48,66,67] we suspected the possibility of *exo*peptidase activity, i.e., enzymatic cleavage of the Phe-Leu peptide bond. We were exactly able to corroborate this unusual pathway for peptide **3** by mass spectrometry (see Supporting Information).

The observed exopeptidase cleavage leads to the formation of Thr-Gly-Ala-Phe-OH as (another) product, one that is not further converted by thermolysin. Therefore, the yield of the expected endopeptidase product (**8**) and the corresponding change in fluorescence response upon the displacement of AO by **8** fall below expectation. In fact, the incomplete conversion, signaled by the plateau being reached at higher fluorescence intensities, can be used to assess the ratio of exo- versus endopeptidase cleavage (2.2:1) which, with the endopeptidase kinetics being directly accessible (Figure 4 and Supporting Information), allows the projection of both rates. This analysis affords a k_{cat}/K_M value of 3.2×10^4 M^{-1} s^{-1} for the endopeptidase activity and a value of 7.0×10^4 M^{-1} s^{-1} for the exopeptidase activity. The higher proteolytic constant for the hydrolysis of the Phe-Leu *exo* bond (i.e., Leu at P1′ position) compared to the hydrolysis of the Ala-Phe *endo* bond (i.e., Phe at P1′) is consistent with the enzyme's preference for hydrophobic P1′ residues, whereby increasing the hydrophobic residue from Leu to Phe increases the interaction of the substrate with the active site of the enzyme, while decreasing the catalytic efficiency,[60,68,69] thereby accounting for the observed lower k_{cat}/K_M value for the *endo* cleavage.

It is interesting that we observed no exopeptidase activity for the nonamidated peptide Thr-Gly-Ala-Phe-Leu-OH (**6**), as confirmed by the plateau region of the fluorescence trace as well as by mass spectrometry (see Supporting Information).

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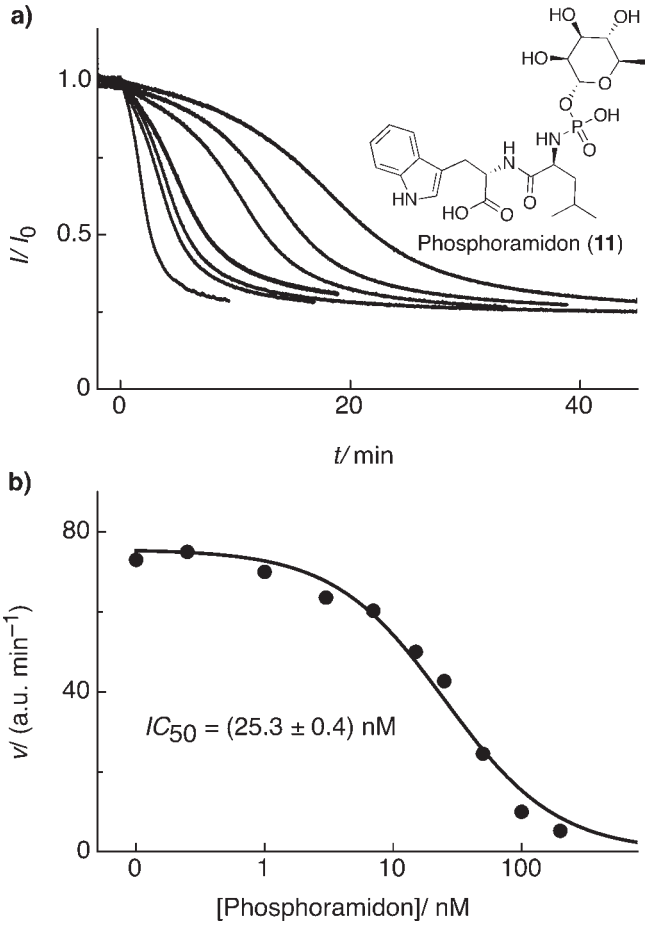

Figure 5. Determination of thermolysin (15 nM) inhibition by phosphoramidon (**11**, 0−100 nM) in the presence of 10 μM Thr-Gly-Ala-Phe-Met-NH_2 (**1**) by using the CB7/AO (0.5 μM/2.5 μM) reporter pair in 10 mM ammonium phosphate, pH 7.2, at 37 °C. (a) Continuous fluorescence traces (λ_{exc} = 485 nm, λ_{obs} = 510 nm) upon addition of 10 μM Thr-Gly-Ala-Phe-Met-NH_2 (**1**) for the determination of the initial rates. (b) Corresponding dose−response curve for inhibition of thermolysin by phosphoramidon (**11**).

This result reveals that the C-terminal charge of the peptide directs endo- versus exopeptidase activity, at least for substrates containing bulky hydrophobic residues (Phe and Leu) at the P_1' position. Conversely, a comparison of substrate **3** (exopeptidase cleavage observed) with its product **8**, for which no conversion by themolysin is observed (Figure 3a and Supporting Information), further exposes that an N-terminal charge directed at phenylalanine suppresses the exopeptidase activity of thermolysin. This observed sensitivity of thermolysin toward adjacent charges supports prior claims based on studies performed by alternative assays with labeled substrates.[48,66,67]

As can be seen from these studies, our label-free protease tandem assay provides information not only on the enzymatic activity, kinetics, and substrate selectivity but also on the chemoselectivity of the proteolytic cleavage, because the plateau region after quantitative conversion is a signature for the identity of the expected product and thus enables direct quantification of the extent of the expected reaction.

Protease Inhibition. Having established the capability of the assay to effectively measure the kinetics of thermolysin activity, we sought to apply the assay to the determination of enzyme inhibition, which is critical to the evaluation of drug candidates. Using Thr-Gly-Ala-Phe-Met-NH_2 (**1**) as the model substrate, inhibition studies for thermolysin were carried out using the product-selective tandem assay principle and the inhibitor phosphoramidon (**11**), a naturally occurring, potent inhibitor of thermolysin.[70−73] Inhibitors can hypothetically interfere with the assay principle by binding to the macrocycle.[24] Fortunately, this can be readily tested by competitive titrations, which afforded a low binding constant for **11** (1200 M^{-1}, Table 1). In the concentration range relevant for studies with potent inhibitors (up to 100 nM), binding of **11** to CB7 (<0.3%) can therefore be safely neglected.

We observed that, as is typical for competitive inhibitors, increasing the concentration of **11** decreases the *rate* of thermolysin hydrolysis via dynamic competition with the substrate for binding to the enzyme, and yet allows the irreversible peptidase reaction on the enzyme·substrate complex to proceed to completion, as observed by similar final steady-state fluorescence intensities at all concentrations of inhibitor (Figure 5a). The change in initial rates of decrease in fluorescence intensity was used to calculate a K_i value of (17.8 ± 0.4) nM,[74] which falls right into the reported range (3.5−60 nM), all determined under slightly varying experimental conditions and with different assay methods employing fluorescently labeled substrates.[70,71,75]

CONCLUSIONS

The addition of macrocyclic host molecules in combination with fluorescent dyes establishes a label-free method for the real-time, continuous monitoring of protease activity by fluorescence spectroscopy. Protease assays using unmodified substrates are important because they enable the detailed characterization of the natural substrate selectivity of a target protease as well as its activity in the presence of inhibitors. We have successfully applied the tandem assay principle to the continuous monitoring of the hydrolysis of enkephalin-based peptides by thermolysin. In doing so, we have established proof-of-principle for the use of cucurbituril-based fluorescent reporter pairs for proteases. The general selectivity of macrocycles (even if moderate in comparison to specific receptors) renders potentially broad transferability of this assay to other exo- and endopeptidases. Furthermore, for the first time, we have extended the applicability of tandem assays toward an in-depth profiling of enzyme activity for a wide range of substrates and toward sensing enzyme substrate stereospecificity, and have demonstrated the potential of this assay for the screening of inhibitors. These applications of tandem assays to monitor proteolytic activity have significant implications for drug design, as well as medical diagnostics, where proteases are important disease markers.

EXPERIMENTAL SECTION

Materials. Peptides **1**−**8** were synthesized by standard Fmoc solid-phase synthesis protocols on Rink amide MBHA resin (for C-terminal amides) or Wang acid resin (for peptide **6** containing a C-terminal carboxylic acid) and purified by reversed phase HPLC. Purity was verified by reversed phase analytical HPLC and ^{1}H NMR spectroscopy. Identity was verified by electrospray mass spectrometry.

Peptide Phe-Leu-OH (**9**) and amino acid Phe-OH (**10**) were used as received from Bachem and Applichem, respectively. Cucurbit[7]uril (CB7) was synthesized according to the literature.[76−78] Acridine orange

(AO), thermolysin (lyophilized powder, 36.5 U/mg), and phosphoramidone (**11**) were used as received from Sigma-Aldrich.

Methods. Absorbance measurements were performed with a Varian Cary 4000 spectrophotometer. The concentrations of peptides **1**−**10** were determined assuming the extinction coefficient of free phenylalanine (**10**) at 257 nm, $\varepsilon_{257} = 195\ M^{-1}\ cm^{-1}$.[79] For thermolysin and phosphoramidon, $\varepsilon_{280} = 61100\ M^{-1}cm^{-1}$ and $\varepsilon_{280} = 5500\ M^{-1}\ cm^{-1}$, respectively, were used.[63,71] A Varian Eclipse spectrofluorimeter was used for steady-state fluorescence measurements and for the enzyme assays.

Continuous assays were performed with 0−55 μM peptide, 2.5 μM CB7, and 0.5 μM AO (λ_{exc} = 485 nm, λ_{obs} = 510 nm) in 10 mM ammonium buffer, pH 7.2, in a variable-temperature cell holder at 37.0 ± 0.1 °C, and the reaction was initiated by addition of thermolysin (15 nM). For the inhibition studies, the mixture of thermolysin (10 nM) and phosphoramidon (0−100 nM) was preincubated for 15 min at 37.0 ± 0.1 °C in the presence of the reporter pair, and the reaction was initiated by addition of 10 μM Thr-Gly-Ala-Phe-Met-NH_2 (**1**).

■ ASSOCIATED CONTENT

Supporting Information. Fluorescence titration plots of peptide substrates, details on the calculations of the enzymatic reaction rates, mass spectra, and supporting evidence for the exopeptidase activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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