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Design of Modular Dual Enzyme-Responsive Peptides

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Abstract

Dual enzyme-responsive peptides were synthesized by masking the ϵ -amine of lysine with various enzyme substrates. Enzymatic cleavage of these sequences unmasked the ϵ -amine, allowing for further digestion by a second enzyme, which was monitored colorimetrically. This modular peptide design should provide substrates for a large combination of clinically relevant enzymes.

Graphical abstract

Keywords

dual-enzyme; multi-enzyme; indirect enzyme detection; multi-enzyme assay; dual-enzyme assay; nitroanilide

INTRODUCTION

Due to their high selectivity and specificity, enzyme responsive systems are commonly used for diagnostic and drug delivery applications.^{1,2} Though many single enzyme-responsive systems show promise in specific targeting, response to more than one enzyme allows for greater target selectivity and indirect enzyme detection, and provides information about cellular environments.^{3–7} For example, caspase sensitive reporters, which respond to peroxide production or to cancer-related matrix metalloproteinases, have been designed to detect cell injury⁸ as well as to monitor reactivation of the apoptotic pathway after anti-

cancer therapy delivery, respectively.^{3,9} Small molecule and protein based probes have also been designed to monitor enzyme cascades, though these often require very specific enzymes, limiting their modularity.^{10,11}

Enzyme responsive systems can also enable selective biodegradation of materials for biomedical applications. By incorporating multiple enzyme cleavage sites, materials will only degrade and release drugs at specific locations *in vivo*. Many of these systems are responsive to the serine protease trypsin due to its widespread presence throughout the body, including in the digestive tract, and its association with various cancers.^{12–16} For example, trypsin sensitive sequences have been grafted into enhanced green fluorescent protein to monitor trypsinogen activation in pancreatic cancer cells.¹⁷ Additionally, trypsin responsive sequences have been incorporated into abuse-deterrent opioid formulations, which allow drug release only in the digestive tract of patients.¹⁸ However, degradation of such formulations could be better controlled if sequences that required digestion by multiple enzymes were installed. To this end, we have designed a dual enzyme-responsive peptide system that requires sequential digestion by two enzymes for cargo release from the C-terminus. In this system, the peptide is first cleaved by an enzyme that unmasks the recognition site for a second enzyme allowing for digestion and release of a colorimetric compound (Scheme 1).

EXPERIMENTAL

Materials and Methods

Fmoc (fluorenylmethyloxycarbonyl)-protected amino acids were purchased from Chem Impex. Chymotrypsin (64.8 units/mg) was purchased from Sigma Aldrich. Trypsin (225 units/mg), papain (30.3 units/mg), and caspases 3 (100 units/ μ L) and 8 (0.2 mg/mL) were purchased from Fisher Scientific. All other chemicals were purchased from Sigma Aldrich. ¹H NMR and ¹³C NMR spectra were obtained on an Avance DRX 400 MHz instrument. ESI mass spectra were obtained using a Waters Acquity LCT Premier XE. Assay measurements were carried out on a Bio-Tek ELx 800 Microplate Reader.

Peptide Synthesis

Protease substrates (Ac-FG, Ac-DEVD, Ac-DVED, Ac-IEPD) were synthesized using standard Fmoc solid-phase chemistry with 2-chlorotrityl chloride resin (0.4 or 1.2 mmol/g substitution). 0.1M 1-Hydroxybenzotriazole (HOBt) in 20% 4-methylpiperidine in *N,N*-dimethylformamide (DMF) was used for Fmoc deprotection to reduce aspartimide formation.¹⁹ N-termini were acetylated prior to cleavage from resin using 50 eq. acetic anhydride and *N,N*-diisopropylethylamine (DIEA) in DMF for 30 min. Peptides were cleaved from the resin using 0.5% trifluoroacetic acid (TFA) in dichloromethane (DCM). Reverse coupling to A was carried out in DMF using 3 eq. 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 6 eq. DIEA. Nitroanilide peptides were purified either by dissolving in ethyl acetate and washing with saturated NaHCO₃, NH₄Cl, and brine, or by preparative reverse-phase HPLC equipped with a C18 column using a linear gradient from 95:5 to 5:95 H₂O/acetonitrile with 0.1% TFA at a flow rate of 20 mL/min. ESI-MS was used to confirm the molecular masses of the desired products.

Assays

Trypsin + Chymotrypsin Assay—Stock solutions of enzymes were prepared by dissolving trypsin (60 mg/mL) in 1 mM HCl and chymotrypsin (60 mg/mL) in pH 7.4 0.035M HEPES buffer + 0.1M NaCl. The peptide substrate, (Ac-AAF)K-pNA, was dissolved in dimethyl sulfoxide (DMSO) to make a 1 mM stock solution. To wells in a 96-well plate, 87.5 μ L pH 7.4 0.035M HEPES + 0.1M NaCl and 2.5 μ L peptide substrate were added. Absorbance was measured using a plate reader at 405 nm. Then, 5 μ L trypsin and 5 μ L chymotrypsin were added to the wells, and absorbance was measured at pre-determined time points. Absorbance values were subtracted from initial reading of only buffer and peptide. When testing only one enzyme, 5 μ L buffer were added to keep total volume per well at 100 μ L. All conditions were measured in triplicate.

Papain + Trypsin Assay—Papain was reconstituted in deionized water at a concentration of 1 mg/mL. A stock solution of trypsin was prepared by dissolving the enzyme (10 mg/mL) in 1 mM HCl. The peptide substrate, (AcFG)K-pNA was dissolved in DMSO to make a 5 mM solution. Separately, 3M NaCl and 20 mM ethylenediaminetetraacetic acid (EDTA) + 50 mM cysteine (pH 6.2) solutions were prepared with deionized water. In a 96-well plate, 5 μ L substrate were mixed with 45 μ L NaCl and 40 μ L EDTA + cysteine solutions. Absorbance was measured at 405 nm to serve as a blank. Then, 5 μ L of both enzyme solutions were added, and absorbance measurements were taken at pre-determined time points. All conditions were prepared and measured in quadruplicate.

Papain + Trypsin Kinetics—Absorbance measurements were carried out using Tecan M1000 plate reader. K_m , K_{cat} , and V_{max} were determined by measuring the absorbance generated by pNA release from enzymatic cleavage of 31.25–1000 μ M (AcFG)K-pNA using previously described assay conditions. For trypsin time points, (AcFG)K-pNA was incubated with papain for 5, 15, and 30 minutes before adding trypsin. Absorbance values were plotted against time for the first fifteen minutes after trypsin addition, during which the slope remained constant, and analyzed using non-linear regression. Absorbance values were recorded against a blank (buffer + substrate). All experiments were carried out in triplicate.

Caspase 3/8 + Trypsin Assay—Caspase 3 was taken directly from a stock solution containing 1000U/ μ L. A stock solution of trypsin was prepared by dissolving the enzyme (5 mg/mL) in 1 mM HCl. The peptide substrates were dissolved in DMSO to make 10 mM solutions. These were then diluted to 2.5 mM with pH 7.5 0.1 M HEPES containing 10 mM dithiothreitol (DTT), 2 mM EDTA, and 10% v/v glycerol. In a 96-well plate, 84 μ L of buffer and 5 μ L peptide were mixed together before measuring the absorbance at 405 nm to serve as a blank. 1 μ L caspase-3 was added, and the plate was incubated at 23°C for 12–24 hours and absorbance was measured again. Then, 5 μ L trypsin were added to the well and absorbance measurements were taken at pre-determined time points. All conditions prepared and measured in triplicate. The same protocol was used for caspase 8 assays, adjusting peptide substrate concentration as needed.

RESULTS AND DISCUSSION

Trypsin is a serine protease that cleaves the C-terminus of positively charged amino acids such as lysine²⁰ but does not cleave if the ϵ -amine of lysine is acetylated, or masked.²¹ Previously, trypsin has been used to indirectly measure histone deacetylase (HDAC) activity by monitoring trypsin digestion of lysine after deacetylation by HDAC.^{5,22} We designed our system to be dual enzyme-responsive by modifying the ϵ -amine of lysine with substrates for four different proteases: chymotrypsin, papain, and caspases 3 and 8 (Figure 1). We chose these substrates to demonstrate that lysine modification can be used to analyze the activity of a broad range of enzymes.

Enzyme substrates chosen for chymotrypsin, papain, and caspases 3 and 8 were AcAAF, AcFG, AcDEVD, and AcIEPD, respectively. Peptides were synthesized using standard Fmoc solid phase chemistry.²³ Initially, the side chain of N₂-acetyl-L-lysine was modified with various protease substrates on resin before coupling the peptide to *p*-nitroaniline (pNA) in solution phase after cleavage from the resin (Scheme S1).²⁴ This approach proved to be challenging to purify on a larger scale due to the large excess of reagents required to drive the reaction to completion. Therefore, a more modular approach was subsequently used in which protease substrates were coupled to a lysine nitroanilide using solution phase conditions (Scheme 2). Peptide identity and purity were confirmed by LC-MS. The synthesized peptides were also analyzed using UV-vis spectroscopy, and the resulting spectra were compared to pNA (Figure S1). The absorbance maxima of the peptides occurred near 310 nm, and no peaks were observed around 405 nm, the wavelength used to monitor pNA release. Trypsin and chymotrypsin were chosen as initial model enzymes because they each require only a single amino acid for recognition and cleavage. The peptide sequence prepared for chymotrypsin/trypsin detection, (Ac-AAF)K-pNA, was designed with phenylalanine at the ϵ -amine of lysine nitroanilide to install chymotrypsin sensitivity since chymotrypsin recognizes and cleaves at the C-terminal side of bulky, aromatic amino acids.²⁵

To test the enzyme responsiveness of (Ac-AAF)K-pNA, the peptide was first incubated with both chymotrypsin and trypsin, and the absorbance at 405 nm, corresponding to pNA release, was monitored over time. A standard curve was then used to convert absorbance values to pNA concentration (Figure S2). A significant absorbance increase was observed, suggesting that both enzymes are required for complete substrate cleavage. Incubation of 50 μ M Ac(AAF)K-pNA with the enzymes showed an absorbance/concentration increase that leveled out at 0.039 ± 0.003 AU after five hours. A two-fold absorbance increase to 0.070 ± 0.002 AU was observed for 100 μ M substrate, and a four-fold absorbance increase to 0.147 ± 0.002 AU was observed for 200 μ M substrate, indicating that release of pNA was substrate concentration dependent as expected (Figure 2). When 100 μ M peptide was incubated with only chymotrypsin, no change in absorbance was observed. However, a minimal absorbance increase (0.016 ± 0.002 AU) was observed when 100 μ M peptide was incubated with trypsin only. This minimal absorbance increase can be explained: it is known that commercial trypsin contains residual chymotrypsin activity, and thus the second enzyme is present at a low concentration.²⁶ Further, our other dual enzyme substrates (*vide infra*) did not show any absorbance increase when incubated with trypsin alone since they do not

contain any chymotrypsin sensitive residues adjacent to lysine, further suggesting residual chymotrypsin activity is the source of the observed slight absorbance increase.

The second substrate, (AcFG)K-pNA, was incubated with papain and trypsin. Papain, a cysteine protease, cleaves the C terminal side one amino acid after an aromatic residue.²⁷ In the presence of papain and trypsin, pNA release from the substrate was observed to be substrate concentration dependent, similar to what we had observed for (AcAAF)K-pNA digestion (Figure 3). Again, no absorbance increase was observed when the substrate was incubated with each enzyme separately. It is interesting to note that papain digestion of basic amino acids, such as lysine, has been reported; however, cleavage rates are drastically lower than those observed for Phe-Gly, which is evidenced by the lack of absorbance increase when papain only was added to the substrate (Figure 3).^{28,29}

To demonstrate the versatility of the peptide design, two other dual-enzyme responsive peptides, (AcDEVD)K-pNA and (AcIEPD)K-pNA, were synthesized by modifying lysine with substrates of two clinically relevant enzymes: caspases 3 and 8, respectively.³⁰ Caspases 3 and 8 are key proteases in apoptotic pathways that are down-regulated in certain cancer cells,^{31,32} and caspase 3 is found at elevated levels after myocardial infarctions.³³ Caspase activity assays are commonly used to monitor delivery of anti-cancer agents since activation of the apoptotic pathway can indicate successful cancer treatment.³⁴ Initial experiments with (AcIEPD)K-pNA revealed little to no absorbance increase in the presence of caspase 8 and trypsin (Figure S3). To ensure the enzyme itself was still active, the activity was confirmed using commercially available single enzyme substrates AcIEPD-pNA and AcDEVD-pNA (Figure S4), which cleaved as expected. These results led us to hypothesize that caspase 8 is not able to efficiently digest our substrate because peptide amide bonds are much less labile than the acyl-nitroanilide in commercial substrates. Further, caspases are sensitive to the structure of the amino acid in the P1' position which is adjacent to the substrate cleavage site, and the presence of an ϵ -amide as P1' may negatively affect enzyme digestion.³⁵ Notably, K_{cat}/K_m values for caspase 8 are reduced approximately 50 times on average when compared to caspase 3, prompting us to design a dual enzyme substrate sensitive to caspase 3 and trypsin.³⁵

Enzyme sensitivity of (AcDEVD)K-pNA was assessed after incubating 125 μ M of substrate first with caspase-3 then with trypsin, which resulted in an absorbance increase of 0.1 ± 0.006 AU over 60 minutes (Figure 4). This indicated that caspase 3 was able to digest the substrate in contrast to our findings for caspase 8. In the presence of trypsin or caspase 3 only, no absorbance increase was observed, as expected. A scrambled version of the caspase 3 substrate, (AcDVED)K-pNA was also synthesized, for which no enzyme digestion was anticipated. As hypothesized, no pNA release occurred when (AcDVED)K-pNA was incubated with trypsin and caspase 3 (Figure 4). Because of the difference in caspase 8 cleavage rates of (AcIEPD)K-pNA and AcIEPD-pNA, we wanted to compare caspase 3 digestion of (AcDEVD)K-pNA to its commercial substrate, AcDEVD-pNA. We found that pNA release from our substrate corresponded to 83% of the release from the commercial substrate (Figure S5), indicating both substrates were digested efficiently by caspase 3. The data together suggest that our peptide system may be useful for enzyme activity screening assays.

Additionally, these dual-enzyme responsive probes can be used to indirectly measure cleavage rates of peptide bonds, which are difficult to detect through single enzyme assays because these typically require an easily detectable analyte to be released instead of an amino acid. A proof-of-concept kinetics experiment using (AcFG)K-pNA was carried out to determine kinetic parameters of papain. K_{cat} values for papain hydrolysis of comparable substrates range from 1.3 (AcFG-pNA) to 5.4 (AcFG-OMe) s^{-1} , and K_{cat} of trypsin hydrolysis of a lysine nitroanilide is 44 s^{-1} .^{27,36} Therefore, we anticipated papain being the rate limiting enzyme, meaning that the rate of pNA release is dependent on papain activity and not trypsin.^{27,36} Initially, various concentrations of substrate were incubated with both enzymes and the resulting pNA release was monitored via UV-Vis. Non-linear regression of substrate concentration versus absorbance increase was carried out to determine K_m (Table 1, Entry 1). However, adding enzymes both at once can give lower cleavage rates for the substrate since the enzymes can digest each other in a competitive reaction.

Thus, the experiment was repeated, this time incubating (AcFG)K-pNA first with papain, then adding trypsin at predetermined time points. The resulting K_m was lower than the first experiment whereas K_{cat}/K_m increased twofold (Table 1, Entry 2). We reasoned that this approach gave a more accurate result since it allowed digestion by the first enzyme to occur without interference by the second. Our determined values fall between the values for two similar papain substrates, indicating the hydrolysis rate of (AcFG)K-pNA lies between that of comparable nitroanilides and methyl esters. Similar trends have been observed by others,³⁷ leading us to believe this strategy may be a useful tool for indirect enzyme kinetic analysis. Alternatively, the cleavage rate of each dual-enzyme substrate could be determined using additional characterization methods, such as LC-MS. This would allow for the monitoring of each enzyme digestion step individually.

Due to the modular design of the dual-enzyme responsive peptides, a wide range of protease substrates could be coupled to the lysine side chain. This could be useful for multi-enzyme screening applications as well as for creating selectively degradable materials for drug delivery. For instance, our system allows for the indirect determination of amide hydrolysis kinetics, which is not possible with traditional single enzyme assay substrates. While we have not yet explored using other proteases in place of trypsin, doing so would broaden the scope of our system even further. Our data also show that the cleavage rates of each enzyme must be taken into account when designing further dual enzyme responsive peptides in this manner, providing important information for successful design.

CONCLUSIONS

In summary, we have designed and synthesized a series of multi-enzyme responsive peptides by modifying the ϵ -amine of lysine with substrates of three different proteases: chymotrypsin, papain, and caspase 3. It was shown that dual protease activity is required for nitroaniline release to occur. Due to the modular design, we envision that these peptides could be used for selective drug delivery, for fundamental studies on dual enzyme activity, as well as for diagnostic enzyme screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Rautio J, Kumpulainen H, Heimbach T, Oliyai R, Oh D, Jarvinen T, Savolainen J. *Nat. Rev. Drug Discov.* 2008; 7:255–270. [PubMed: 18219308]
2. Hu JM, Zhang GQ, Liu SY. *Chem. Soc. Rev.* 2012; 41:5933–5949. [PubMed: 22695880]
3. Tian X, Baek KH, Shin I. *Chem. Sci.* 2013; 4:947–956.
4. Tucking KS, Grutzner V, Unger RE, Schonherr H. *Macromol. Rapid Commun.* 2015; 36:1248–1254. [PubMed: 25940300]
5. Wegener D, Wirsching F, Riester D, Schwienhorst A. *Chem. Biol.* 2003; 10:61–68. [PubMed: 12573699]
6. Huang R, Wang XJ, Wang DL, Liu F, Mei B, Tang AM, Jiang J, Liang GL. *Anal. Chem.* 2013; 85:6203–6207. [PubMed: 23789580]
7. Kimura Y, Komatsu T, Yanagi K, Hanaoka K, Ueno T, Terai T, Kojima H, Okabe T, Nagano T, Urano Y. *Angew. Chem. Int. Ed. Engl.* 2017; 56:153–157. [PubMed: 27933714]
8. Van de Bittner GC, Bertozzi CR, Chang CJ. *J. Am. Chem. Soc.* 2013; 135:1783–1795. [PubMed: 23347279]
9. Li SY, Liu LH, Cheng H, Li B, Qiu WX, Zhang XZ. *Chem. Commun.* 2015; 51:14520–14523.
10. Mulder MPC, Witting K, Berlin I, Pruneda JN, Wu KP, Chang JG, Merckx R, Bialas J, Groettrup M, Vertegaal ACO, Schulman BA, Komander D, Neeffjes J, El Oualid F, Ovaas H. *Nat. Chem. Biol.* 2016; 12:523–533. [PubMed: 27182664]
11. Peters RJRW, Marguet M, Marais S, Fraaije MW, van Hest JCM, Lecommandoux S. *Angew. Chem. Int. Ed.* 2014; 53:146–150.
12. Ohta T, Terada T, Nagakawa T, Tajima H, Itoh H, Fonseca L, Miyazaki I. *Br. J. Cancer.* 1994; 69:152–156. [PubMed: 8286198]
13. Vilen ST, Suojanen J, Salas F, Risteli J, Ylipalosaari M, Itkonen O, Koistinen H, Baumann M, Stenman UH, Sorsa T, Salo T, Nyberg P. *Cancer Invest.* 2012; 30:583–592. [PubMed: 22909050]
14. Koivunen E, Itkonen O, Halila H, Stenman UH. *Cancer Res.* 1990; 50:2375–2378. [PubMed: 2180568]
15. Radhakrishnan K, Tripathy J, Gnanadhas DP, Chakravorty D, Raichur AM. *RSC Adv.* 2014; 4:45961–45968.
16. van Dijk M, Nollet ML, Weijers P, Dechesne AC, van Nostrum CF, Hennink WE, Rijkers DTS, Liskamp RMJ. *Biomacromolecules.* 2008; 9:2834–2843. [PubMed: 18817441]
17. Chen N, Zou J, Wang SM, Ye YM, Huang Y, Gadda G, Yang JJ. *Biochemistry.* 2009; 48:3519–3526. [PubMed: 19271729]
18. Moorman-Li R, Motycka CA, Inge LD, Congdon JM, Hobson S, Pokropski B. *P & T.* 2012; 37:412–418. [PubMed: 22876107]
19. Lauer JL, Fields CG, Fields GB. *Lett. Pept. Sci.* 1994; 1:197–205.
20. Olsen JV, Ong SE, Mann M. *Mol. Cell. Proteomics.* 2004; 3:608–614. [PubMed: 15034119]
21. Zee BM, Garcia BA. *Essays Biochem.* 2012; 52:147–163. [PubMed: 22708569]
22. Roessler C, Tuting C, Meleshin M, Steegborn C, Schutkowski M. *J. Med. Chem.* 2015; 58:7217–7223. [PubMed: 26308971]
23. Merrifield RB. *J. Am. Chem. Soc.* 1963; 85:2149–2154.

24. Gorske BC, Bastian BL, Geske GD, Blackwell HE. J. Am. Chem. Soc. 2007; 129:8928–8929. [PubMed: 17608423]
25. Appel W. Clin. Biochem. 1986; 19:317–322. [PubMed: 3555886]
26. Groleau PE, Gauthier SF, Pouliot Y. Int. Dairy J. 2003; 13:887–895.
27. Lowe G, Yuthavong Y. Biochem. J. 1971; 124:107–115. [PubMed: 5126466]
28. Tesser GI, Gruber M, Nivard RJF. Biochim. Biophys. Acta. 1964; 89:303–308. [PubMed: 14203176]
29. Mackenzie NE, Malthouse JPG, Scott AI. Biochem. J. 1985; 226:601–606. [PubMed: 3922354]
30. Stennicke HR, Salvesan GS. J. Biol. Chem. 1997; 272:25719–25723. [PubMed: 9325297]
31. Devarajan E, Sahin AA, Chen JS, Krishnamurthy RR, Aggarwal N, Brun AM, Sapino A, Zhang F, Sharma D, Yang XH, Tora AD, Mehta K. Oncogene. 2002; 21:8843–8851. [PubMed: 12483536]
32. Fulda S. Cancer Lett. 2009; 281:128–133. [PubMed: 19111387]
33. Agosto M, Azrin M, Singh K, Jaffe AS, Liang BT. J. Am. Coll. Cardiol. 2011; 57:220–221. [PubMed: 21211695]
34. Olsson M, Zhivotovsky B. Cell Death Differ. 2011; 18:1441–1449. [PubMed: 21455218]
35. Stennicke HR, Renatus M, Meldal M, Salvesen GS. Biochem. J. 2000; 350:563–568. [PubMed: 10947972]
36. Briand L, Chobert JM, Tauzin J, Declerck N, Leonil J, Molle D, Tran V, Haertle T. Protein Eng. 1997; 10:551–560. [PubMed: 9215573]
37. Smith EL, Parker MJ. J. Biol. Chem. 1958; 233:1387–1391. [PubMed: 13610847]

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Scheme 1.

Scheme 2.

Table 1

Kinetic parameters of Phe-Gly papain substrates.

Substrate	Enzyme	K_m (μM)	K_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
(AcFG)K-pNA	Papain + Trypsin	256 ± 29	$6.5 \pm 0.5 \times 10^3$
(AcFG)K-pNA	Papain then Trypsin	127 ± 48	$1.4 \pm 0.5 \times 10^4$
AcFG-pNA ¹⁶	Papain	880 ± 100	$1.5 \pm 0.2 \times 10^3$
AcFG-OMe ¹⁶	Papain	32 ± 1	$1.7 \pm 0.15 \times 10^5$