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The Synthesis and Application of Fmoc-Lys(5-Fam) Building Blocks

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Abstract

Fluorescence resonance energy transfer (FRET) peptide substrates are often utilized for protease activity assays. This study has examined the preparation of FRET triple-helical peptide (THP) substrates using 5-carboxyfluorescein (5-Fam) as the fluorophore and 4,4-dimethylamino-azobenzene-4'-carboxylic acid (Dabcyl) as the quencher. The N^α-(9-fluorenylmethoxycarbonyl)-N^ε-(5-carboxy-fluorescein)-L-lysine [Fmoc-Lys(5-Fam)] building block was synthesized utilizing two distinct synthetic routes. The first involved copper complexation of Lys while the second utilized Fmoc-Lys with microwave irradiation. Both approaches allowed convenient production of a very pure final product at a reasonable cost. Fmoc-Lys(5-Fam) and Fmoc-Lys(Dabcyl) were incorporated into the sequence of a THP substrate utilizing automated solid-phase peptide synthesis protocols. A second substrate was assembled where (7-methoxycoumarin-4-yl)-acetyl (Mca) was the fluorophore and 2,4-dinitrophenyl (Dnp) was the quencher. Circular dichroism spectroscopy was used to determine the influence of the fluorophore/quencher pair on the stability of the triple-helix. The activity of the two substrates was examined with three matrix metalloproteinases (MMPs), MMP-1, MMP-13, and MT1-MMP. The combination of 5-Fam as fluorophore and Dabcyl as quencher resulted in a triple-helical substrate that, compared with the fluorophore/quencher pair of Mca/Dnp, had a slightly destabilized triple-helix but was hydrolyzed more rapidly by MMP-1 and MMP-13 and had greater sensitivity.

Keywords

fluorescence resonance energy transfer; matrix metalloproteinase; fluorescein; triple-helix; collagen

INTRODUCTION

Fluorogenic peptide substrates utilizing fluorescence resonance energy transfer (FRET) are very popular tools for protease activity assays. FRET may be achieved when the emission spectrum of a donor (fluorophore) is overlapped by the absorption spectrum of an acceptor (quencher). In a FRET peptide substrate the donor moiety needs to be at the appropriate

distance from the acceptor moiety to accommodate the distance dependence of the energy transfer. During enzyme-catalyzed cleavage of the peptide the distance between the donor and acceptor group increase and the energy is no longer transferred as the cleaved peptide fragments diffuse away. Reaction progress can be monitored by a decrease in the fluorescence of the acceptor or by an increase in the fluorescence of the donor. Some FRET substrates take advantage of an acceptor that does not emit light (dark quencher). Such approaches are usually preferred as there is only one fluorescent moiety (the donor) thus avoiding the potential disturbance of the donor signal caused by another fluorescent group.

Significant advantages of FRET-based assays are that they provide the opportunity of monitoring enzyme activity continuously and at a relatively low working concentration range. Peptides that incorporate FRET have made high-throughput screening (HTS) for protease inhibitors much more efficient and convenient. FRET peptide substrates have been used in different applications of matrix metalloproteinase (MMP) research, including HTS of collagenolytic MMPs,¹ identification and development of selective, exosite-binding MMP inhibitors,^{1,2} and measurement of MMP activity in biological fluids and cellular environments.³⁻⁹

Most of the FRET substrates applied for MMP research have incorporated (7-methoxycoumarin-4-yl)-acetyl (Mca)/2,4-dinitrophenyl (Dnp) or (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid) (Edans)/4,4-dimethylamino-azobenzene-4'-carboxylic acid (Dabcyl) fluorophore/quencher pairs.¹⁰ However, the relatively short absorption/emission wavelengths and weak fluorescence intensities (low extinction coefficients) of the fluorophores make assays that use these substrates prone to background interference and thus limit their usefulness especially in applications such as HTS and cell-based assays. In consideration of these properties, fluorophores with higher (longer) excitation/emission wavelengths and larger extinction coefficients have been investigated. 5-Carboxyfluorescein (5-Fam) has an excitation wavelength of $\lambda = 490$ nm and emission wavelength of $\lambda = 520$ nm. The 5-Fam extinction coefficient is 13- and 4-fold higher than Edans and Mca, respectively, allowing for more sensitive assays.¹¹ Fluorescein and its derivatives have been widely applied for many biological studies that utilize a fluorescent label. Fluorescein has been incorporated into FRET peptide substrates, usually paired with a QXL520TM or Dabcyl quencher, for MMP assays.^{8,9,11,12}

Several 5-Fam substrates and various forms of 5-Fam are commercially available. However, the building block approach, where the fluorophore (or quencher) is attached to the side chain of an Fmoc-protected amino acid such as Lys, allows for incorporation of 5-Fam into any substrate via automated solid-phase peptide synthesis (SPPS). Fmoc-Lys(5-Fam) is commercially available but rather expensive. Herein, we examined synthetic routes to produce Fmoc-Lys(5-Fam) economically, one using a similar approach described previously for Fmoc-Lys(Mca)¹³ and one (shorter) route that utilized Fmoc-Lys and microwave irradiation. As the QXL520TM quencher is only available already incorporated into a peptide, our choice for the quencher was the Dabcyl group, which is commercially available as Fmoc-Lys(Dabcyl) and reasonably priced.

5-Fam and Dabcyl were incorporated into the triple-helical MMP substrate (Gly-Pro-Hyp)₅-Gly-Pro-Lys(fluorophore)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(quencher)-Gly-Val-Arg-(Gly-Pro-Hyp)₅-NH₂. This peptide contained a consensus sequence from the MMP hydrolysis sites in types I-III collagen.¹⁴ The supersecondary structure of the substrate was evaluated by circular dichroism (CD) spectroscopy. Kinetic parameters for hydrolysis of the substrate by several MMPs were determined, and substrate stability and hydrolysis was compared to an analogous substrate bearing the Mca/Dnp fluorophore/quencher pair.

MATERIALS AND METHODS

The reagents and solvents used for building block and peptide synthesis were purchased as follows: *N,N'*-diisopropylcarbodiimide (DIC), *N,N*-diisopropylethylamine (DIPEA), *N*-methylmorpholine (NMM), acetonitrile high-performance liquid chromatography (HPLC) grade, water (HPLC grade), and Na⁺Chelex 100 resin from Sigma-Aldrich; 5-Fam, *N*-hydroxybenzotriazole·H₂O (HOBt H₂O) and N^α-(9-fluorenylmethoxycarbonyl)-N^ε-(2,4-dinitrophenyl)-L-lysine [Fmoc-Lys(Dnp)] from Anaspec (Fremont, CA); L-Lys monohydrochloride and 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimidyl ester (Fmoc-OSu) from Novabiochem; Fmoc-Lys from Bachem; methyl *tert*-butyl ether (MTBE) from J.T. Baker; Fmoc-amino acids, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 1-hydroxy-7-azabenzotriazole (HOAt), *N*-methyl-2-pyrrolidone (NMP), and *N,N*-dimethylformamide (DMF) (AR grade) from AGTC Bioproducts (Wilmington, MA); and trifluoroacetic acid (TFA) from Thermo Fisher Scientific (Waltham, MA).

The enzymes and reagents for enzyme assays were acquired as follows: human recombinant proMMP-1 and *p*-aminophenylmercuric acetate (APMA) from CalBiochem; human recombinant proMT1-MMP from Millipore (Billerica, MA); human recombinant proMMP-13, furin, trypsin-3, and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) from R&D Systems (Minneapolis, MN).

Synthesis of N^α-(9-Fluorenylmethoxycarbonyl)-N^ε-(5-carboxyfluorescein)-L-lysine [Fmoc-Lys(5-Fam)]

L-Lys monohydrochloride (Scheme 1; 1) (730 mg; 4 mmol) was dissolved in 5 mL water and refluxed with 1105.5 mg (5 mmol) of basic cupric carbonate for 6 h (Scheme 1). The reaction mixture was filtered and the precipitate washed with hot water until the filtrate was colorless. The combined filtrates were lyophilized and gave 1002 mg (2.82 mmol) of copper-complexed Lys (2).

The copper-complexed Lys (1002 mg, 2.82 mmol) was suspended in 25 mL of NMP and 491 μL (2.82 mmol) of DIPEA was added. 5-Fam (1062 mg, 2.82 mmol) (3) was dissolved in 10 μL of NMP with 381 mg (2.80 mmol) of HOAt. The solution was cooled to 0–4 °C, 437 μL (2.82 mmol) of DIC was added, and the solution stirred. After 15 min the mixture of copper-complexed Lys and DIPEA was added. The reaction proceeded at 0–4 °C for 2 h followed by room temperature overnight.

The Chelex ion-exchange resin was washed 6 times with 1 N acetic acid, six times with water, and six times with 1 N sodium hydroxide. Ion-exchange resin (5 g) was added to the reaction mixture and stirred overnight at room temperature. The resin was removed by filtration and washed with water. The filtrate was added to a mixture of 100 mL water and 100 mL ethyl acetate. The resulting precipitate from the organic layer was centrifuged and decanted. The precipitate was dried in a desiccator in the presence of anhydrous Na₂SO₄, dissolved in water, and lyophilized to give 1231 mg (2.44 mmol) of Lys(5-Fam) (4).

Lys(5-Fam) was dissolved in 20 mL water. Fmoc-OSu (1234.5 mg, 3.66 mmol) was dissolved in 25 mL acetonitrile and was added to the Lys(5-Fam) solution. DIPEA (424 μ L, 2.44 mmol) was added and the reaction proceeded overnight at 30°C. To stop the reaction, the solution was acidified with diluted HCl to pH ~6 and acetonitrile was evaporated in vacuo. The remaining aqueous suspension was extracted five times with chloroform. The organic layer was evaporated in vacuo and dried in a desiccator in the presence of anhydrous Na₂SO₄ to provide 1590 mg (2.19 mmol) of Fmoc-Lys(5-Fam) (5). The crude product was purified using normal phase chromatography with an elution gradient of 30–70% ethyl acetate in toluene containing 5% acetic acid. Fractions from purification were evaporated to dryness in vacuo, dissolved in 5% acetone in water, and analyzed for purity on an Agilent 1260 Infinity analytical HPLC system with a Vydac C₁₈ column (5 μ m, 300Å, 150 $\times$ 4.6 mm²). The elution gradient was 2–98% B in 30 min (where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile), UV-vis detection was at λ = 220 nm, and fluorescent detection was at $\lambda_{\text{excitation}}$ = 490 nm and $\lambda_{\text{emission}}$ = 520 nm. The desired fractions were identified by MALDI-TOF MS analysis on a Voyager DETM PRO instrument. The combined pure fractions were suspended in water, frozen, and lyophilized to give 465 mg (0.64 mmol, 16% overall yield) of pure product.

Microwave Assisted Synthesis of Fmoc-Lys(5-Fam)

5-Fam (250 mg, 0.665 mmol) (3) and 87 mg (0.64 mmol) of HOAt was dissolved in 2 mL of DMF (Scheme 2). DIC (104.25 μ L; 0.665 mmol) was added and the mixture was stirred for 15 min. Fmoc-L-Lys monohydrochloride (269 mg, 0.665 mmol) (6) in 2 mL of DMF and 116 μ L (0.665 mmol) of DIPEA was added to the preactivated 5-Fam. The reaction proceeded for 45 min (microwave power of 25 W at 45°C). The reaction mixture was diluted with water 15:1 (v/v), centrifuged, and decanted. The remaining precipitate was suspended in water, frozen, and lyophilized. The crude product was dissolved in 5% acetone in water and purified by Reversed Phase flash chromatography (RP C-18 column) on a CombiFlash® Companion® (Teledyne Isco), using a linear gradient of 2–98% B in 60 min (where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile). Purified fractions were analyzed using RP-HPLC (analytical gradient 2–98% B in 30 min, flow rate 1 mL/min, detection at λ = 220 nm) and by MALDI-TOF MS. The pure fractions were combined, frozen, and lyophilized to provide 152 mg (0.21 mmol, 31.5% overall yield) of Fmoc-Lys(5-Fam) (5).

Peptide Synthesis

Fluorogenic triple-helical peptide fTHP-15(5-Fam/Dabcyl) [sequence (Gly-Pro-Hyp)₅-Gly-Pro-Lys(5-Fam)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dabcyl)-Gly-Val-Arg-(Gly-Pro-

Hyp)₅-NH₂, where Hyp is 4-hydroxy-L-proline] was synthesized on NovaPEG Rink amide resin, substitution level 5 0.48 mmol/g, using Fmoc chemistry. Peptide synthesis was carried out on the Liberty (CEM) automated microwave assisted peptide synthesizer equipped with microwave module Discover.

Fmoc-amino acids were coupled using 5 equiv. of each amino acid, 4.9 equiv. HCTU, and 8 equiv. NMM (microwave power of 25 W at 50°C, 300 s) with the exception of Fmoc-Pro₁₂, Fmoc-Gln(Trt)₂₀, Fmoc-Pro₂₁, Fmoc-Gly₂₂, Fmoc-Gly₂₈, and Fmoc-Lys (DabcyI), where coupling time was increased to 600 sec. Coupling reactions were repeated for Fmoc-Pro₁₄, Fmoc-Gly₂₅, and Fmoc-Gln(Trt)₂₆.

Fmoc-Lys(Fam) was incorporated manually using 3 equiv. of amino acid, 3 equiv. DIC, and 6 equiv. DIPEA, reaction time 17 h. Unreacted amino groups were capped using 0.5M Ac₂O, 0.125M DIPEA, and 0.015M HOBt solution.

Deprotection of side chains and peptide cleavage were carried out for 3 h in an atmosphere of ambient gas (Ar) using 7 mL of cleavage cocktail (5% H₂O, 5% thioanisole, 2.5% phenol, 2.5% 1,2-ethanedithiol in TFA). Cleaved peptide was precipitated in cold MTBE. The solvent was decanted and the precipitate dissolved in water, frozen, and lyophilized.

Crude peptide was purified using RP-HPLC on an Agilent 1260 Infinity series preparative HPLC equipped with a Vydac C₁₈ column (15–20 µm, 300Å, 250 × 22 mm²). The elution gradient was 20–40% B in 60 min (where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile), flow rate 15 mL/min, detection at λ = 220 and 490 nm. The HPLC homogenous fractions were combined, frozen, and lyophilized.

Peptide purity was evaluated on an Agilent 1260 Infinity analytical HPLC using a Vydac C₁₈ column (5 µm, 300Å, 150 × 4.6 mm²), analytical gradient 2–98% B in 30 min, flow rate 1 mL/min, detection at λ = 220 and 490 nm. MALDI-TOF MS analysis indicated [M+H]⁺ = 4818.5 Da (theoretical [M+H]⁺ = 4818.3 Da). (Gly-Pro-Hyp)₅-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)₅-NH₂ (fTHP-15) was synthesized, purified, and characterized as previously described.^{15,16}

CD Spectroscopy

Peptide triple-helical structure was evaluated by CD spectroscopy. Spectra were obtained on a JASCO J-810 spectropolarimeter over the range of λ = 180–250 nm using a 0.1 cm quartz cuvette. The peptide concentration was 25 µM in 1% aqueous acetic acid solution. The thermal transition curve was obtained by recording the molar ellipticity ([θ]) at λ = 225 nm with temperature increasing by 20°C/h from 5 to 80°C. Temperature was controlled by a JASCO PTC-348WI temperature control unit. The melting temperature (*T_m*) was defined as the inflection point in the transition region. The spectra were normalized by designating the highest [θ]_{225 nm} as 100% folded, and the lowest [θ]_{225 nm} as 0% folded.^{17–20}

FRET MMP Assays

Peptide substrate stock solutions were prepared in the concentration range of 0.03–1.5 µM [for fTHP-15(5-Fam/DabcyI)] and 0.05–8.5 µM (for fTHP-15) in Tris buffer (50 mM Tris,

100 mM NaCl, 10 mM CaCl₂, 0.05% Brij-35, 0.02% NaN₃, pH 7.5). ProMMP-1 and proMMP-13 were activated by reacting with 2 mM APMA for 3 and 1 h, respectively, at 37°C. ProMT1-MMP was activated by incubation with 0.1 equiv. furin for 1 h at 37°C. After activation of MT1-MMP, furin inhibitor I (Sigma) was added (10 equiv. per equiv. of furin). Immediately after activation the enzymes were diluted to desired concentrations with Tris buffer and incubated with substrates at 25°C at a final enzyme concentration of 5 nM. The activity of each MMPs was determined using the Knight substrate [Mca-Lys-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH₂].

Fluorescence was monitored continuously at $\lambda_{\text{excitation}} = 490$ nm and $\lambda_{\text{emission}} = 520$ nm (for 5-Fam) and $\lambda_{\text{excitation}} = 324$ nm and $\lambda_{\text{emission}} = 394$ nm (for Mca) on a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek). All measurements were done in triplicate. Rates of hydrolysis ($\mu\text{M/s}$) were determined from plots of fluorescence over time, but only data points from the linear part of the plot were used. Slopes of hydrolysis curves were divided by the fluorescence change from the beginning to the completion of the enzymatic reaction and multiplied by substrate concentration. Kinetic parameters were calculated using Lineweaver-Burk, Hanes-Woolf, and Eadie-Hofstee linear plots and by nonlinear regression, Michaelis-Menten model with GraphPad Prism 5 software.

RP-HPLC MMP-13 Assay

Stock solutions of fTHP-15(5-Fam/Dabcyl) peptide were prepared in the concentration range of 1.67–6.67 μM in Tris buffer. ProMMP-13 was activated as described above. Immediately after activation the enzyme was diluted to 12.5 nM, giving an enzyme concentration of 0.625 nM in the assay. MMP-13 was incubated with the substrate at 25°C and chromatograms recorded at 0, 0.5, 1, 1.5, 2, and 24 h reaction time for each substrate concentration. The HPLC elution gradient was 2 - 98% B in 25 min (where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile), UV-vis detection was at $\lambda = 220$ nm, and fluorescent detection was at $\lambda_{\text{excitation}} = 490$ nm and $\lambda_{\text{emission}} = 520$ nm.

For each sample the concentration of product formed (μM) was plotted over reaction time (s) and the initial velocities (V_i 5 [$\mu\text{M/s}$]) for each substrate concentration were calculated as $y/ x \times V_i$. The obtained values of initial velocities for each substrate concentration were fitted into the Michaelis–Menten nonlinear regression model in GraphPad Prism 5 software to calculate the kinetic parameters.

RESULTS AND DISCUSSION

Synthesis of Fmoc-Lys(5-Fam)

We examined two synthetic routes to obtain Fmoc-Lys(5-Fam). The first route utilized copper complexation of Lys, 5-Fam addition, disruption of the copper complex, and Fmoc addition (Scheme 1). Unfortunately, the yield from this route was low (16%) so a different approach was investigated. The second route utilized commercially available Fmoc-Lys, reacted with a preformed HOAt ester of 5-Fam (Scheme 2). This approach did not require the generation and removal of the copper complex. Fmoc addition was not required, either. However, this approach had one serious drawback, the bulkiness of the two reactants. To

improve the reaction efficiency microwave irradiation was applied. Several different conditions were tested (15, 30, and 45 min at 45°C and the same reaction times at 60°C; for all reactions power was set to 25 W) and the best results were observed for 45 min at 45°C, whereas without microwave irradiation no product was obtained (even after overnight reaction and traditional heating). Temperatures higher than 60°C were avoided to limit the risk of 5-Fam deg-radation,²¹⁻²³ although it is worth noting that one prior report indicated that 5-Fam could be acylated with microwave irradiation at 80°C.²⁴ All reactions that involved 5-Fam were conducted in a covered reaction vessel to minimize fluorophore decomposition.²² The microwave assisted approach proved to be a convenient, less time consuming, and more effective way to synthesize Fmoc-Lys(5-Fam), as the reaction gave a high yield and no side products were observed in the crude product. MALDI-TOF mass spectra of the crude product obtained after 45 min at 45°C microwave assisted reaction showed peaks corresponding to Fmoc-Lys(5-Fam) (727.45 Da; theoretical 726.6 Da) and unreacted 5-Fam (377.27 Da; theoretical 376.32 Da).

Both synthetic routes provided Fmoc-Lys(5-Fam) of very high purity (Figure 1) and yielded products that were less expensive to produce than commercially available Fmoc-Lys(5-Fam). fTHP-15(5-Fam/DabcyI) was synthesized using the Fmoc-Lys(5-Fam) building block. The synthesis was successful using standard protocols, although the Fmoc-Lys(5-Fam) building block required prolonged (overnight) coupling.

There have been contrary reports in the literature about the necessity of protecting the phenolic hydroxyl group of 5-FAM during SPPS.^{25,26} Use of tritylchloride to tritylate the hydroxyl group increased the purity of several peptides, with side products of +14 and +68 Da observed more prominently when nontritylated 5-Fam was incorporated.²⁵ The side product resulting in an increased mass of 14 Da was reported when a 5-Fam-containing peptide was treated with hydrazine to remove the Dde protecting group from Lys.²⁵ The synthesis herein did not require the use of Fmoc-Lys(Dde) and thus hydrazine treatment was not pursued. The second side product, resulting in an increased mass of 68 Da, was hypothesized to be a piperidine adduct.²⁵ Alternatively, a prior study in which a 26-residue peptide was synthesized using C-terminal 5-Fam with and without trityl protection showed no difference in peptide purity.²⁶ Piperidine was used to remove the Fmoc group in this prior study. In our SPPS, we used piperazine for Fmoc removal instead of piperidine, and we did not observe any mass corresponding to a piperazine-based adduct during the SPPS of fTHP-15(5-Fam/DabcyI). It is worth noting that efficient tritylation of resin-bound 5-Fam can be difficult to achieve.²⁴

Utilizing the building block approach for incorporating the fluorophore moiety into a peptide sequence has several advantages over other strategies. Fluorophores are often incorporated by selective side-chain deprotection of an assembled peptideresin, followed by covalent attachment of the fluorophore to the deprotected amino acid side-chain. This process is subject to the relative efficiencies of selective deprotection and attachment, which may be additionally dependent upon the position in the peptide chain. Coupling of Fmoc-Lys(5-Fam) as a standard amino acid derivative at any given position in the peptide sequence avoids additional synthetic steps and subsequent strenuous purification often needed with peptide labeling approaches. Use of Fmoc-Lys(5-Fam) also avoids an

additional step in which piperidine treatment is used to remove additional Fam molecules produced during labeling approaches.²⁵ MMP FRET substrates containing Fam have been synthesized using an *O*-(methoxyethoxy-methyl)-5-amino-fluorescein 2-chlorotrityl resin.⁹ However, this approach does not allow for internal incorporation of the fluorophore during the synthesis of longer substrates.

The CD spectrum of fTHP-15(5-Fam/Dabcyl) showed a maximum at $\lambda = 225$ nm and a minimum at $\lambda = 200$ nm (Figure 2), characteristic for triple-helical structure.²⁷ The peptide maintained its triple-helical structure despite the bulky fluorophore/quencher pair of 5-Fam/Dabcyl. However, the T_m values calculated from the thermal transition curves were 42°C for fTHP-15(5-Fam/Dabcyl) and 51°C for fTHP-15 (Figure 3). Thus, the incorporation of 5-Fam/Dabcyl decreased the thermal stability of the THP substrate compared with Mca/Dnp.

Hydrolysis of Triple-Helical Substrates by MMPs

Hydrolysis of fTHP-15 and fTHP-15(5-Fam/Dabcyl) was quantified for MMP-1, MMP-13, and MT1-MMP. All three enzymes cleaved both substrates, and MMP-13 had the highest activity of the three enzymes (Table I). Incorporation of the bulkier fluorophore (5-Fam) and quencher (Dabcyl) into the peptide sequence did not disturb the ability of the enzyme to recognize and hydrolyze the substrate (Table I). In fact, k_{cat}/K_M values for MMP-1 and MMP-13 were higher for the 5-Fam/Dabcyl containing peptide than for the peptide with the Mca/Dnp groups (Table I). In the case of MMP-1, this might be explained by the lower thermal stability of the triple-helix allowing for easier processing of the 5-Fam fTHP.²⁸ In turn, the improved rate of hydrolysis by MMP-13 may be based on the enhanced ability of MMP-13 (compared with the other collagenolytic MMPs) to hydrolyze interrupted triple-helices,²⁹ as the thermally destabilized fTHP-15(5-Fam/Dabcyl) has its folded $\leftrightarrow$ unfolded equilibrium shifted toward the nontriple-helical state compared with fTHP-15.

The difference in size between the fluorophore/quencher pairs can affect the interaction of the substrate with the enzyme active and/or secondary binding sites. The most significant differences in K_M values were observed for MMP-1 and MT1-MMP. The bulkier fluorophore/quencher decreased the affinity of MMP-1 toward the substrate as the K_M value was 3.2-fold higher for fTHP-15(5-Fam/Dabcyl). Conversely, the affinity of MT1-MMP increased threefold when 5-Fam/Dabcyl was present. This would suggest that MT1-MMP tolerates or even prefers bulkier residues in the P₅ and/or P₅' subsites. For MMP-13 there was no significant difference in the K_M values.

To evaluate if inner filter effects (self quenching or quenching by the acceptor moiety of a neighboring substrate molecule) compromised the kinetic parameters obtained from the FRET assays, an RP-HPLC hydrolysis assay was performed. The RP-HPLC derived K_M , k_{cat}/K_M , and k_{cat} values were in good agreement with the values obtained from monitoring the fluorescence signal (data not shown).

fTHP-15(5-Fam/Dabcyl) proved to have a higher signal to background (*S/B*) ratio compared with fTHP-15 (Table II and Figure 4). The *S/B* ratio was calculated as fluorescence signal at the 24 h time point of the enzymatic reaction divided by the fluorescence signal of the substrate sample without the enzyme. The improved *S/B* ratio (compared with Mca/Dnp)

will allow the use of a lower concentration of the fluorogenic substrate, thus making the whole assay more cost effective especially in HTS campaigns. The higher fluorescence intensity together with longer excitation/emission wavelengths for the 5-Fam/Dabcyl substrate may allow for the development of a quantitative assay measuring collagenolytic protease activity in the cell surface environment, analogous to recent studies of MMP-11.⁹

Abbreviations

Ac₂O	acetic anhydride
AEBSF	4-(2-aminoethyl)benzensulfonyl fluoride hydrochloride
APMA	<i>p</i> -aminophenyl mercuric acetate
CD	circular dichroism
Dabcyl	4,4-dimethylamino-azobenzene-4'-carboxylic acid
Dde	1-[(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl]
DIC	<i>N,N'</i> -diisopropylcarbo-diimide
DIPEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
Dnp	2,4-dinitrophenyl
Edans	(5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid)
5-Fam	5-carboxyfluorescein
Fmoc	9-fluorenylmethoxycarbonyl
FRET	fluorescence resonance energy transfer
fTHP	fluorogenic triple-helical peptide
HCTU	2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexa-fluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzo-triazole
HTS	high throughput screening
MALDI-TOF	matrix-assisted laser desorption/ionization time of flight
Mca	(7-methoxycoumarin-4-yl)-acetyl
MMP	matrix metalloproteinase
MTBE	methyl <i>tert</i> -butyl ether
NMM	<i>N</i> -meth-ylmorpholine
NMP	<i>N</i> -methyl-2-pyrrolidone
OSu	<i>N</i> -hydroxysuccinimidyl ester

RP-HPLC	reversed-phase high performance liquid chromatography
SPPS	solid-phase peptide synthesis
TFA	trifluoroacetic acid

REFERENCES

1. Lauer-Fields JL, Minond D, Chase PS, Baillargeon PE, Saldanha SA, Stawikowska R, Hodder P, Fields, G B. *Bio-org Med Chem.* 2009; 17:990–1005.
2. Roth J, Minond D, Darout E, Liu Q, Lauer J, Hodder P, Fields GB, Roush WR. *Bioorg Med Chem Lett.* 2011; 21:7180–7184. [PubMed: 22018790]
3. Neumann U, Kubota H, Frei K, Ganu V, Leppert D. *Anal Biochem.* 2004; 328:166–173. [PubMed: 15113693]
4. Beekman B, van El B, Drijfhout JW, Rondan HK, TeKop-pele JM. *FEBS Lett.* 1997; 418:305–309. [PubMed: 9428733]
5. Beekman B, Wouter J, Bloemhoff W, Rondan K, Tak PP, te Koppele JM. *FEBS Lett.* 1996; 390:221–225. [PubMed: 8706864]
6. Giricz O, Lauer JL, Fields GB. *Anal Biochem.* 2011; 409:37–45. [PubMed: 20920458]
7. Miller MA, Barkal L, Jeng K, Herrlich A, Moss M, Grif-fith LG, Lauffenburger DA. *Integr Biol.* 2011; 3:422–438.
8. Roy R, Zurakowski D, Pories S, Moses MA, Moss ML. *Clin Biochem.* 2011; 44:1434–1439. [PubMed: 22001071]
9. Meyer BS, Rademann J. *J Biol Chem.* 2012; 287:37857–37867. [PubMed: 22927434]
10. Fields, GB. In *Methods in Molecular Biology*, Vol. 622: *Matrix Metalloproteinase Protocols*. 2nd ed.. Clark, IM., editor. Humana Press; Totowa, NJ: 2010. p. 393–433.
11. Rakhmanova V, Meyer R, Tong X. *Gen Eng Biotechnol News.* Oct 15.2007 :40.
12. Moss ML, Rasmussen FH. *Anal Biochem.* 2007; 366:144–148. [PubMed: 17548045]
13. Malkar NB, Fields GB. *Lett Peptide Sci.* 2001; 7:263–267.
14. Robichaud TK, Steffensen B, Fields GB. *J Biol Chem.* 2011; 286:37535–37542. [PubMed: 21896477]
15. Lauer-Fields JL, Broder T, Sritharan T, Nagase H, Fields GB. *Biochemistry.* 2001; 40:5795–5803. [PubMed: 11341845]
16. Minond D, Lauer-Fields JL, Cudic M, Overall CM, Pei D, Brew K, Visse R, Nagase H, Fields GB. *J Biol Chem.* 2006; 281:38302–38313. [PubMed: 17065155]
17. Beck K, Chan VC, Shenoy N, Kirkpatrick A, Ramshaw JAM, Brodsky B. *Proc Natl Acad Sci USA.* 2000; 97:4273–4278. [PubMed: 10725403]
18. Persikov AV, Ramshaw JAM, Brodsky B. *Biopolymers (Peptide Sci).* 2000; 55:436–450.
19. Persikov AV, Ramshaw JAM, Kirkpatrick A, Brodsky B. *Biochemistry.* 2000; 39:14960–14967. [PubMed: 11101312]
20. Persikov AV, Xu Y, Brodsky B. *Protein Sci.* 2004; 13:893–902. [PubMed: 15010541]
21. Leonhardt H, Gordon L, Livingston R. *J Phys Chem.* 1971; 75:245–249.
22. Smith SA, Pretorius WA. *Water SA.* 2002; 28:403–406.
23. Fara MA, D iaz-Mochon JJ, Bradley M. *Tetrahedron Lett.* 2006; 47:1011–1014.
24. Kowalczyk R, Harris PWR, Dunbar RP, Brimble MA. *Synthesis.* 2009:2210–2222.
25. Fischer R, Mader O, Jung G, Brock R. *Bioconj Chem.* 2003; 14:653–660.
26. Tong X, Fu W, Sheng L. *J Biomol Tech.* 2007; 18:47.
27. Fields GB, Prockop D. J. *Biopolymers (Peptide Sci).* 1996; 40:345–357.
28. Minond D, Lauer-Fields JL, Nagase H, Fields GB. *Bio-chemistry.* 2004; 43:11474–11481.
29. Minond D, LauerFields JL, Cudic M, Overall CM, Pei D, Brew K, Moss ML, Fields GB. *Biochemistry.* 2007; 46:3724–3733. [PubMed: 17338550]

FIGURE 1.

MALDI-TOF mass spectra of purified Fmoc-Lys(5-Fam) obtained by the copper complexation route (A) ($[M+H]^+$ 5 726.9 Da, theoretical $[M+H]^+$ 5 726.6 Da) and by the microwave assisted reaction (B) ($[M+H]^+$ 5 727.0 Da, theoretical $[M+H]^+$ 5 726.6 Da). Analytical chromatogram of purified Fmoc-Lys(5-Fam) obtained by copper complexation route (C) and by the microwave assisted synthesis (D) (retention time was ~19 min).

FIGURE 2.

CD spectrum of fTHP-15(5-Fam/Dabcyl). The sample was dissolved in 1% acetic acid at a concentration of 25 μ M.

FIGURE 3.

Thermal transition curves for fTHP-15 (closed squares) and fTHP-15(5-Fam/Dabcyl) (closed diamonds). The samples were dissolved in 1% acetic acid at a concentration of 25 μ M. Molar ellipticity ($[\theta]$) was recorded at $\lambda = 225$ nm while the temperature was increased from 5 to 80°C by 20°C/h.

FIGURE 4.

Fluorescence signal increase obtained after 24 h incubation of fTHP-15 or fTHP-15(5-Fam/Dabcyl) with no enzyme or MMP-13. $\lambda_{\text{emission}} = 324 \text{ nm}$ for fTHP-15 and 520 nm for fTHP-15(Fam/Dabcyl).

SCHEME 1.
Synthesis of Fmoc-Lys(5-Fam) using copper complexation.

SCHEME 2.

Synthesis of Fmoc-Lys(5-Fam) using microwave assistance.

Table I
Kinetic Parameters for MMP Hydrolysis of fTHPs

Substrate	MMP-1			MMP-13			MT1-MMP		
	k_M (μM)	k_{cat}/K_M ($s^{-1} M^{-1}$)	k_{cat} (s^{-1})	k_M (μM)	k_{cat}/K_M ($s^{-1} M^{-1}$)	k_{cat} (s^{-1})	k_m (μM)	k_{cat}/K_M ($s^{-1} M^{-1}$)	k_{cat} (s^{-1})
fTHP-15(5-Fam/Dabcyl)	7.3	2968	0.0217	16.76	65,964	1.1	4.37	1249	0.0055
fTHP-15	2.31	1833	0.0042	20.25	46,815	0.948	13.1	1981	0.0259

Table II
Signal to Background Ratio for MMP Hydrolysis of fTHPs

Substrate	MMP-1	MMP-13	MT1-MMP
fTHP-15(5-Fam/Dabcyl)	21.0	17.3	11.3
fTHP-15	5.2	5.2	4.2