Highly Sensitive FRET Substrates for the Assay of MMPs

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Introduction

Matrix metalloproteinases (MMPs) are involved in the degradation of extracellular matrix and play an important role in apoptosis, embryogenesis, and reproduction tissue remodeling and repair. The development of agonists or inhibitors in controlling MMP activity continues to be of great interest for drug discovery and life science research.

FRET peptide-based MMP assays have been widely used for measuring MMP activity. However, most of these FRET peptides use Mca/Dnp or EDANS/DABCYL pairs [1], which have relatively weak fluorescence signals with short wavelengths. We designed and synthesized sixteen MMP substrates that incorporated 5-FAM (donor) and QXL™520 (quencher) by Fmoc solid phase synthesis method. QXL™520 is an effective quencher for fluoresceins such as FAM and FITC. These novel 5-FAM/QXL™520 FRET peptides offer several advantages.

Results

MMP Substrates (Table 1) were synthesized by Fmoc solid phase synthesis methods, using Rink amide MBHA resin. For normal amino acid, the couplings were performed with fourfold excess of activated amino acids. Fmoc-amino acids were activated using the ratio of Fmoc-amino acid:HBTU:HOBt:DIEA, 1:1:1:2. For special amino acids [Dap(Mtt), Dab(Mtt), Cys(Me), Cha, Smc and D-Arg(Pbf)], couplings were performed with two-threefold excess of activated amino acids. 5-FAM and QXL™ 520 were activated using DIC:HOBt (1:1). Upon completion of the chain assembly, the peptide-resin was treated with 1% TFA and 3% TIPS in DCM to remove the Mtt group. QXL™ 520 and 5-FAM were coupled to the side chain amino group of Dap, Dab and Lys using DIC:HOBt 1:1. Complete deprotection of the peptide and cleavage was performed with TFA:thioanisole:water:phenol:EDT for 2h. Crude peptides were purified to homogeneity by RP-HPLC using 0.1% (v/v) TFA/water and 0.1% (v/v) TFA/ acetonitrile. Compared to EDANS and Mca, the extinction coefficient of 5-FAM is 13-fold higher and shows less interference from the short wavelength auto-fluorescence of drug candidates. Additionally, 5-FAM is much brighter and less sensitive to the environment than EDANS and Mca. These characteristics of 5-FAM prompted us to design 5-FAM containing MMP FRET peptide substrates in order to increase the sensitivity. QXL™ 520 serves as an excellent quencher for the 5-FAM, since its absorption spectrum perfectly overlaps with the emission spectrum of 5-FAM (Figure 1). Additionally, QXL™ 520 is a hydrophilic compound, which unlike DABCYL and Dnpare are hydrophobic. This property of QXL™ 520 increases the solubility of the peptide substrate in aqueous buffers, alleviating the problem caused by the hydrophobic nature of many fluorescent donors and quenchers.

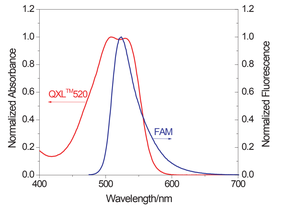

Figure 1. *The absorption spectrum of QXL™520 perfectly overlaps with the emission spectrum of 5-FAM.*

Table 1: 5-FAM/QXL™520 FRET MMP substrates.

Seq# (Catalog No.)	FRET Peptide Sequence
#1 (60568-01)	QXL™520-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-Lys(5-FAM)-NH_2
#2 (60569-01)	QXL™520 -Pro-Leu-Ala-Leu-Trp-Ala-Arg-Lys(5-FAM)-NH_2
#3 (60570-01)	QXL™520-Pro-Leu-Gly-Cys(Me)-His-Ala-D-Arg-Lys(5-FAM)-NH_2
#4 (60571-01)	5-FAM-Pro-Leu-Ala-Nva-Dap(QXL™520)-Ala-Arg-NH_2
#5 (60572-01)	5-FAM-Pro-Leu-Gly-Leu-Dap(QXL™520)-Ala-Arg-NH_2
#6 (60573-01)	QXL™520-Pro-Leu-Gly-Met-Trp-Ser-Arg-Lys(5-FAM)-NH_2
#7 (60574-01)	QXL™520 -Pro-Tyr-Ala-Tyr-Trp-Met-Arg-Lys(5-FAM)-NH_2
#8 (60575-01)	QXL™520-Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(5-FAM)-NH_2
#9 (60576-01)	QXL™520-Arg-Pro-Leu-Ala-Leu-Trp-Arg-Lys(5-FAM)-NH_2
#10 (60577-01)	QXL™520 -Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys(5-FAM)-NH_2
#11 (60578-01)	5-FAM-Pro-Cha-Gly-Nva-His-Ala-Dap(QXL™520)-NH_2
#12 (60579-01)	5-FAM-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(QXL™520)-NH_2
#13 (60580-01)	5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(QXL™520)-NH_2
#14 (60581-01)	QXL™520 -γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2
#15 (60582-01)	QXL ™520-γ-Abu-Pro-Gln-Gly-Leu-Dab(5-FAM)-Ala-Lys-NH_2
#16 (60583-01)	QXL ™520 -Arg-Pro-Lys-Pro-Gln-Gln-Phe-Trp-Lys(5-FAM)-NH_2

Sixteen 5-FAM/QXL™520 FRET peptides were synthesized and screened by MMP-1, 2, 3, 7, 8, 9, 12, 13 and 14. All labeled peptides were incubated at room temperature with activated MMPs in assay buffer (pH 7.5). Each peptide was found to be cleaved by certain MMPs.

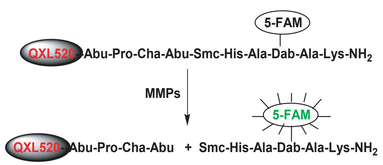

Figure 2. *Schematic representation of the proteolytic cleavage of Seq#14.*

Seq# 14 was cleaved by all the MMPs tested and was shown to have the highest kinetic slope (RFU/min).

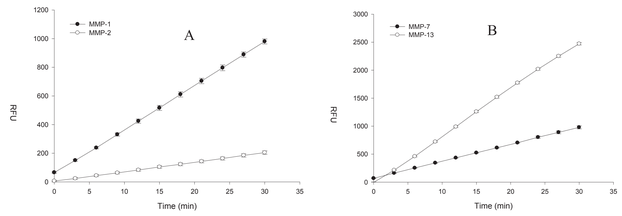

Figure 3. *Fluorescence intensity of 5-FAM increased with reaction time when Seq# 14 was cleaved by MMPs.*

Seq# 14 was cleaved by MMP-1, 2, 7 and 13 (Figure 3). It was also cleaved by MMP-3, 8, 11, 12 and 14 (data not shown). This substrate showed the highest kinetic slope (RFU/min) among all the 5-FAM/QXL™520 FRET peptides listed in Table 1. Fluorescence signal was monitored at Ex/Em=492 nm/518 nm for 30 mins. (n=2 independent samples, mean ± S.D.)

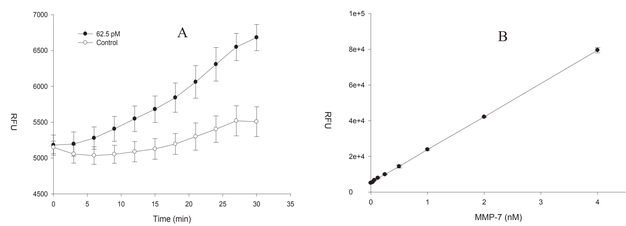

Figure 4. *Analysis of Seq# 14 sensitivity for MMP-7.*

Serially diluted MMP-7 solutions were incubated with Seq# 14. Fluorescence signal was monitored at Ex/Em = 492nm/518nm for 30 min. (n=2 independent samples, mean±S.D.) The results (Figure 4A.B) showed that this substrate can detect 62.5 pM of MMP-7 with a linear range of up to 4 nM.

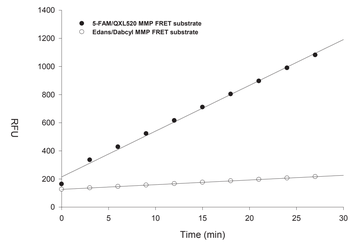

Figure 5. *Hydrolysis of Seq# 14 and EDANS/DABCYL based FRET peptide with MMP-1.*

Two FRET peptides, DABCYL-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Glu(EDANS)-Ala-Lys-NH_2 (Cat# 27061) and QXL™520-γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2 (Seq# 14), were incubated with MMP-1(4 nM). Seq# 14 showed higher sensitivity than the EDANS/ DABCYL based FRET peptide because of its more intense fluorescence emission and longer wavelength which avoids autofluorescence.

Conclusion

- We developed sixteen MMP substrates incorporating 5-FAM and QXL™520 which can be applied to high throughput screening for MMP activity assays.
- FAM/QXL™520 substrates have stronger absorption and emission intensity at longer wavelengths (492 nm/518 nm) compared to DABCYL/EDANS and Mca/Dnp substrates.
- Assays using FAM/QXL™520 substrates exhibit lower background due to less autofluorescent interference from cellular components and test compounds.
- QXL™520 is more water soluble than DABCYL, thus improving the observed hydrolysis rate.
- Seq# 14 shows the highest proteolytic kinetics to all of `the MMPs tested. A submicromolar concentration of this substrate is adequate to detect picomolar level of MMPs.

Reference:

1. Gregg B. Fields (2001) Using fluorogenic peptide substrates to assay matrix metalloproteinases, in *Matrix Metalloproteinase Protocols* (Ian M. Clark ed.) pp.495 518, Humana Press, Totowa, NJ.