Highly Sensitive FRET Substrate for Assay of HCV Protease

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Introduction

The alarming spread of hepatitis C viral (HCV) infections and the consequences associated with chronic hepatitis C have resulted in a world-wide medical problem affecting 170 million patients [1]. The inhibition of HCV protease activity serves as an important method for preventing HCV infection caused by mutiplication of the HCV virus. Although a fluorescence resonance energy transfer (FRET) depsipeptide, **Ac-DED(Edans)EE-αAbuψ[COO]ASK(Dabcyl)-NH$_2$** (substrate **I**) is widely used for detecting HCV NS3/4A serine protease activity [2], its low sensitivity and short detection wavelength limit its use for high throughput screening.

We have recently developed a sensitive FRET HCV protease substrate for high throughput screening of HCV protease inhibitors. This new FRET substrate, **Ac-DE-Dap(QXL™520)EE-αAbuψ[COO]AS-C(5-FAMsp*)-NH$_2$** (substrate **II**), incorporates 5-FAM (donor) and QXL™520 (quencher). QXL™520 is proven to be the most effective quencher for fluoresceins such as FAM and FITC. In comparison to substrate **I**, this new FRET peptide offers several advantages.

Compared to Edans, the extinction coefficient of 5-FAM is 13-fold higher and its fluorescence receives less interference from the short wave-length auto-fluorescence of drug candidates. Additionally, 5-FAM is much brighter and less sensitive to the environment than Edans. These characteristics of 5-FAM prompted us to design a more sensitive 5-FAM FRET peptide substrate for HCV NS3/4A protease. We developed the QXL™520 to serve as a quencher for the 5-FAM. Its absorption spectrum perfectly overlaps with the emission spectrum of 5-FAM (Figure 1). Additionally, QXL™520 is a hydrophilic compound unlike Dabcyl which is hydrophobic. This property of QXL™520 increases the solubility of the peptide substrate. The problem caused by the hydrophobic nature of many fluorescent donors and quenchers is thus alleviated.

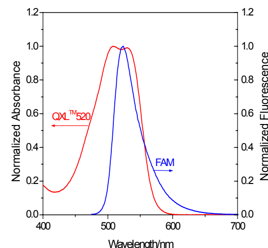

Figure 1. The absorption spectrum of QXL™520 perfectly overlaps with the emission spectrum of 5-FAM. QXL™520 is an excellent quencher when paired with 5-FAM.

Results

Substrate **II** was synthesized by a combination of Fmoc solid phase and solution phase synthesis methods. The resin used was Rink amide MBHA resin. All couplings, including Dap(Mtt), were performed with fourfold excess of activated amino acids over the resin-free amino groups, using the ratio of Fmoc-amino acid:HBTU:HOBt:DIEA (1:1:1:2). L-(+)-lactic acid was activated using DIC:HOBt (1:1). Esterification of Abu to the free hydroxyl of lactic acid was performed using the Fmoc-Abu-F in the presence of a catalytic amount of DMAP. At the end of the assembly, the peptide-resin was treated with 1% TFA and 3% TIPS in DCM to remove the Mtt group. QXL™520-OH was coupled to β-amino group of Dap with DIC:HOBt. Complete deprotection of the peptide was performed with TFA:water:TIPS (93:4:3) for 2 h to obtain crude **Ac-DE-Dap(QXL™520)EE-αAbuψ[COO]ASC-NH$_2$ (III)**. Peptide **III** was incubated with 5-FAMsp to obtain crude substrate **II.** The crude peptide was purified by RP-HPLC using as eluents (A) 50 mM ammonium acetate (pH 6.5), and (B) acetonitrile.

Substrate **II** peptide was derived from the sequence of the NS4A/NS4B cleavage site (DEMEECASHL). In comparison with the sequence of substrate **I** peptide, Ac-Asp-Glu-Asp(Edans)-Glu-Glu-αAbuψ[COO] Ala-Ser-Lys(Dabcyl)-NH2, we have changed the Asp(Edans) to Dap-(QXL™520) and Lys(Dabcyl) to Cys(5-FAMsp).

In the intact substrate **II** FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520 (Figure 2). Upon cleavage, the fluorescence of 5-FAM is recovered and can be continuously monitored at Excitation/Emission = 490 nm/520 nm over time (Figure 3).

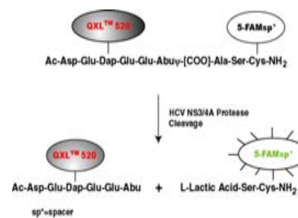

Figure 2. The scheme of the proteolytic cleavage of substrate II peptide by HCV NS3/4A protease.

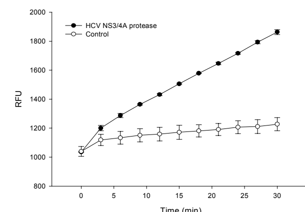

***Figure 3.** The fluorescence intensity of 5-FAM increased with reaction time when the 5-FAM/ QXL™520 FRET peptides were cleaved by HCV NS3/4A protease.*

The substrate **II** peptide showed significantly less inner filter effect than substrate **I** peptide. The new 5-FAM/QXL 520-based substrate **II** has inner filter effect < 5% when the peptide concentration is <50 µM. The inner filter effect is the phenomenon in which light emitted by the fluorophore is absorbed by nearby quencher on intact substrates or cleaved products, so that only a fraction of its fluorescent signal can be detected by a fluorometer. As shown in Figure 4, when the substrate **I** peptide concentration reaches 20 µM, 50% of Edans's fluorescence is quenched. The inner filter effect significantly reduces the accuracy of enzymatic kinetic parameters (*Km* and *K*cat et al).

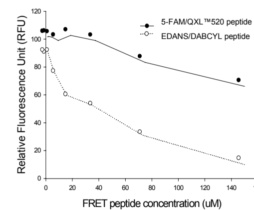

***Figure 4.** 5-FAM/QXL™520 FRET peptide showed less inner filter effect compared to Edans/Dabcyl FRET peptide.*

The enzyme detection dynamic range of 5-FAM/QXL™520 FRET peptide is from 8.27 to 0.064 pmole, while that of Edans/Dabcyl FRET peptide is from 8.27 to 0.52 pmole (Figure 4). These results demonstrate 5-FAM/QXL™520 FRET peptide is eight times more sensitive than Edans/Dabcyl FRET peptide.

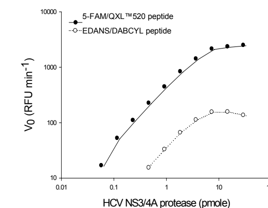

***Figure 4.** The sensitivity comparison of 5-FAM/QXL™520 FRET peptide and Edans/Dabcyl FRET peptide*

Substrate **II** has smaller *Km* and higher *Kcat/Km* value compared to substrate **I** (Table 1). Individual kinetic parameters (K_m and K_{cat}) are determined over a substrate concentration range of 0-100 mM and calculated by double reciprocal plots.

***Table 1.** The comparison of kinetic parameters of two FRET substrates.**

	K_m(µM)	K_{cat}(min^{-1})	K_{cat}/K_m(M^{-1}s^{-1})
Substrate **II**	3.2	2.7	14127.3
Substrate **I**	69.4	16.5	3961.0

* ***HCV NS3/4A protease is incubated with the substrates in 50 mM Tris, pH 7.5, 30 mM DTT, 1% Chaps, 15% glycerol at room temperature.***

Conclusion

- We have developed a highly sensitive FRET substrate **II** for HCV NS3/4A protease assay which can be applied to high throughput screening of anti -HCV NS3/4A protease drugs.
- Substrate **II** has stronger absorption and emission intensity at longer wavelengths (490 nm/520 nm) compared to substrate **I**.
- Assays using substrate **II** exhibit lower background due to less auto-fluorescent interference from cell components and test compounds.
- QXL™520 is more water-soluble than Dabcyl. This property increases the solubility of substrate **II**.
- Substrate **II** provides better assay sensitivity. K_m is 21-fold lower than substrate **I**. Substrate **II** is 8 times more sensitive than substrate **I**, and can detect < 0.1 pmol of HCV NS3/4A protease.

Reference:

1. Wesley, A., and Alter, M.J. (2000) *Semin. Liver Dis.* **20**, 1-16.
2. Taliani, M., et al., (1996) *Anal. Biochem.* **240**, 60-67.