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Novel Blue- and Red-Shifted Internally Quenched Fluorogenic Substrates for Continuous Monitoring of SARS-CoV 3CL^{pro}

Pamela Hamill[1], Martin Richer[1], Derek Hudson[2], Hongyan Xu[3] and François Jean[1]

[1]Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, V6T 1Z3, Canada; [2]Biosearch Technologies Inc., Novato, CA, 94949-5750, USA; [3]GL Biochem Ltd., Shanghai, 201203, China

Introduction

The SARS coronavirus (SARS-CoV)-encoded main protease, 3CL^{pro} or M^{pro}, plays an essential role in the viral life cycle and is currently the main focus for the development of anti-coronaviral therapies. The genome of SARS-CoV is translated in the cytoplasm of infected cells to generate several large polyproteins. For viral replication to occur, these polyproteins must be proteolytically processed into individual proteins, a process that is mediated by two viral proteases; PL2^{pro} and 3CL^{pro} [1]. 3CL^{pro} is considered the main viral protease as it releases the key replicative proteins of the virus, including the viral RNA polymerase and helicase proteins. Since the emergence of SARS-CoV in late 2002, the development of sensitive assays to detect recombinant 3CL^{pro} activity in formats amenable to high-throughput screening (HTS) has been a priority for the generation of novel 3CL^{pro} inhibitors as anti-viral therapies. Here, we report our success in developing blue- and red-shifted internally quenched fluorogenic substrates (IQFSs) based on resonance energy transfer between the donor and acceptor pairs Abz/Tyr(3-NO_2) [IQFS-1], and CAL Fluor Red 610 (CalRed 610)/Black Hole Quencher-2 (BHQ-2) [IQFS-2], using a decapeptide sequence corresponding to that of the cleavage site between the non-structural protein (nsp)4/nsp5 in the SARS-CoV polyprotein pp1ab.

Fig. 1. Amino acid sequences of SARS-CoV 3CL^{pro} IQFSs (A) IQFS-1 and (B) IQFS-2. The chemical structures of the donor group o-aminobenzoyl (Abz) and acceptor groups 3-nitrotyrosine (3-NO_2) [2-4] and Black Hole Quencher-2 (BHQ-2) are shown.

Results and Discussion

SARS-CoV 3CL^{pro} quenched fluorescent substrate IQFS-1 (Fig. 1) was synthesized using Fmoc solid phase peptide chemistry [2-4]. The synthesis protocol for IQFS-2 will be presented elsewhere (Hamill P. *et al.,* manuscript in preparation). In both cases, peptide purity and composition were demonstrated by RP-HPLC, mass spectrometry and amino acid analysis. Following expression in *E. coli,* His-tagged SARS-CoV 3CL^{pro} was purified to > 90% homogeneity by nickel chromatography.

The ability of purified SARS-CoV 3CL^{pro} to cleave both IQFSs was then tested by using a continuous protease assay (Fig. 2A) and RP-HPLC assay (Fig. 2B and 2C).

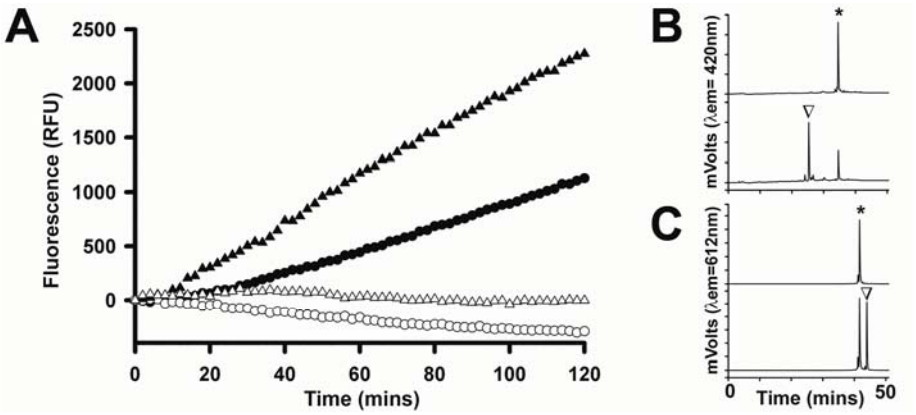

Fig. 2. Processing of blue- and red-shifted quenched fluorescent peptidyl substrates by recombinant SARS-CoV 3CL^{pro} (A) Continuous protease assay using a fluorescence plate reader [3-4]. IQFS-1 and IQFS-2 were added at concentrations of 36 μM or 18 μM, respectively, to assay reactions containing 500 nM of 3CL^{pro} in reaction buffer (50 mM Hepes, 100 mM NaCl, [pH 7.3], 10 mM DTT and 10 μg/well of BSA) at 30°C. The fluorescence emitted from reactions in the presence (closed symbols) or absence (open symbols) of 3CL^{pro} was measured using λex/λem of 320/420 nm or 584/612 nm for IQFS-1 (circles) and IQFS-2, (triangles) respectively. RP-HPLC analysis [4] of fluorescent peptide products present in reactions containing (B) IQFS-1 and (C) IQFS-2 in the absence (top panel) or presence (lower panel) of 3CL^{pro}. Intact IQFS (*) and N-terminal cleavage products (∇) are indicated.

Our results show that both the blue- and red-shifted IQFSs are sensitive substrates that are efficiently processed at low micromolar concentration by recombinant SARS-CoV 3CL^{pro} in our continuous assay. In addition, we demonstrate that both are cleaved in one position only following the glutamine residue, consistent with the known proteolytic specificity of SARS-CoV 3CL^{pro}. Our novel *in vitro* continuous assay system utilizing both blue- and red-shifted IQFS is advantageous since both the efficacy and mode of inhibition of putative 3CL^{pro} inhibitors can be determined. Moreover, our dual-substrate continuous 3CL^{pro} assay may offer advantages for HTS of small molecule natural-colored compounds, since the number of false positives resulting from non-specific interference with the fluorophore group may be reduced.

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