

## Development of fluorescent peptide substrates for improved monitoring of SARS-CoV-2 main protease 3CLpro activity based on active site specificity by degradomics approach

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SARS-CoV-2 main protease (3CLpro) is crucial for processing the viral polyprotein. While drug development targeting 3CLpro often focuses on the catalytic non-prime (P) side for specificity and potency, the significance of the prime (P') side in substrate specificity and drug development is often overlooked. We analyzed the P6–P6' specificity of 3CLpro by identifying over 800 cleavage sites using Proteomic Identification of Cleavage site Specificity (PICS). Cleavage typically occurred after the canonical P1-Gln and non-canonical P1-His and P1-Met residues, with a preference for Arg/Lys at P3 and His at P3'. Critical hydrogen bonds between the N-terminal Ser1 of protomer-B in 3CLpro dimers form with P1-His but not with P1-Met. However, cleavage at P1-Met456 in native MAP4K5 still occurs. Elevated reactive oxygen species during SARS-CoV-2 infection oxidize methionines. Molecular simulations showed that oxidized P1-Met (P1-MetOX) forms a hydrogen bond with Ser1, and strong positive cooperativity between P1-Met and P3'-His enhances peptide cleavage rates. The adaptable S3' subsite accommodates P3'-His, which stabilizes through backbone hydrogen bonds with Thr25, central to a "threonine trio" (Thr24-Thr25-Thr26) in the P'-binding domain I. Molecular docking simulations highlighted structure-activity relationships affecting 3CLpro-substrate interactions. These structural determinants were confirmed by MALDI-TOF-MS cleavage assays of P1'- and P3'-positional scanning peptide libraries with an internal positive control. These insights guided the design of two new, highly soluble 3CLpro quenched-fluorescent peptide substrates, resulting in a 15-fold improvement in sensitivity for FRET monitoring of 3CLpro activity compared to current assays. These findings should inform medicinal and chemical improvement of new small-molecule inhibitor compounds potent against SARS-CoV-2 3CL<sup>pro</sup>.

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