Design and construction of the HKU5 nsp5 expression plasmid

The DNA sequence encoding HKU5 nsp5 (3CLpro) with an N-terminal His_6-tag followed by the nsp4/5 cleavage site was codon optimized for optimal expression in *E.coli* and was synthesized by BioBasic. The gene was then sub-cloned into a pET-11a expression vector. This construct generates leads to auto cleavage of the nsp4/5 site during expression and produces the authentic HKU5 catalytic domain without any N-terminal or C-terminal extensions or deletions. The enzyme was expressed in *E.coli* BL21-DE3 cells through auto-induction in Super-LB medium with 100 µg/ml carbenicillin for 24 hours at 25°C. Cells were harvested by spinning at 5000 rpm for 20 min at 4°C.

Purification

Cells were resuspended in Buffer A (20 mM Tris pH-7.5, 0.05 mM EDTA, 10% glycerol and 5 mM β-mercaptoethanol (BME)) followed by lysis with one pass through a French-press at 1200 psi. Lysed cells were centrifuged at 29,000g rcf to separate cell debris from the cleared lysate. 1 M ammonium sulfate was added to the cleared lysate through gradual mixing at 4°C. The sample was then loaded onto a Phenyl Sepharose high-sub fast-flow column pre-equilibrated in Buffer B (50 mM Tris pH-7.5, 1 M ammonium sulfate, 0.05 mM EDTA, 10% glycerol and 5 mM BME). A gradient of 100 % Buffer A was used to elute the protein. Fractions containing HKU5 3CLpro were pooled and loaded onto a DEAE anion-exchange column after being dialyzed in Buffer A. DEAE column was pre-equilibrated in Buffer A. Gradient of 50 % Buffer C (50 mM Tris pH-7.5, 1 M sodium chloride, 0.05 mM EDTA, 10% glycerol and 5 mM BME) was used to elute the protein. Fractions containing HKU5 3CLpro, as judged by SDS-PAGE analysis and kinetic activity, were pooled and dialyzed in Buffer A. A Mono-Q anion-exchange column (pre-equilibrated in Buffer A) was used as a final chromatography step to obtain purified

HKU5 3CLpro, which eluted in the fractions from 50 % gradient of Buffer C. A final yield of 28 mg of pure HKU5 3CLpro protein was obtained from 1 liter of cell culture.

Purification statistics table

Sample	Protein (mg)	Total activity Units	Specific activity (Units/mg)	Fold purification	% Yield
Lysate	486	9407	19	1	100
Phenyl-Sepharose	84	4857	58	3	52
DEAE	37	4459	122	6	47
Mono-Q	28	3767	136	7	40

HKU5 3CLpro Kinetic and Inhibition assays

The enzymatic activity of HKU5 3CLpro was measured in an assay buffer containing 50 mM HEPES pH-7.5, 0.1 mg/ml BSA, 0.01% Triton X-100 and 1 mM DTT in a final reaction volume of 100 µl in a well of a 96-well plate. The HKU5 3CLpro peptide substrate used in the assay is as follows: Hilyte Fluor ™ -488-ESATLQSGLRKAK (QXL™520). This FRET-based substrate contains a HiLyte fluor ™ 488 fluorophore attached at the *N*-terminus of a canonical 3CLpro peptide substrate. The fluorophore is internally quenched by the QXL™520 dye. The kinetic activity was determined by following the increase in fluorescence over time on a BioTek Synergy H1 plate reader that resulted from cleavage of the FRET-based peptide substrate. The excitation wavelength used was 480 nm and emission wavelength was 540 nm. The rate of reaction in arbitrary fluorescence units (AFU) per sec, determined by measuring the initial slope of the progress curve, was converted to units of µM product/min using experimentally determined value of fluorescence extinction coefficient determined by the maximum change in fluorescence after the reaction was completed at a series of substrate concentrations.

For inhibition assays, the progress curves for cleavage of 1 μM substrate by 250 nM HKU5 3CLpro was determined in the presence of a range of GRL-001-13S concentrations (10 to 0 μM). Progress curve analysis was used to determine the values of inhibition constant (K_i) and inactivation rate constant (k_{inact}).