Supplemental data for

The Maturation Mechanism of SARS Coronavirus 3C-like Proteinase *

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Experimental Procedures

Plasmid construction of the large fusion proteins His-C-XX(Q/E)-3CLP-Y, His-C-XX-C145A-Y, 3CLP-Y-His and 3CLP-MBP-His- The process of plasmid construction was depicted in Fig. S1. For pET28a-CY2.0 construction, CFP and YFP were cloned into the vector pET28a in sequence after separately double-digestion by *Nco*I/*BamH*I and *EcoR*I/*Hind*III.. This N-terminal his-tagged pET28a-CY2.0 was then used as cloning vector to construct His-C-XX-C145A-Y and His-C-XX(Q/E)-3CLP-Y for protein purification *in vitro*. To construct His-C-XX-C145A-Y, DNA sequence of C145A with 8 extra amino acids SITSAVLQ at N-terminal was obtained by PCR using pET 21x C145A (1) as template with forward primer *BamH*I-8aa-3CLP-F and reverse primer 3CLP-*EcoR*I-R (Table S1). For His-C-XX(Q/E)-3CLP-Y construction, $3CL^{pro}$ with 8 extra amino acids SITSAVLE (here, Q was replaced by E to get XX(Q/E)) were also obtained by PCR using plasmid pET 3clp-21h (1) as template with primers Q-E-F and 3CLP-*EcoR*I-R (Table S1). All the PCR products were digested with *BamH*I/ *EcoR*I and inserted into the same double-digested vector pET28a-CY2.0.

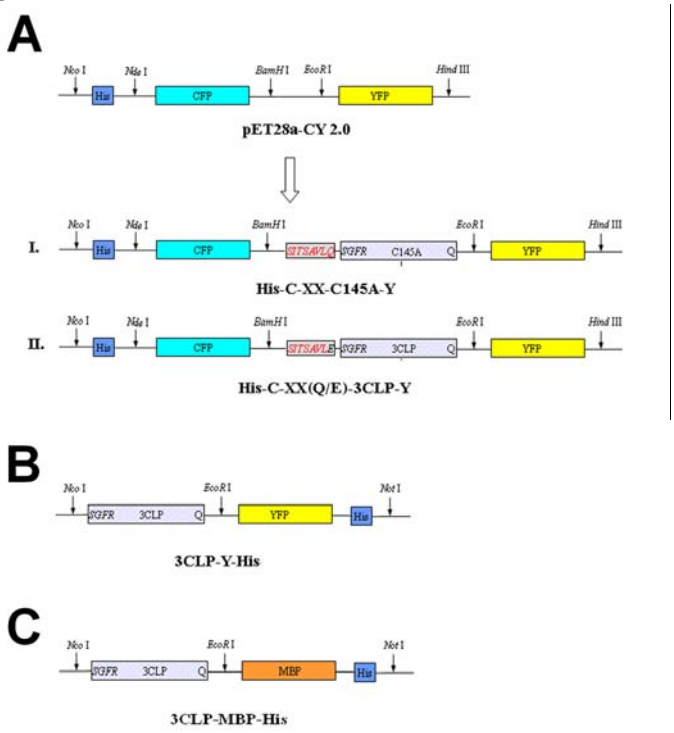

Figure S1. Schematics of plasmid construction. A) Plasmid frameworks of the large fusion proteins for purification; B) Plasmid framework of 3CLP-Y-His; C) Plasmid framework of 3CLP-MBP-His.

Table S1. Primers used for preparing the large fusion proteins

Primer	Oligonucleotide sequence (5'-3')	Polarity	Remark
*BamH*I-8aa-3CLP-F	5'-GGTATCGGATCCATTACGAGCGCAGTCCTGCAGAGCGGTTTTAGGAAAATGGC-3'	Forward	Introduce 8aa to N-terminal of 3clp and C145A,
Q-E-F	5'-GGTATCGGATCCATTACGAGCGCAGTCCTGGAGAGCGGTTTTAGGAAAATGGC-3'	Forward	Gln to Glu in 8aa
3CLP-*EcoR*I-R	5'-CCCTTGGAATTCGCACCGGTTTGGAAGGTAACACCAGAGC-3'	Reverse	Insert to pET28a CY2.0
*Nco*I-3CLP-F	5'-GATTCGCCATGGGCAGTGGTTTTAGGAAAATGGC-3'	Forward	Amplify
YFP-his-R(*Not*I)	5'-CCCTTGGCGGCCGCTTAGTGGTGGTGGTGGTGGTGCTTGTACAGCTCGTCC-3'	Reverse	Amplify
*EcoR*I -MBP-F	5'-CGGTGCGAATTCCGTCAAAATCGAAGAAGGTAAACTGGT-3'	Forward	Amplify
MBP-His-R(*Not*I)	5'-CGAGTGCGGCCGCTTAGTGGTGGTGGTGGTGGTGAGTCTGCGCGTCTTTCAGGGC-3'	Reverse	Amplify

3CLP-Y-His was constructed by PCR using His-C-XX(Q/E)-3CLP-Y as a template with the forward primer *Nco*I-3CLP-F and reverse primer YFP-his-R(*Not*I) (Table S1). The PCR product was digested with *Nco*I and *Not*I and then ligated into pET28a (also cut with *Nco*I and *Not*I). 3CLP-MBP-His was a plasmid in which the YFP of 3CLP-Y-His was replaced by maltose binding protein (MBP). The plasmid of 3CLP-Y-His was double-digested with *EcoR*I and *Not*I to remove the YFP fragment, and MBP was obtained by PCR with primers *EcoR*I -MBP-F and MBP-His-R(*Not*I) (Table S1) and double-digested with *EcoR*I and *Not*I. Two linear fragments were ligated together by T4 DNA ligase (Promega).

Fusion protein expression and purification- The plasmids of large polyproteins with 6×his tag were transformed into *E. coli* strain Rosetta <DE3> for protein expression and purification. Cells were grown at 37°C in 1 liter LB medium containing 30 μg/ml kanamycin and 34 μg/ml clarithromycin until the A_{600} reached 0.6-0.8, and then induced with 0.5 mM IPTG at 25°C for 4-5 hours. The cells were harvested by centrifugation at 6000 rpm for 10 min at 4°C and suspended in buffer B (20 mM PBS, PH 7.3, 500 mM NaCl, 20 mM imidazole) with 5 mM DTT at 2% of the original LB medium volume. Cell lysis was achieved by ultrasonication and then centrifuged at 17,000 rpm for 25min at 4°C. The supernatant was filtered, applied to a nickel-nitrilotriacetic acid column (GE Healthcare),

and equilibrated with buffer B. Then the protein bound to the resin was eluted with the gradient of 1-80% buffer C (20 mM PBS, pH 7.3, 500 mM NaCl, 500 mM imidazole). Fresh DTT (5 mM) was added to the eluted protein and concentrated by centrifugation. The concentrated protein was loaded on a gel filtration column (Sephacryl S-200 HR; GE Healthcare) that had been pre-equilibrated with 180 ml buffer A (40 mM PBS, pH 7.3, 100 mM NaCl, 1 mM EDTA). After elution with another 180 ml buffer A, 95% purified protein was received and fresh DTT (5 mM) was immediately added to the fractions containing the purified proteins. The molecular weights of the proteins were confirmed by SDS/PAGE on a 10% (w/v) polyacrylamide gel.

RESULTS

Analytic gel filtration analysis. Both His-C-XX(Q/E)-3CLP-Y and His-C-XX-C145A-Y, showed high FRET efficiency (Fig. S2), indicating that they were intact.

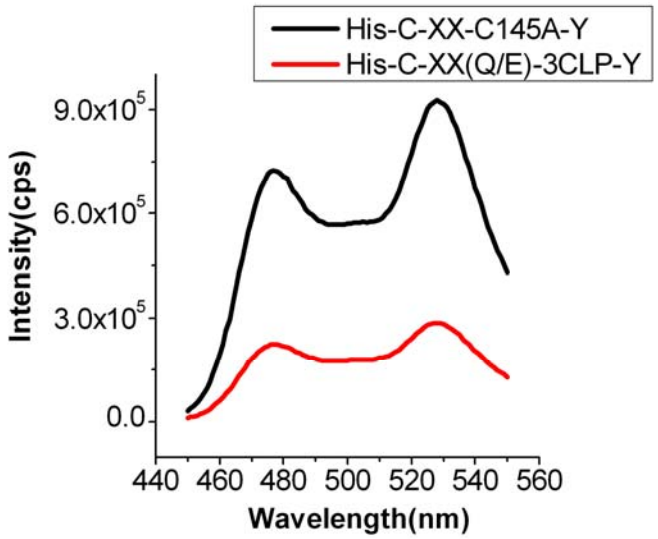

Figure S2. The FRET spectrum of 7.5 μM His-C-XX(Q/E)-3CLP-Y and 22.5 μM His-C-XX-C145A-Y.

Five gel filtration molecular weight markers (Table S2) were applied to a Superdex 200 HR 10/300 GL column (GE Health) and a standard curve was fitted by plotting the retention volume (x-axis) against the logarithm of the molecular weight (y-axis) (Fig. S3). Two purified polyproteins, His-C-XX(Q/E)-3CLP-Y and His-C-XX-C145A-Y, were also loaded to the column to estimate their apparent molecular weight. For both of the fusion proteins, only one peak appeared with almost the same retention volume at a concentration of 4.4 mg/ml or 10 mg/ml (Fig. S3). The apparent molecular weights of both polyproteins deduced from the standard curve mentioned above were about 90 kDa, corresponding to theoretical molecular weight (Table S3).

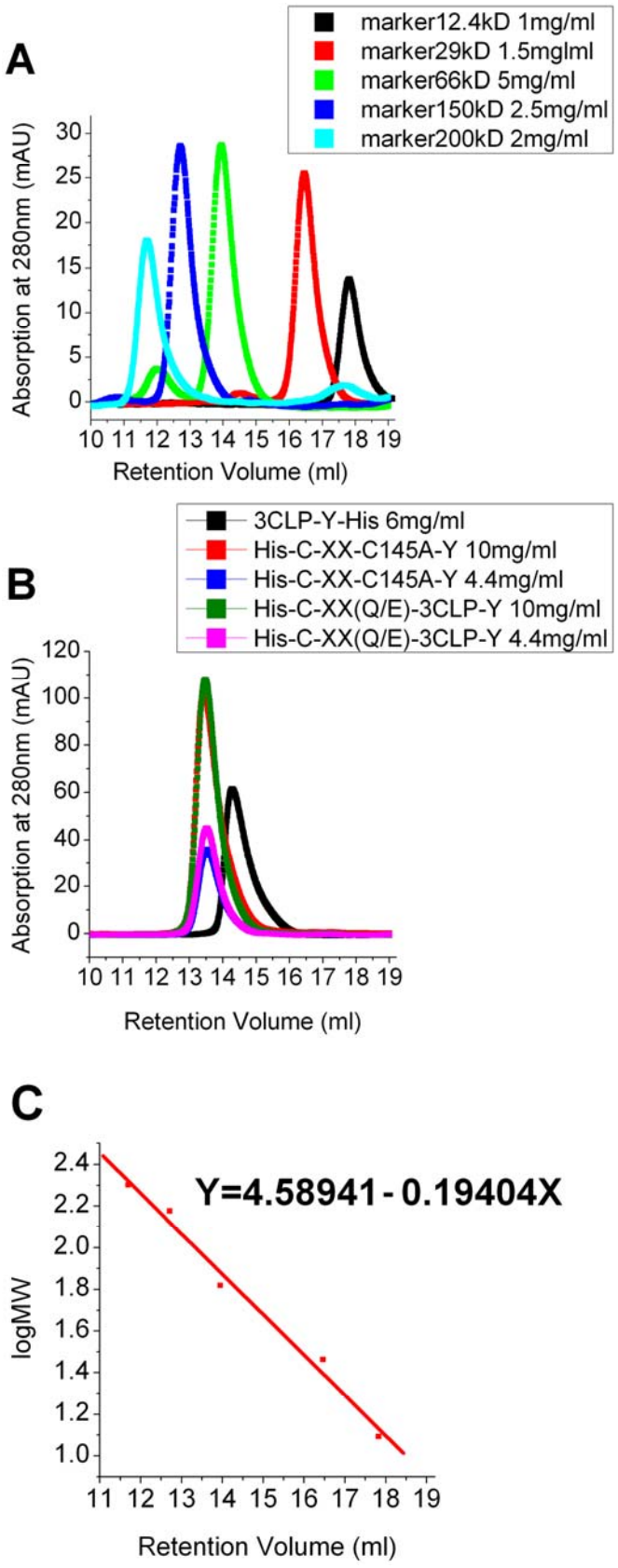

Figure S3. Analytic gel filtration profile of A) the standard protein molecular weight markers; B) the polyproteins at different concentrations; C) standard curve calculated by plotting the logarithm of standard protein molecular weight markers versus the retention volume.

Table S2. Retention volume of all gel filtration molecular weight markers

Name of marker	MW (kDa)	Log MW	Retention Volume (ml)
β-amylase	200	2.301	11.691
alcohol dehydrogenase	150	2.176	12.715
albumin from bovine serum	66	1.819	13.952
carbonic anhydrase	29	1.462	16.465
cytochrome c	12.4	1.093	17.822

Table S3. Retention volumes of the polyproteins at different concentrations and their putative molecular weights (MW) deduced from the standard calculated curve.

Concentration of polyprotein	Retention volume (ml)	Putative MW (kDa)	Theoretical MW (kDa)
His-C-XX-C145A-Y 10 mg/ml	13.443	95.704	91.163
His-C-XX-C145A-Y 4.4 mg/ml	13.538	91.727	
His-C-XX(Q/E)-3CLP-Y 10 mg/ml	13.478	94.219	91.163
His-C-XX(Q/E)-3CLP-Y 4.4 mg/ml	13.519	92.509	
3CLP-Y-His 6 mg/ml	14.278	65.903	62.125

Analytic ultracentrifugation analysis. Sedimentation coefficient of His-C-XX(Q/E)-3CLP-Y and His-C-XX-C145A-Y were listed in Table S4. Based on the c (M) distribution model (Fig. S4), the molecular masses of His-C-XX(Q/E)-3CLP-Y and His-C-XX-C145A-Y were estimated to be 82.7 kDa and 86.0 kDa (Table S4), indicating both of them were monomeric.

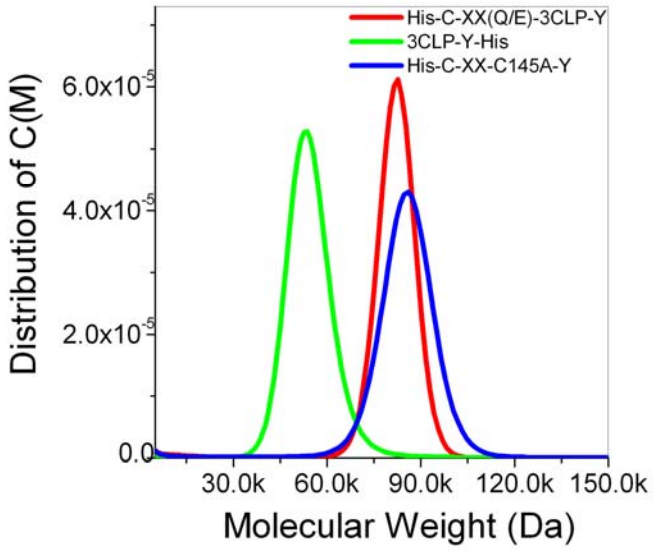

Figure S4. Molecular weight distribution c(M) profile of His-C-XX(Q/E)-3CLP-Y,

His-C-XX-C145A-Y, 3CLP-Y-His at 40,000 rpm and 20℃

Table S4. The sedimentation coefficient (S) and molecular masses (Da) of the fusion proteins.

Fusion protein	Sedimentation Coefficient (S)	Molecular masses (Da)
His-C-XX(Q/E)-3CLP-Y	4.93	82735.1
His-C-XX-C145A-Y	4.98	85999.2
3CLP-Y-His	4.08	53561.5

Inhibition efficiency of an isatin inhibitor on His-Y-XX(Q/E)-3CLP-Y. The inhibition of **5f** on the enzymatic activity of His-Y-XX(Q/E)-3CLP-Y at 50 μM show in Fig. S5 and Table S5, implying that the substrate binding pocket conformation of $3CL^{pro}$ in the polyprotein is similar to that in the free $3CL^{pro}$.

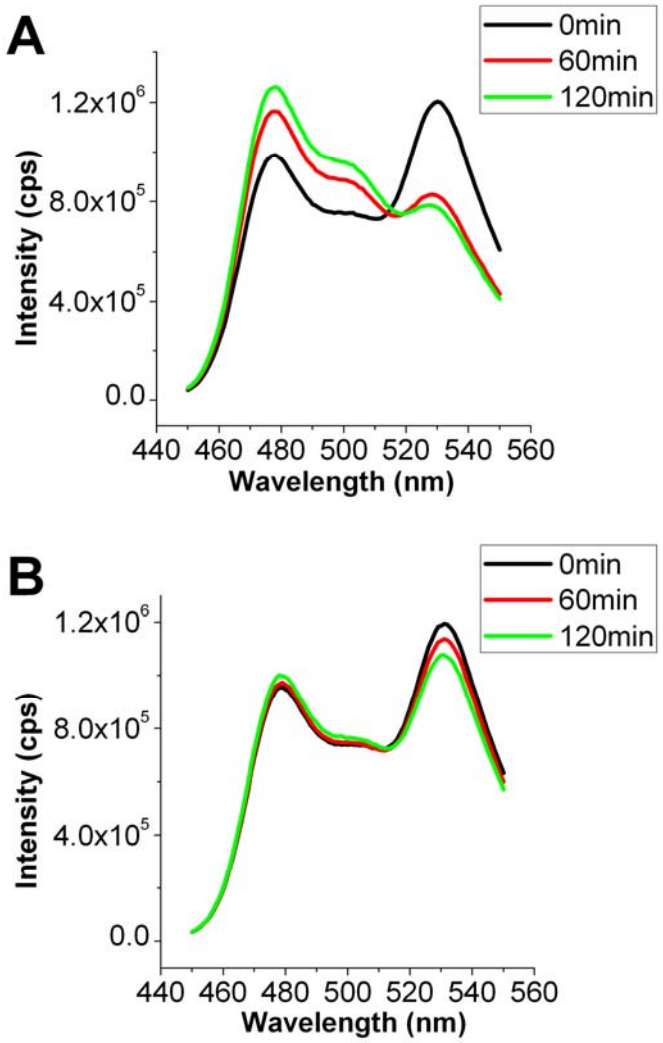

Figure S5. The inhibition activity of **5f** against enzyme His-Y-XX(Q/E)-3CLP-Y (His-C-XX-C145A-Y was served as the substrate). A) Control assay: the enzymatic activity of His-Y-XX(Q/E)-3CLP-Y in buffer A with 5% DMSO. B) Inhibition assay: the inhibition activity of 50 μM **5f** against His-Y-XX(Q/E)-3CLP-Y in buffer A with 5% DMSO.

Table S5. Value of peak 527nm/475nm of control and inhibition assay

	Value of peak 527nm/475nm		
	0min	60min	120min
Control assay	1.22	0.71	0.62
Inhibition assay	1.25	1.17	1.08

1. Huang, C., Wei, P., Fan, K., Liu, Y., and Lai, L. (2004) *Biochemistry* **43**(15), 4568-4574