

Video Article

Quantitative FRET (Förster Resonance Energy Transfer) Analysis for SENP1 Protease Kinetics Determination

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Abstract

Reversible posttranslational modifications of proteins with ubiquitin or ubiquitin-like proteins (UBIs) are widely used to dynamically regulate protein activity and have diverse roles in many biological processes. For example, SUMO covalently modifies a large number of proteins with important roles in many cellular processes, including cell-cycle regulation, cell survival and death, DNA damage response, and stress response 1-5. SENP, as SUMO-specific protease, functions as an endopeptidase in the maturation of SUMO precursors or as an isopeptidase to remove SUMO from its target proteins and refresh the SUMOylation cycle ^{1,3,6,7}.

The catalytic efficiency or specificity of an enzyme is best characterized by the ratio of the kinetic constants, k_{cat}/K_M . In several studies, the kinetic parameters of SUMO-SENP pairs have been determined by various methods, including polyacrylamide gel-based western-blot, radioactive-labeled substrate, fluorescent compound or protein labeled substrate ⁸⁻¹³. However, the polyacrylamide-gel-based techniques, which used the "native" proteins but are laborious and technically demanding, that do not readily lend themselves to detailed quantitative analysis. The obtained k_{cat}/K_M from studies using tetrapeptides or proteins with an ACC (7-amino-4-carbamoylmethylcoumarin) or AMC (7-amino-4-methylcoumarin) fluorophore were either up to two orders of magnitude lower than the natural substrates or cannot clearly differentiate the iso- and endopeptidase activities of SENPs.

Recently, FRET-based protease assays were used to study the deubiquitinating enzymes (DUBs) or SENPs with the FRET pair of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) ^{9,10,14,15}. The ratio of acceptor emission to donor emission was used as the quantitative parameter for FRET signal monitor for protease activity determination. However, this method ignored signal cross-contaminations at the acceptor and donor emission wavelengths by acceptor and donor self-fluorescence and thus was not accurate.

We developed a novel highly sensitive and quantitative FRET-based protease assay for determining the kinetic parameters of pre-SUMO1 maturation by SENP1. An engineered FRET pair CyPet and YPet with significantly improved FRET efficiency and fluorescence quantum yield, were used to generate the CyPet-(pre-SUMO1)-YPet substrate ¹⁶. We differentiated and quantified absolute fluorescence signals contributed by the donor and acceptor and FRET at the acceptor and emission wavelengths, respectively. The value of k_{cat}/K_M was obtained as $(3.2 \pm 0.55) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ of SENP1 toward pre-SUMO1, which is in agreement with general enzymatic kinetic parameters. Therefore, this methodology is valid and can be used as a general approach to characterize other proteases as well.

Video Link

The video component of this article can be found at <https://www.jove.com/video/4430/>

Protocol

1. Plasmid Constructs

1. Amplify the open reading frames of the genes by PCR, and clone the PCR products into PCRII-TOPO vector.
2. Confirm the products by sequencing, and clone the cDNA encoding CyPet-(pre-SUMO1)-YPet, CyPet-SUMO1, YPet and catalytic domains of SENP1 into the pET28 (b) vector with an N-terminal hexahistidine tag.

2. Protein Expression and Purification

1. Transform *Escherichia coli* cells of strain BL21 (DE3) with pET28 (b) vectors encoding CyPet-(pre-SUMO1)-YPet, CyPet-SUMO1, YPet and the catalytic domains of SENP1.
2. Grow the transformed bacteria in 2xYT medium for 3 hr at 37 °C (shaking at 250 rpm) to reach an optical density of 0.5 at 600 nm.
3. Add 100 μM isopropyl- β -D-thiogalactoside (IPTG) (final concentration) to induce protein expression and shake for 16 hr at 25 °C at 200 rpm.

- Harvest the bacteria by centrifugation at 10,000 rpm at 4 °C for 5 min and resuspend them in a buffer of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 5 mM imidazole.
- Sonicate the cells for 10 min in 5-sec intervals at a power setting of 25 W using MiSonics 4,000 sonicator and collect them by centrifugation at 35,000 x g at 4 °C for 30 min.
- Set up the column with 500 ml Ni-NTA beads for 1 L culture and transfer the supernatant into the column.
- Wash the resin with buffer of 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 10 mM imidazole twice.
- Elute protein with buffer of 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 500 mM imidazole and dialysis into a buffer of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl and 1 mM dithiothreitol (DTT) at 4 °C overnight.
- Determine the concentrations of the purified proteins by the Bradford assay. Alternative method for protein concentration measurements will be SDS-PAGE gel electrophoresis and then stained with Coomassie blue followed with imaging quantitative.

3. Quantitative FRET Spectrum Analysis

- The general strategy for the assay was based on FRET signaling (**Figure 1**). The FRET pair, CyPet and YPet, was tagged to the N- and C-termini, respectively, of pre-SUMO1. SENP1 cleaves the fusion protein CyPet-(pre-SUMO1)-YPet at the Gly-Gly site in SUMO1's C-terminus and, thus, releases the SUMO tail with YPet. The FRET signal is disrupted, resulting in an increase of the emission from CyPet and a dramatic decrease of YPet's emission at the CyPet excitation wavelength.
- When excited by light of wavelength 414 nm, the total fluorescence emission of CyPet-(pre-SUMO1)-YPet at 530 nm can be derived from three sources: the absolute FRET-induced YPet's emission, CyPet direct emission and YPet direct emission (**Figure 2**).

$$FL_{530/414} = FL_{FRET} + FL_{CyPet(cont)} + FL_{YPet(cont)}$$

where $FL_{530/414}$ is the total fluorescence emission at 530 nm when excited at 414 nm, FL_{FRET} is the absolute FRET signal, $FL_{CyPet(cont)}$ is the CyPet direct emission when excited at 414 nm, and $FL_{YPet(cont)}$ is the YPet direct emission when excited at 414 nm. The subscript of (cont) stands for contribution.

- The direct emission of CyPet at 530 nm was proportional to its emission at 475 nm when excited at 414 nm with a constant ratio of α . CyPet-SUMO1 was prepared at concentrations of 50, 100, 200, 500, and 750 nM and 1 mM, and emissions at 475 and 530 nm were measured after excitation at 414 nm to determine α (**Figure 3-1**).
- The direct emission of YPet at 530 nm under excitation at 414 nm was proportional to its emission at 530 nm when excited at 475 nm with a constant ratio of β . YPet was prepared at concentrations of 50, 100, 200, 500, 750 and 1,000 nM, and the emission at 530 nm was measured when the samples were excited by wavelengths of 414 and 475 nm to determine β (**Figure 3-2**).
- When CyPet-(pre-SUMO1)-YPet was digested by SENP1, the cleavage released CyPet-SUMO1 and the SUMO1 tail with YPet. When the compound was excited at 414 nm, the fluorescence emission at 530 nm ($FL'_{530/414}$) was decreased but can still be divided into three parts as:

$$FL'_{530/414} = FL'_{FRET} + \alpha FL'_{CyPet(475/414)} + \beta FL_{YPet(530/475)}$$

where $FL'_{530/414}$ is the total fluorescence emission at 530 nm after digestion when excited at 414 nm, FL'_{FRET} is the remaining absolute FRET signal, $FL'_{CyPet(475/414)}$ is the CyPet emission at 475 nm after digestion when excited at 414 nm (here the CyPet emission is from two parts: undigested CyPet-(pre-SUMO1)-YPet and digested CyPet-SUMO1), and $FL_{YPet(530/475)}$ is the YPet emission when excited at 475 nm, which is constant whether CyPet-(pre-SUMO1)-YPet is digested or not.

- After digestion by SENP1, the remaining FRET emission (FL'_{FRET}) is:

$$FL'_{FRET} = \frac{C-x}{C} (FL_{530/414} - \alpha FL_{CyPet(475/414)} - \beta FL_{YPet(530/475)})$$

where C is the total concentration of CyPet-(pre-SUMO1)-YPet and x is the concentration of digested CyPet-(pre-SUMO1)-YPet.

- By combining all of the items, the detected fluorescence emission at 530 nm under excitation of 414 nm ($FL'_{530/414}$) is:

$$FL'_{530/414} = \frac{C-x}{C} (FL_{530/414} - \alpha FL_{CyPet(475/414)} - \beta FL_{YPet(530/475)}) + \alpha FL'_{CyPet(475/414)} + \beta FL_{YPet(530/475)}$$

4. FRET-based Protease Assay for Enzyme Kinetic Study

- CyPet-(pre-SUMO1)-YPet was incubated with the catalytic domain of SENP1 at 37 °C in a buffer of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1% (v/v) Tween-20 and 1 mM DTT to a total volume of 80 ml and transferred into 384-well plate.
- Runs were conducted by measuring the fluorescence emission at 475 and 530 nm after an excitation at 414 nm in a fluorescence multiwell plate reader for 5 min with 15-sec intervals.
- The reaction rate (v) was correlated with the change in the amount of substrate (S) as:

$$v = -\frac{d[S]}{dt} = \frac{d[P]}{dt}$$

- The concentration of product increased exponentially from 0 as $[S]_0$ (original substrate concentration) when $t=0$:

$$[P] = [S]_0(1 - e^{-kt})$$

5. When $t=0$, the original velocity (V_0) is:

$$V_0 = \frac{d[P]}{dt} = k[S]_0$$

- The concentration of SENP1 was fixed, and the concentration of CyPet-(pre-SUMO1)-YPet was increased from 100 times more than the concentration of enzyme, which is required by Michaelis-Menten equation.
- All of the fluorescent readings were analyzed by the quantitative FRET analysis method and plotted in GraphPad Prism V Software to fit the Michaelis-Menten equation. The nonlinear regression can also be performed with the aid of more common software packages like spreadsheet programs (such as Microsoft Excel).

5. Representative Results

Maturation of pre-SUMO1 by SENP1 can be determined by monitoring the changes in the fluorescence signal at 475 and 530 nm during the process. The result showed that the velocity of pre-SUMO1 digestion by SENP1 in a substrate-dose dependent manner (**Figure 4**). This suggests that the catalytic domain of SENP1 exhibits excellent activity for pre-SUMO1's maturation. The initial reaction velocities were calculated by the above analysis with different substrate concentrations (**Table 1**).

The k_{cat}/K_M ratio is generally used to compare the efficiencies of different enzymes with one substrate or a particular enzyme with different substrates. K_M and V_{max} can be obtained from the Michaelis-Menten equation by plotting the various initial velocities, corresponding to the different concentrations of CyPet-(pre-SUMO1)-YPet (**Figure 5**). k_{cat} was obtained as:

$$k_{cat} = \frac{V_{max}}{[E]}$$

According to the above analysis, the calculated K_M was $0.21 \pm 0.04 \mu\text{M}$, the k_{cat} was $6.90 \pm 0.28 \text{ s}^{-1}$, and the k_{cat}/K_M ratio was $(3.2 \pm 0.55) \times 10^7 \text{ M}^{-1}\text{s}^{-1}$.

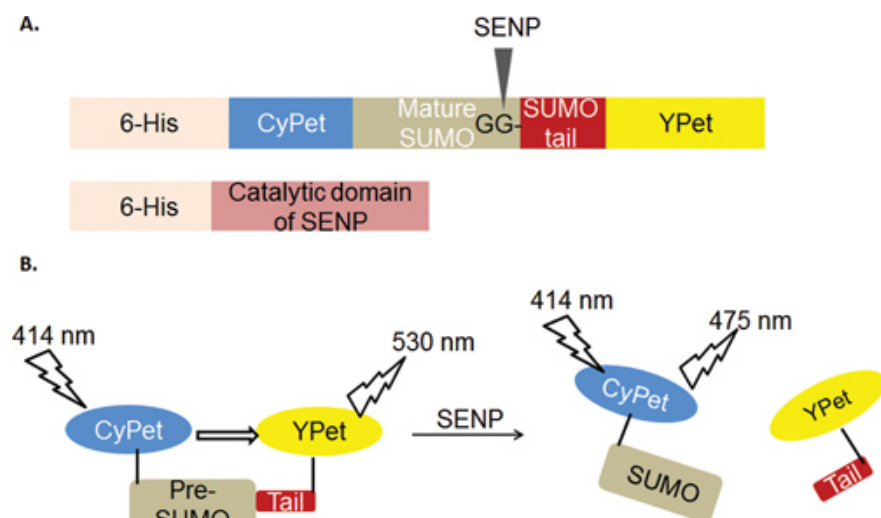


Figure 1. Graph of FRET-based protease assay for SENP's pre-SUMOs maturation.

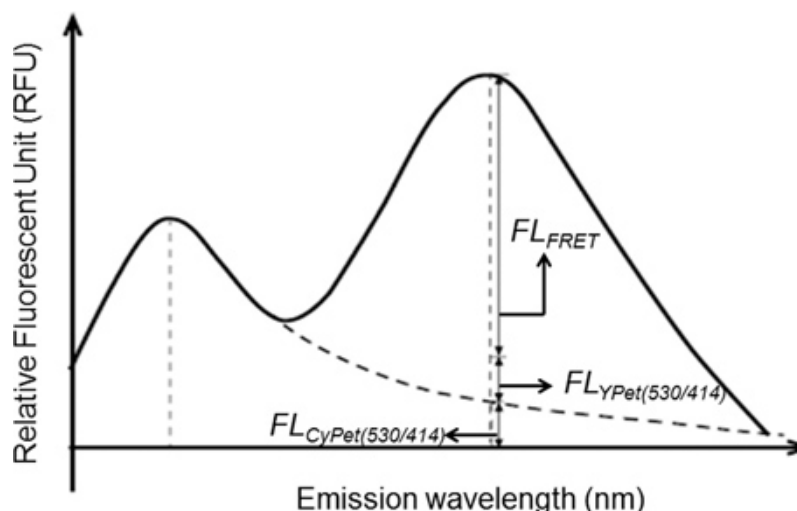


Figure 2. Quantitative analysis of fluorescent signal as contributions by the donor, acceptor and FRET. Dissection of emission spectra from CyPet-(pre-SUMO1)-YPet under excitation at 414 nm. $FL_{CyPet(530/414)}$ is CyPet's emission at 530 nm under excitation of 414 nm, FL_{FRET} is the FRET-induced YPet emission at 530 nm under excitation of 414 nm, and $FL_{YPet(530/414)}$ is YPet's emission at 530 nm under excitation of 414 nm.

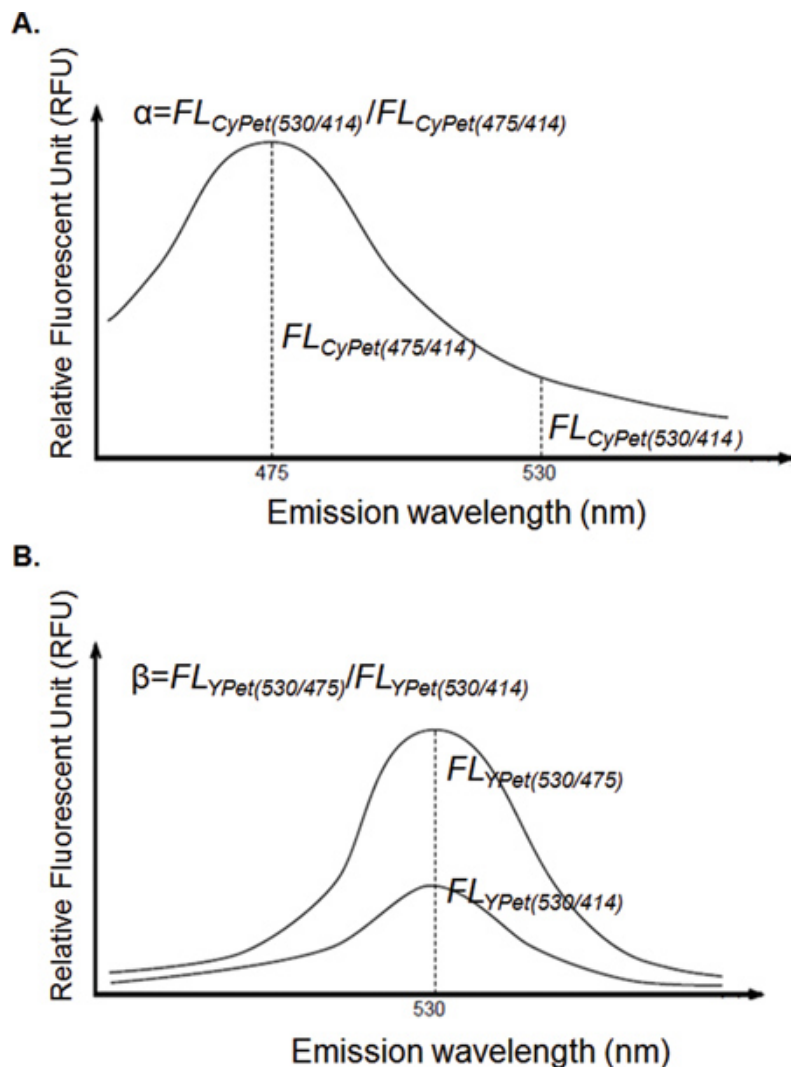


Figure 3. Calculation of direction emission factor α and β .

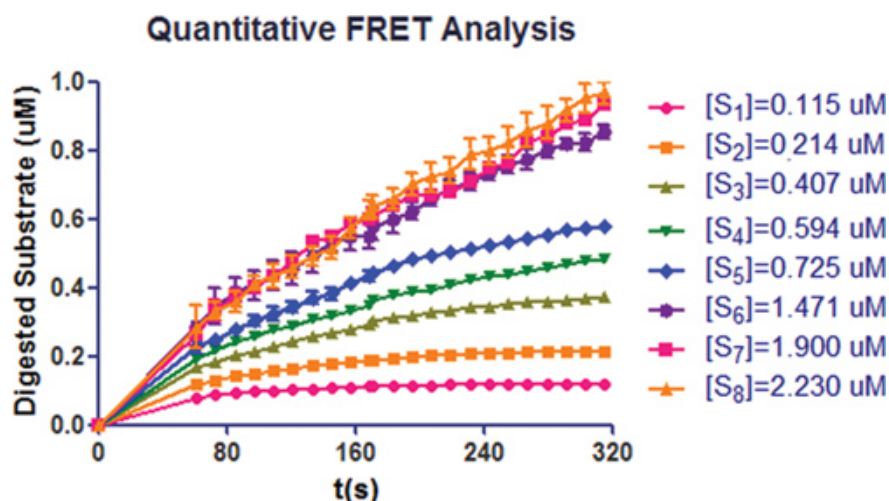


Figure 4. Quantitative analysis of CyPet-(pre-SUMO1)-YPet digested by different ratios of the catalytic domain of SENP1. Reactions were monitored within the first 5 min.

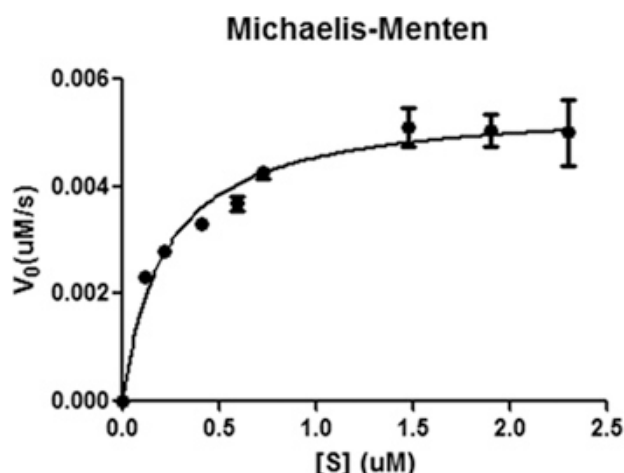


Figure 5. Michaelis-Menten graphical analysis of CyPet-(pre-SUMO1)-YPet's digestion by SENP1. Data were plotted and analyzed by GraphPad Prism V and nonlinear regression.

[S](μM)	$V_0(\mu\text{M/s})$
0.115	0.0023 \pm 0.00005
0.214	0.0028 \pm 0.00004
0.407	0.0033 \pm 0.00007
0.594	0.0037 \pm 0.00013
0.725	0.0043 \pm 0.00012
1.471	0.0051 \pm 0.00036
1.899	0.0050 \pm 0.00031
2.300	0.0050 \pm 0.00062

Table 1. Initial velocities determination of pre-SUMO1's maturation by SENP1. In each substrate concentration, four samples were used to measure the digestion. The standard deviation came from the variations of these four samples.

$K_M(\mu\text{M})$	$k_{cat}(s^{-1})$	$k_{cat}/K_M(M^{-1}\cdot s^{-1})$
0.21 \pm 0.04	6.90 \pm 0.28	3.2 \pm 0.55 $\times 10^7$

Table 2. Kinetic parameters of pre-SUMO1's maturation by SENP1 by quantitative FRET analysis. The standard deviation came from the four samples in each substrate concentration.

Discussion

FRET technology has been used to study pre-SUMO1's maturation by SENP1⁹. CFP-YFP was used as the FRET pair and ratiometric analysis, which is the ratio of acceptor to donor emissions, was used to characterize the kinetic properties. However, there is no consideration of donor and acceptor self-fluorescence in the traditional ratiometric FRET analysis. The ratio does not directly correlate with the amount of digested substrate.

Here we report a developed highly sensitive FRET-based protease assay to study the kinetic of pre-SUMO1's maturation by SENP1. In contrast to the previous ratiometric approach, we fundamentally improved the method with a new theory of FRET signal for kinetic analysis and an experimental procedure to derive kinetic parameters by determining the quantitative contributions of self-fluorescence from donor and acceptor, and the real FRET-induced acceptor's emission. Ratiometric analysis cannot do this. The ignorance of self-fluorescent emissions of donor and acceptor may lead to an overestimation of the FRET signal and the donor's emission. The overestimations might not greatly affect the final k_{cat}/K_M ratio ($3.81 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for the ratiometric analysis, $3.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ by our quantitative FRET analysis), but the effect is more obvious when studying the individual parameters, K_M (0.098 vs 0.21 μM) and k_{cat} (3.43 s^{-1} vs 6.90 s^{-1}), which are important in determining the rate-limiting step and inhibitor potency of enzymes.

The method we report here is a one-step assay of protease kinetics parameters and requires only molecular cloning and protein expression without radioactive labeling or expensive instruments. The one-step procedure not only simplifies the experimental procedure but also limits a lot of variations. The fluorescent-tagged proteins are in the aqueous phase, which is typically similar to their natural environment in cells. Fluorescence intensity can be determined by general fluorescence spectroscopy or fluorescence plate readers, which are widely available. Compared with the traditional "gel-based" method, our FRET-based protease assay offers several advantages, including increased sensitivity, real-time measurement, and less time and labor needed. Furthermore, the methodology and procedure of protease kinetics parameter determinations are environmentally friendly and non-hazardous materials, such as radioisotopes or harsh chemicals. In addition, the highly sensitive FRET-based assay can be used in high-throughput biological assays, such as protease inhibitor screenings. The kinetic study can also be used to characterize the properties of the inhibitors (e.g. K_i , IC_{50}).

Therefore, the highly sensitive quantitative FRET-based protease assays could be a powerful approach in developing genome-wide protease-substrate profiling and inhibitor screenings.

Disclosures

No conflicts of interest declared.

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