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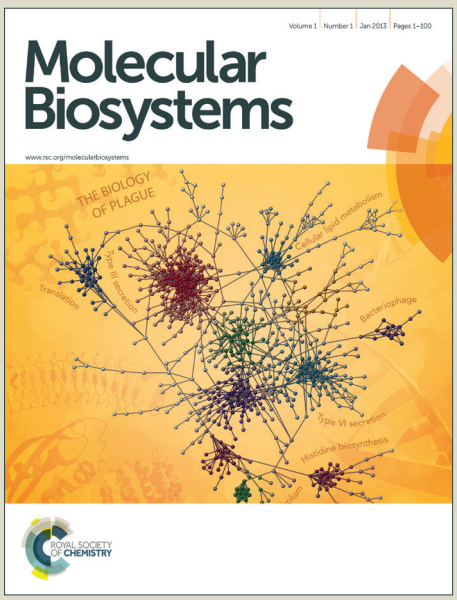

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A Novel Robust Quantitative Förster Resonance Energy Transfer Assay for Protease SENP2 Kinetics Determination Against its all Natural Substrates

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Short Title: SENP Protease Kinetics Determinations by Quantitative FRET Assay in Single Solution

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Abstract

SUMOylation (the process of adding SUMO [small ubiquitin-like modifier] to substrates) is an important post-translational modification of critical proteins in multiple processes. Sentrin/SUMO-specific proteases (SENPs) act as endopeptidases to process pre-SUMO or as isopeptidases to deconjugate SUMO from its substrate. Determining the kinetics of SENPs is important for understanding their activities. Förster resonance energy transfer (FRET) technology has been widely used in biomedical research and is a powerful tool for elucidating protein interactions. In this paper we report a novel quantitative FRET-based protease assay for SENP2 endopeptidase activity that accounts for the self-fluorescent emissions of donor (CyPet) and acceptor (YPet). The kinetic parameters, k_{cat}, K_M, and catalytic efficiency (k_{cat}/K_M) of catalytic domain SENP2 toward pre-SUMO1/2/3, were obtained by this novel design. Although we use SENP2 to demonstrate our method, the general principles of this quantitative FRET-based protease kinetic determination can be readily applied to other proteases.

Abbreviations and symbols

FRET: Förster Resonance Energy Transfer

SUMO: Small Ubiquitin-like modifier

SENP: Sentrin/SUMO-specific proteases

ACC: 7-amino-r-carbamoylmethylcoumarin

CFP: Cyan fluorescent protein

YFP: Yellow fluorescent protein

Introduction

Proteases are one of the most important enzyme classes in various signaling pathways, including proliferation and apoptosis, cellular signal transductions, protein maturation and trafficking, and are involved in many human diseases, ranging from cardiovascular disorders, autoimmune diseases, metabolic diseases to cancers [1,2]. The accuracy of proteases' kinetic parameters is not only important for understanding protease activity in normal physiological processes but also critical in drug discovery and development in estimating inhibitor potency and efficacy. In this paper we focus on the kinetics determination for Sentrin/SUMO-specific proteases (SENPs).

SUMO (small ubiquitin-like modifier) covalently modifies and regulates the activities of proteins with important roles in diverse cellular processes, including regulation of cell cycle, cell survival and death, DNA damage responses, and stress responses [3-7]. Mammalian cells usually express four SUMO paralogues (SUMO1–4) [8-11]. Like ubiquitination, SUMO conjugation occurs through a cascade of reactions by an activating enzyme (E1), a conjugating enzyme (E2) and, usually, a SUMO ligase (E3). The SENPs perform two critical functions via an encoded cysteinyl proteinase activity. The first one involves proteolysis of SUMO C-terminal amino acid residues to release a mature form of the SUMO terminated with a di-Gly motif, the only known form of SUMO that can be activated and conjugated to other proteins. The second protease activity catalyzes SUMO deconjugation from the target protein, releasing the target lysine and SUMO [12]. SENPs participate in diverse biological pathways, including transcriptional regulation, development, cell growth and differentiation, cancer, and ribosome biogenesis [13]. Different SENPs demonstrate various specificities toward SUMO substrates. In particular, SENP1 and SENP2 process all three SUMO isoforms. SENP2 prefers pre-SUMO2 more than the SUMO1 precursor while SENP1 cleaves the SUMO1 precursor more efficiently than the SUMO2 precursor [14].

Recently Förster resonance energy transfer (FRET) has been widely used in biological and biomedical research, including cell biology, medical diagnostics, optical imaging and drug discovery [15-18]. FRET occurs when the donor fluorophore and acceptor fluorophore are close to each other (1–10 nm) with favorable orientations. Excitation of the donor elicits an energy transfer that induces emission from the acceptor and results in the quenching of the donor and excitation of the acceptor. Fluorescent proteins are being increasingly used in FRET

systems due to the ease of genetic labeling. FRET-based protease assays have been used to study the de-ubiquitinating enzymes (DUBs) or SENPs [19-23]. However, these assays have significant shortcomings. First, the low FRET efficiencies of the fluorescent proteins and the complexity of fluorescent emissions of the donor and acceptor limit their reliability and sensitivity. Second, the ratio of acceptor emission to donor emission, which is always used as the quantitative characterization for protease activities and kinetics [21,22], is not an accurate measurement for FRET analysis because there is no consideration of the donor and acceptor's self-fluorescence. Therefore, this ratiometric analysis is not directly correlated with the amount of digested substrate. So far various approaches have tried to correct the FRET signal contamination from the donor and acceptor, including the "three-cube FRET" fluorescence microscopy/spectroscopy. However, none of them has been completely successful.

Here we report a novel quantitative FRET-based protease assay which is highly sensitive for the study of SENP2 endopeptidase kinetics. In our proposed assay, an engineered FRET pair, CyPet and YPet, with significantly improved FRET efficiency and fluorescent quantum yield [24], was used to generate the CyPet-(pre-SUMO1/2/3)-YPet substrate. The absolute fluorescent signals contributed by the donor, the acceptor and FRET at the acceptor's emission wavelengths were then differentiated and quantified. Unlike previous FRET methods [25], we used the real-time fluorescence at both the donor and acceptor's emission wavelength instead of creating standard curves. Our method yields k_{cat}/K_M values for SENP2 that agree with other methods. More importantly, our methodology can be also used as a general approach to characterize other proteases.

Materials and methods

Designing a sensitive FRET-based protease assay for SENP2 endopeptidase activity

The general strategy for the assay is based on FRET and fluorescent protein–tagged substrates (Fig. 1A). More specifically, Pre-SUMO1/2/3 is flanked by a FRET pair CyPet (donor) and YPet (acceptor), which has an energy transfer 20-fold more efficient than that of CFP and YFP, to yield a high dynamic range and sensitivity for the assay [24]. When CyPet–(pre-SUMO1/2/3)–YPet is mixed with SENP2, it is cleaved by the protease to yield two products: the CyPet–SUMO/1/2/3 and the SUMO tail with YPet. Therefore, FRET signaling will be disrupted, resulting in an increase of CyPet's emission and a dramatic decrease of YPet's

emission when CyPet is excited. The change of fluorescent emission after the cleavage can be used to characterize kinetic properties of SENP2 in real time.

To test the sensitivity and dynamic of the developed FRET-based protease assays, we incubated CyPet-(pre-SUMO2)-YPet with the catalytic domain of SENP2 (8 μM and 0.6 nM, respectively) at 37°C in a low-salt Tris buffer. The emission spectrum under excitation at 414 nm was monitored every 2–5 min until the substrate was totally processed (Fig. 1C). The result shows that CyPet's emission increases and YPet's emission decreases (disruption of FRET) when the pre-SUMO2 is matured by SENP2. The catalytic domain of SENP2 shows excellent activity even at a 13,333:1 ratio of substrate to enzyme.

Quantitative FRET analysis and determination of FRET signal

Because the emission of acceptor at 475nm when exited at 441nm can be neglected(less than 0.1% as compared with the donor emission when excited at 441nm, data not shown), therefore, the contribution of acceptor at 475n,m when excited at 441nm is not considered in the following FRET assay. We first define the cross-talk ratio of CyPet's self-fluorescence (α) as the ratio of CyPet-SUMO1/2/3's emissions at 530 ($I_{d530/414}$) to 475 nm ($I_{d475/414}$) when excited at 414 nm (Fig. 2A), i.e.,

$$\alpha = \frac{I_{d530/414}}{I_{d475/414}} \qquad (1)$$

The calculated values of α were 0.332±0.07, 0.278±0.06, 0.265±0.05 for CyPet-SUMO1/2/3, respectively. The slight difference among the cross-talk ratios is probably due to the structure differences of these fusion proteins. Although the fluorescent proteins, CyPet and YPet, are same for all of them, the middle portion of the fusion proteins, pre-SUMO1/2/3, have slightly structural difference and may result in different configurations and distances of FRET pair.

Similarly, because the emission of donor is almost not be able to be excited at 475nm (Iad530/475~0, data not shown), the contribution of donor at 530nm when excited at 475nm can be neglected. We define the cross-talk ratio of YPet's self-fluorescence (β) as the ratio of Ypet's emission at 530 nm when excited at 414 nm ($I_{a530/414}$) to that at 530 nm when excited at 475 nm ($I_{a530/475}$) (Fig. 2B), i.e.,

$$\beta = \frac{I_{a530/414}}{I_{a530/475}} \qquad (2)$$

The calculated value of β was 0.026.

We further decompose the detected fluorescence signal at 530 nm, under excitation at 414 nm ($FL_{530/414}$), into three parts: the FRET-induced acceptor emission (I_{da}), the donor direct emission ($I_{d530/414}$), and acceptor direct emission ($I_{a530/414}$) (Fig. 2C)

$$FL_{530/414} = I_{da} + I_{d530/414} + I_{a530/414}$$

Based on (1) and (2),

$$FL_{530/414} = I_{da} + \alpha I_{d475/414} + \beta I_{a530/475} \quad (3)$$

After hydrolysis by SENP2, the fluorescent signal at 530 nm is decreased, and the fluorescent signal at 475 nm is increased by the disruption of FRET. The remaining fluorescent emission at 530 nm ($FL'_{530/414}$) can be still divided into three parts similar to those in (3):

$$FL'_{530/414} = I'_{da530/414} + \alpha I'_{d475/414} + \beta I'_{a530/475} \quad (4)$$

Here $I'_{da530/414}$ is the remaining FRET-induced acceptor's emission. $I'_{d475/414}$ is the fluorescent emission of CyPet, which can divided into two parts: one from the undigested CyPet-(pre-SUMO1/2/3)-YPet and the other from digested CyPet-SUMO1/2/3. $I'_{a530/475}$ is the fluorescent emission of YPet, which is a constant whether the substrate has been digested or not.

After treatment with SENP2, the remaining FRET-induced acceptor's emission ($I'_{da530/414}$) is:

$$I'_{da530/414} = \frac{C-x}{C} \times I_{da} = \frac{C-x}{C} \times (FL_{530/414} - \alpha I_{d475/414} - \beta I_{a530/475}) \quad (5)$$

where C is the concentration of CyPet-(pre-SUMO1/2/3)-YPet (μM) in 80 μl and x is the concentration of digested CyPet-(pre-SUMO1/2/3)-YPet (μM) in 80 μl.

Combining (3)-(5), the detected fluorescent signal at 530 nm under excitation at 414 nm can be expressed as:

$$FL'_{530/414} = \frac{C-x}{C} \times \left(FL_{530/414} - \alpha I_{d475/414} - \beta I_{a530/475}\right) + \alpha I'_{d475/414} + \beta I_{a530/475} \quad (6)$$

The detected total fluorescent emission at 530 nm, CyPet and YPet direct emission as well as the FRET-induced YPet's emission analyzed by the above method and by the standard curve method [26] were compared for CyPet-(pre-SUMO1)-YPet at a concentration of 3.6 μM and CyPet-(pre-SUMO2)-YPet of 0.096 μM (Fig. 3). As we can see from Fig. 3, the fluorescent emission detected at 530 nm is not equal to the FRET-induced YPet's emission, while they are always considered the same in the ratiometric analysis. During the pre-SUMO's maturation process, the CyPet's direct emission increases and the FRET-induced acceptor's emission decreases due to the disruption of the donor's quenching. The results of CyPet's direct

emission and FRET-induced YPet's emission analyzed by real-time detection and standard curved method are comparable (Fig. 3).

Initial velocity determination of SENP2 to pre-SUMO1/2/3

The pre-SUMO1/2/3's maturation by SENP2 can be measured by monitoring the changes of fluorescent signals at 475 and 530 nm when excited at 414 nm during the maturation process. In our experiment, different amounts of substrate CyPet-(pre-SUMO1/2/3)-YPet were incubated with 6, 0.15, and 7.5 nM catalytic domain of SENP2, respectively. The concentrations of digested substrate, *x*, were calculated according to (6). The digested substrate concentrations show very good dose-dependent digestions with the amount of substrate (Fig. 4).

The initial velocity (V_o) of CyPet-(pre-SUMO1/2/3)-YPet's maturation by SENP2 was determined as described in [26]:

$$V_o = \frac{d[P]}{dt}|_{t=0} = k[S]_o$$

The calculated initial velocities display a good substrate dose-dependent relationship (Table 1).

Plasmid constructs

The open reading frames of the genes were amplified by PCR, and the PCR products were cloned into the PCRII-TOPO vector (Invitrogen). After confirming the constructs by sequencing, the cDNAs encoding CyPet-(pre-SUMO1/2/3)-YPet, CyPet-SUMO1/2/3, YPet, and the catalytic domains of SENP2 were cloned into the pET28 (b) vector (Novagen), engineered with an N-terminal polyhistidine tag.

Protein expression and purification

Escherichia coli cells of strain BL21(DE3) were transformed with pET28 vectors encoding CyPet-(pre-SUMO1/2/3)-YPet, CyPet-SUMO1/2/3, YPet, and the catalytic domains of SENP2. Transformed bacteria were grown in 2xYT medium to an optical density at 600 nm of 0.4–0.5, by induction with 100 μM isopropyl-β-D-thiogalactoside for 16 h at 25ºC. The polyhistidine-tagged recombinant proteins were purified from bacterial lysates with nickel agarose affinity chromatography (QIAGEN) and eluted in 20 mM Tris-HCl, pH7.4, 50 mM NaCl, 1 mM DTT. Protein purity was examined by SDS-PAGE, and concentrations of the purified proteins were determined by the Bradford assay (Thermo Scientific).

Self-fluorescence cross-talk ratio determination

To determine the cross-talk ratios of CyPet and YPet's self-fluorescence, purified CyPet-SUMO1/2/3 and YPet were incubated individually at 37°C in a buffer containing 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 µl in the concentration of 10, 20, 50, 100, 200, and 500 nM for 10 min and added to each well of a 384-well plate (Greiner, glass bottom).

Fluorescent emissions of CyPet at 475 and 530 nm were detected in a fluorescence multi-well plate reader (Molecular Devices, Flexstation II^{384}) at an excitation wavelength of 414 nm to determine the cross-talk ratio α; fluorescent emissions of YPet at 530 nm were detected under excitation at 414 and 475 nm to determine the cross-talk ratio β. Three samples were repeated for each concentration.

Protease kinetics assay

FRET-based SUMO processing assays were conducted by measuring the emission intensities of CyPet at 475 nm and of YPet at 530 nm with an excitation wavelength of 414 nm in a fluorescence multi-well plate reader (Molecular Devices, Flexstation II^{384}).

CyPet-(pre-SUMO1/2/3)-YPet was incubated with recombinant catalytic domain of SENP2 at 37°C in buffer of 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.1% (v/v) Tween-20 and 1 mM DTT in a total volume of 80 µl and added to each well of a 384-well plate (Greiner, glass bottom).

To validate the protease assay of pre-SUMO2 maturation by SENP2, 8 µM CyPet-(pre-SUMO2)-YPet and 0.6 nM catalytic domain of SENP2 were mixed in the low-salt Tris buffer in a total volume of 80 µl. Reactions were tested every 2–5 minutes until all the pre-SUMO2 was processed.

To study the kinetics of the CyPet-(pre-SUMO1/2/3)-YPet processing by SENP2, different concentrations of substrates were digested with 6, 0.15 and 7.5 nM SENP2 individually. Reactions were tested within the first 5 minutes. A one-phase association model was used to fit the exponentially increased reaction velocity. Data were analyzed by the developed method and plotted in GraphPad Prism V software to fit the Michaelis-Menten equation. Five samples were repeated for each concentration.

Results

Enzyme kinetics determination by Michaelis-Menten analysis

The catalytic specificity and efficiency of an enzyme for a specific substrate is best defined by the ratio of the kinetic constants, k_{cat}/K_M. This ratio is generally used to compare the efficiencies of different enzymes with one substrate or for different substrates by a particular enzyme.

The K_M and V_{max} values can be obtained from the Michaelis-Menten equation by plotting various velocities of SENP2 digestion versus the corresponding concentrations of the substrate. The initial velocities (Table 1) are plotted in the Michaelis-Menten model (Fig. 5). The maturation V_{max} is 0.021 ± 0.0022 μM/s for pre-SUMO1, 0.0006 ± 0.000035 μM/s for pre-SUMO2 and 0.011± 0.0042 μM/s for pre-SUMO3. *The* k_{cat} was obtained from:

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

The derived values of K_M, k_{cat} and their ratio for SENP2-(pre-SUMO1/2/3) are listed in Table 2. Similar to previous studies using western blots [21], our data show that the key step in pre-SUMOs maturation is the binding (different K_M) rather than the catalysis (similar k_{cat}), although the individual K_M, k_{cat} and their ratios are quite different. Also, the k_{cat}/K_M ratio is in agreement with the preference of SENP2 to pre-SUMO's maturation, i.e., pre-SUMO2>pre-SUMO1>pre-SUMO3 [27].

Discussion

Accurately determining kinetics parameters is critical for understanding enzymatic activity. In this paper, we describe a novel quantitative FRET-based protease assay which is highly sensitive for studying the kinetics of the SENP2 endopeptidase. Unlike traditional ratiometric FRET signal analytic methods, we differentiated and quantified the absolute fluorescent signals contributed by the donor, the acceptor and FRET at the acceptor's emission wavelengths. We also used the real-time fluorescent reading at both the donor and acceptor's emission wavelengths instead of creating standard curves. By using this approach, we determined that the K_M values of SENP2 are 0.048 ± 0.011 μM, 4.49 ± 0.99 μM and 12.06 ± 7.46 μM for pre-SUMO2, pre-SUMO1 and pre-SUMO3, respectively. The k_{cat}/K_M values of SENP2 to pre-SUMO1/2/3 are (7.83 ± 1.9) x 10^5 $M^{-1}{\cdot}s^{-1}$, (8.2 ± 1.87) x 10^7 $M^{-1}{\cdot}s^{-1}$, and (1.21 ± 0.88) x 10^5 $M^{-1}{\cdot}s^{-1}$ respectively. The k_{cat}/K_M values agree well with the general enzymatic

kinetic parameters, and they also agree with earlier methods in that the key step for pre-SUMOs maturation is binding (different K_M), not catalysis (similar k_{cat}) [28].

Several methods have been used to determine k_{cat}/K_M. Examples include the enzymatic digestion in solution, followed by the polyacrylamide gel–based western blot method, radioactive-labeled substrate, dialysis of digested substrate, fluorescent compound-labeled peptide substrate, and fluorescent protein-labeled substrate. The activities of SENP2 to process pre-SUMO1/2/3 and remove SUMO1/2 from SUMO substrate RanGAP1 can then be characterized in solution, followed by SDS-PAGE and western blot analysis. Using this method, the K_M values are similar for pre-SUMO2 (2.0 ± 0.6 μM) and pre-SUMO3 (2.2 ± 0.3 μM) with SENP2, but they are much smaller than the value of pre-SUMO1 (27.9 ± 3.7 μM). Those K_M values of the binding step are not consistent with the discovery of the nonproductive crystal structures of SENP2-(pre-SUMO1/2/3). The results are also not in agreement with other studies [27]. The k_{cat}/K_M values determined by this method are 2.6 x 10^4 $M^{-1}{\cdot}s^{-1}$ for pre-SUMO1, 3.7 x 10^5 $M^{-1}{\cdot}s^{-1}$ for pre-SUMO2 and 5 x 10^4 $M^{-1}{\cdot}s^{-1}$ for pre-SUMO3 [28]. Another study used tetrapeptides with di-Gly motif and labeled an organic fluorophore ACC (7-amino-4-carbamoylmethylcooumarin), which can emit fluorescence signal when cleaved by SENPs. The range of the k_{cat}/K_M values determined by this method is 17-325 $M^{-1}{\cdot}s^{-1}$, which is up to two orders of magnitude lower than the natural substrates. *The substrate difference outside of the catalytic cleft (di-Gly motif) significantly affect the binding step (K_M) of SUMO substrates.”” [29,30]. In another study, mature SUMO1/2 were tagged with a similar organic fluorophore AMC (7-amino-4-methylcoumarin). Although the determined k_{cat}/K_M value is 1.8 x 10^5 $M^{-1}{\cdot}s^{-1}$ for SENP2-SUMO1 and 9.9 x 10^4 $M^{-1}{\cdot}s^{-1}$ for SENP2-SUMO2, there is no specific sequence of either SUMO tail or SUMO-specific substrate followed by the AMC. Furthermore, the method cannot clearly distinguish the iso- or endopeptidase function of SENPs [31].

We improve the methodology with a new theory of FRET signal analysis for kinetic determination and an experimental procedure to derive kinetic parameters. We achieve this by quantifying the individual contributions of absolute fluorescent signals from the donor’s direct emission, the acceptor’s direct emission and the real FRET-induced acceptor’s emission. The large numeric differences between the developed quantitative FRET analysis and ratiometric analysis reflect a fundamental difference of the FRET data process. The discrepancy between these two approaches might be due to the inclusion of direct emissions from the donor and

acceptor in the ratiometric analysis. We compared the ratiometric FRET analysis with our quantitative FRET analysis in studying pre-SUMO1's maturation by SENP1 [26]. Although the overestimations of FRET signal in the ratiometric analysis might not greatly affect the final k_{cat}/K_M ratio, the effect is more obvious when studying the individual parameters, K_M and k_{cat}, which are important in determining the rate-limiting step and inhibitor potency of enzymes.

Unlike previous quantitative FRET analysis [26], the current approach detects direct donor's emission in real time without standard curves. As noted by a review of different quantitative FRET analysis [32], more accurate and robust results can be obtained by observing multiple channels instead of only one channel. In our approach, real-time fluorescent signal at 475 and 530 nm were detected, whereas the previous methods only analyzed the fluorescent signal from 530 nm. We compared our method with the standard curve method for SENP2 and pre-SUMO1/2 (the kinetics study of pre-SUMO3's maturation by the standard curve method cannot fit the Michaelis-Menten equation due to the large variations). The results are presented in the supplementary figure and table. From this comparison, we find that the standard errors of standard curve method are larger than those of the real-time detection. While the ultimate source of the errors is unclear, detecting the emissions at two wavelengths (i.e., 475 and 530 nm) may help reduce errors.

Our highly sensitive quantitative FRET-based protease assays can be also used as a robust general approach for other protease kinetics determinations. 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. It is environmentally friendly and requires only molecular cloning and protein expression without radioactive labeling or expensive instruments. The fluorescent-tagged proteins are in the aqueous phase, which closely approximates their natural environment in cells. Fluorescence intensity can be determined by general fluorescence spectroscopy or fluorescence plate readers, which are widely available.

Conclusions

A novel methodology has been developed for protease kinetics determination based on the quantitative FRET assay in one sample. Absolute FRET signal is derived from the subtraction of free fraction FRET donor and acceptor from the total fluorescent emission. This methodology provides a convenient and robust assay platform for protease kinetics

determination. The proposed quantitative FRET analysis can be easily extended to other FRET-based assays.

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Declarations of interest

The authors declare that they have no conflict of interest that would affect the present study.

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Author contribution statement

YL conducted the experiments, analysis and manuscript writing; YS conducted some analysis and major discussion; SZ contributed discussions and manuscript writing; JL designed the experiments, analysis and manuscript writing.

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Table 1. Initial velocity determinations of Pre-SUMO1,2,3 by quantitative FRET assay. In each condition, three samples were measured.

[S] (μM)	V_o (μM/s)
0.6	0.0029 ± 0.00010
1.2	0.0046 ± 0.00039
1.8	0.0066 ± 0.00040
2.4	0.0082 ± 0.00050
3.6	0.0085 ± 0.00057
4.5	0.0091 ± 0.00174
6	0.0126 ± 0.00101
7.5	0.0128 ± 0.00140
9	0.0149 ± 0.00240

Table 1-2. Initial velocities determination of SENP2 for SUMO2

[S] (μM)	V_o (μM/s)
0.016	0.00011 ± 0.00001
0.032	0.00029 ± 0.00001
0.048	0.00032 ± 0.00003
0.064	0.00033 ± 0.00004
0.096	0.00042 ± 0.00002
0.12	0.00037 ± 0.00004
0.16	0.00039 ± 0.00013
0.24	0.00050 ± 0.00006
0.32	0.00058 ± 0.00007
0.4	0.00055 ± 0.00011
0.48	0.00050 ± 0.00022

Table 1-3. Initial velocities determination of SENP2 for SUMO3

[S] (μM)	V_o (μM/s)
0.75	0.00075 ± 0.0001
1.5	0.00157 ± 0.0002
2.25	0.00227 ± 0.0006
3	0.00240 ± 0.0004
4.5	0.00174 ± 0.0010
7.5	0.00469 ± 0.0010
9.375	0.00446 ± 0.0016
11.25	0.00551 ± 0.0023

Table 2. Kinetics parameters of SENP2 for substrate SUMO 1/2/3 . In each condition, three samples were measured.

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	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
SUMO1	4.49 ± 0.99	3.52 ± 0.37	7.83 (± 1.90) x 10^5
SUMO2	0.048 ± 0.011	3.93 ± 0.23	8.20 (± 1.87) x 10^7
SUMO3	12.06 ± 7.46	1.46 ± 0.55	1.21 (± 0.88) x 10^5

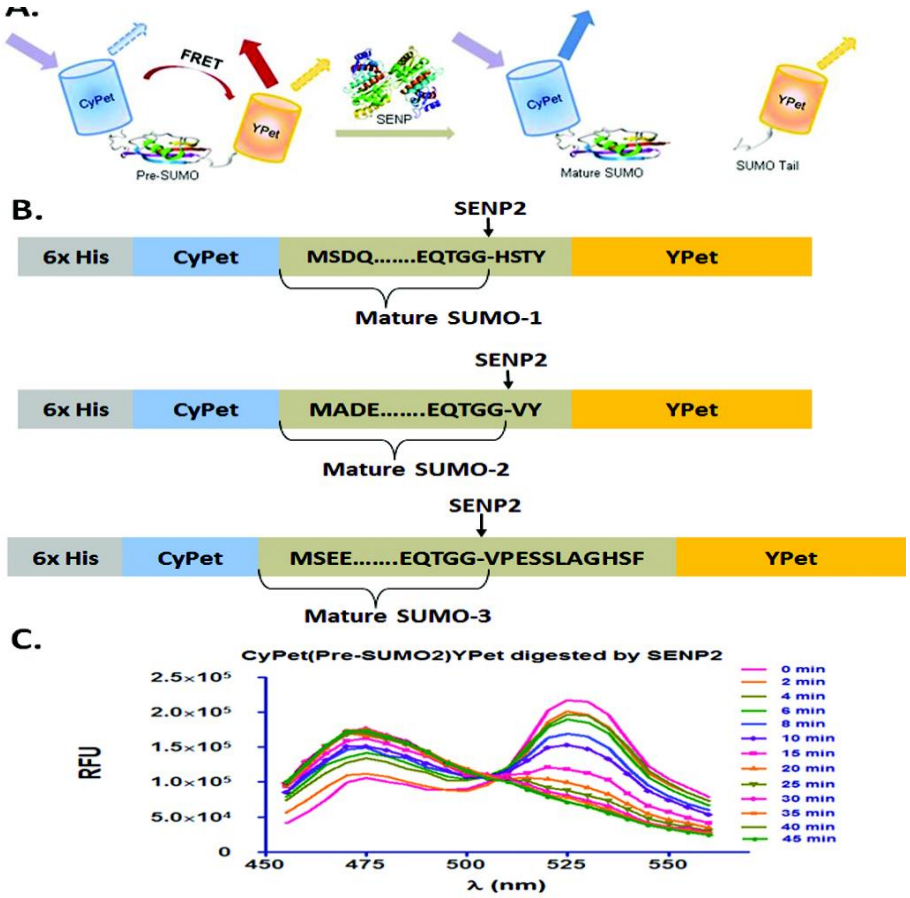

Figure 1. Overeview of the FRET-based SENP endopeptidase assay. A. Schematic depiction of the CyPet-(pre-SUMOs)-YPet substrate and the principle of FRET from CyPet (donor, excitation with light of 414 nm) to YPet (acceptor, emission measured at 530 nm). Once cleaved by SENP, the distance between the fluorescent proteins is increased beyond a FRET-sensitive distance, and thus, CyPet emission measured at 475 nm is increased and the YPet FRET-induced emission is reduced. B. Schematic depiction of the 6His-CyPet-(pre-SUMO1/2/3)-YPet constructs. C. Emission spectrum of CyPet-(pre-SUMO2)-YPet (8 μM) digested by catalytic domain of SENP2 (0.6 μM) under excitation of 414 nm. The process was monitored every 2–5 minutes until the substrate was totally matured.

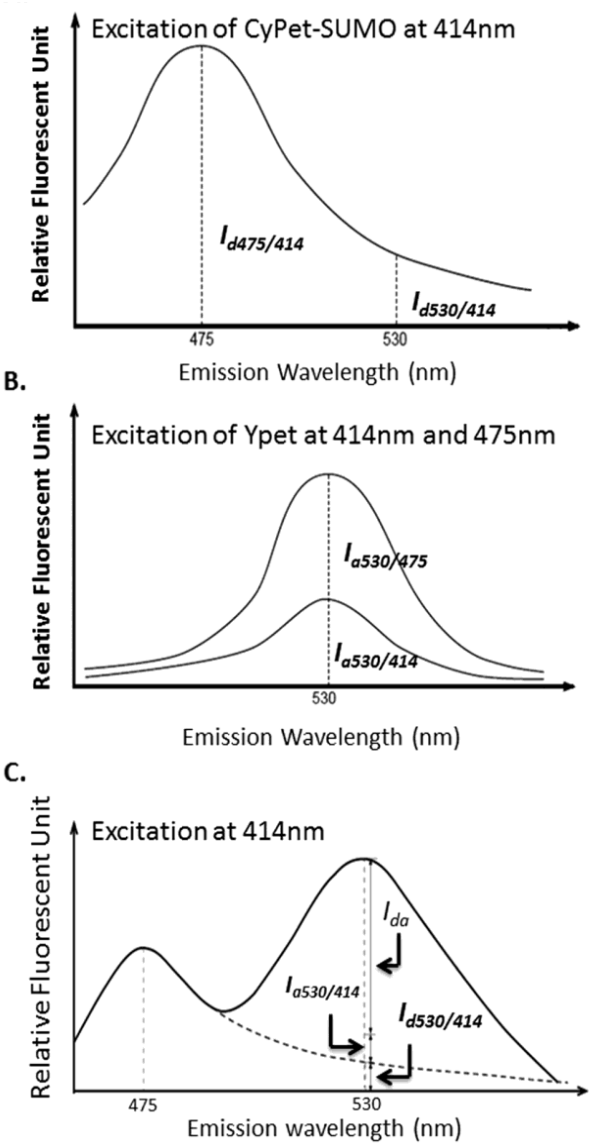

Figure 2. Spectrum analysis of FRET spectrum. A. Spectrum analysis of direct emission of donor (CyPet). $I_{d475/414}$ is the fluorescent emission of CyPet-SUMO1/2/3 at 475 nm under the excitation of 414 nm; $I_{d530/414}$ is the fluorescent emission of CyPet-SUMO1/2/3 at 530 nm when excited at 414 nm. B. Spectrum analysis of direct emission of acceptor (YPet). $I_{a530/475}$ is the fluorescent emission of YPet at 530 nm under the excitation of 475 nm; $I_{a530/414}$ is the fluorescent emission of YPet at 530 nm when excited at 414 nm. C. Spectrum analysis of detected emission at 530 nm. Dissection of emission spectra from engineered protein CyPet–(pre-SUMOs)–YPet when excited at 414 nm. $I_{d530/414}$ is CyPet fluorescence at 530 nm under excitation of 414 nm, I_{da} is FRET-induced YPet emission at 530 nm under excitation of 414 nm, and $I_{a530/414}$ is direct YPet emission at 530 nm under excitation of 414 nm.

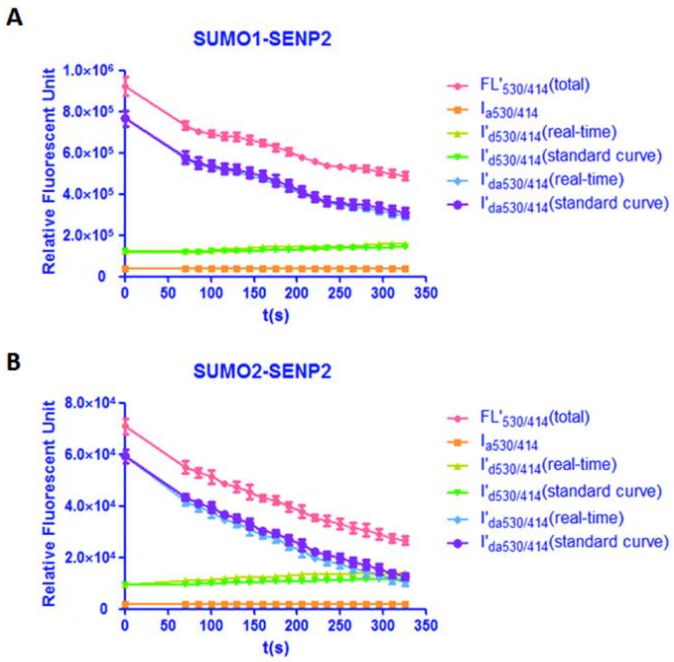

Figure 3. Timecourse of fluorescence component changes during digestion by SENP2. A. Changes to fluorescence components of CyPet-(pre-SUMO1)-YPet during maturation by SENP2. B. Changes to fluorescence components of CyPet-(pre-SUMO2)-YPet during maturation by SENP2. **In each condition, three samples were measured.**

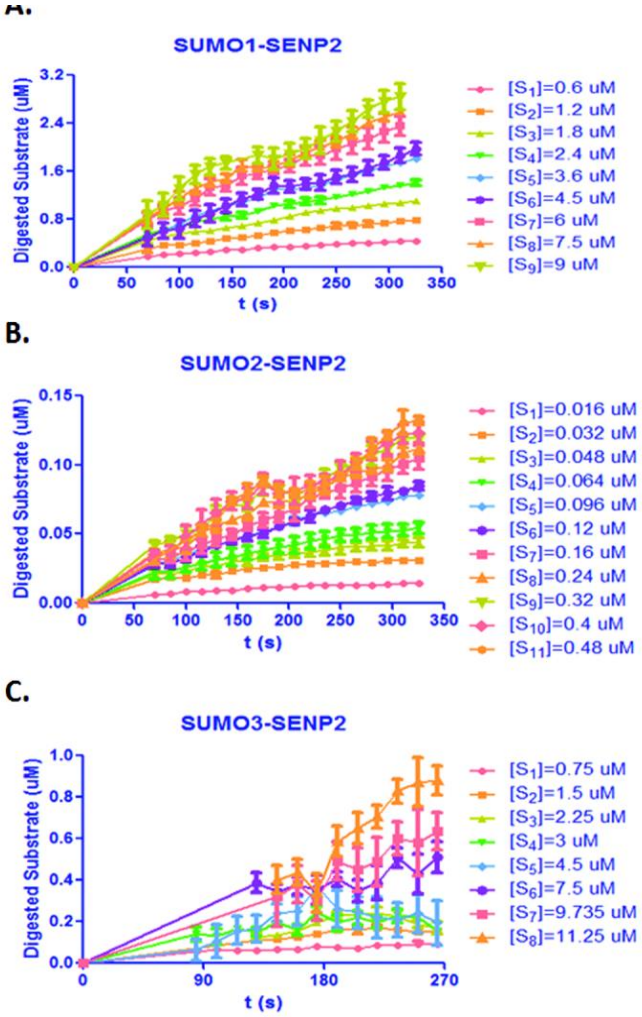

Figure 4. The velocities of pre-SUMO1, 2, and 3 substrates digested by SENP2. A. The velocity of various concentrations of pre-SUMO1 digested by SENP2. B. The velocity of various concentrations of pre-SUMO2 digested by SENP2. C. The velocity of various concentrations of pre-SUMO3 digested by SENP2. **In each condition, three samples were measured.**

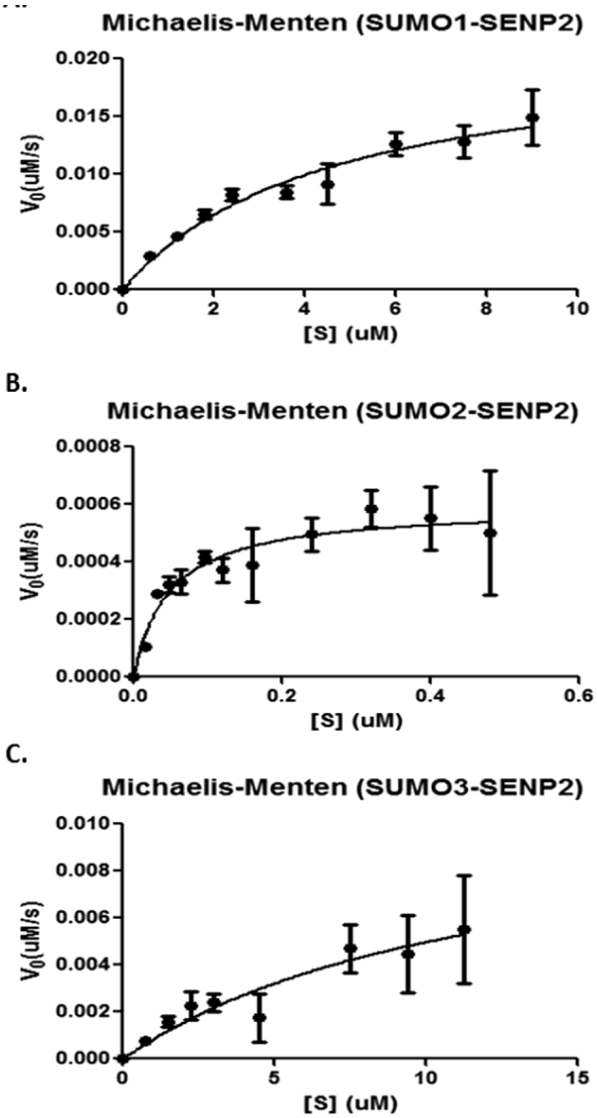

Figure 5. Michaelis-Menten graphs of pre-SUMO1, 2, and 3 digestions by SENP2 by an internal calibration method. A. The Michaelis-Menten graph of pre-SUMO1 digestion by SENP2. B. The Michaelis-Menten graph of pre-SUMO2 digestion by SENP2. C. The Michaelis-Menten graph of pre-SUMO3 digestion by SENP2. **In each condition, three samples were measured.**