



FRET-based assay of the processing reaction kinetics of stimulus-responsive peptides: influence of amino acid sequence on reaction kinetics

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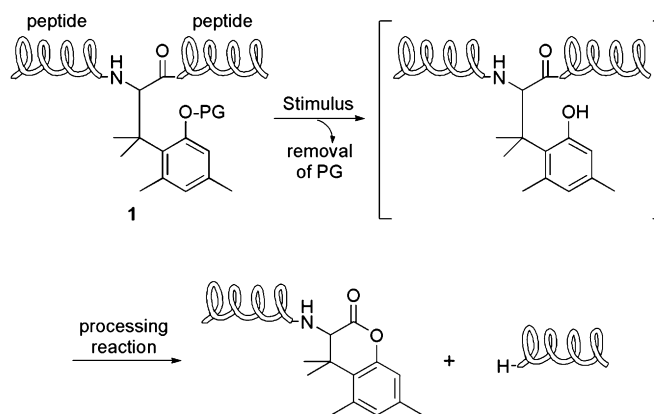
ABSTRACT

In the field of chemical biology, methods for controlling peptidyl function by a stimulus are attracting increasing attention. Recently, we reported a stimulus-responsive peptide, which can be cleaved after exposure to a stimulus. In this study, we developed a FRET-based assay system to estimate the kinetics of the stimulus-induced processing (peptide bond cleavage) reaction. Based on the FRET system, it was clarified that introduction of a sterically less-hindered or polar residue at the position adjacent to the stimulus-responsive amino acid accelerates the processing reaction.

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1. Introduction

In the field of chemical biology, it is important to develop technology to control the function of intracellular peptides and proteins by an external stimulus. In this context, photo-responsive bond cleavage reaction¹ or conformational change² of a peptide/protein backbone has been successfully applied for spatiotemporal control of its function. Whereas these stimulus-responsive peptides and proteins can respond to specific stimuli, we developed a stimulus-responsive peptide, which is applicable to arbitrary stimuli. Based on the trimethyl lock system,³ we previously reported a stimulus-responsive processing (peptide bond cleavage) device with successful application for controlling peptidyl function that induces processing reaction after exposure to a stimulus such as UV irradiation or enzymatic reaction.⁴ When peptide **1** possessing the stimulus-responsive processing device was exposed to stimulus, removal of PG (protective group removable by a stimulus) followed by nucleophilic involvement of the generated phenolic hydroxyl group afforded processing products in good purity (Scheme 1). In a previous report, we briefly mentioned that the rate of the processing reaction was dependent on the amino acid adjacent to the stimulus-responsive residue. In the present paper, we report details of the influence of amino acid sequence on the kinetics of the processing reaction.



Scheme 1. Stimulus-responsive processing system (PG: protective group removable by a stimulus).

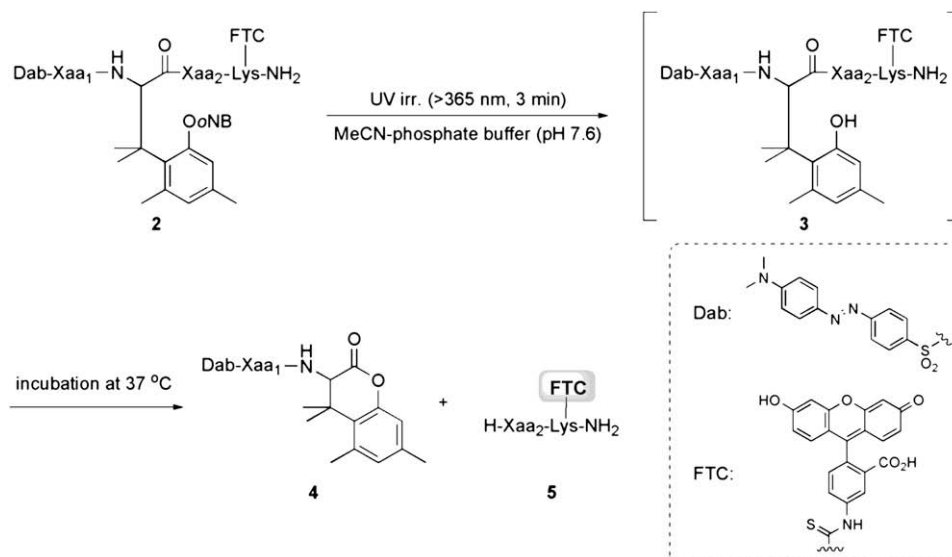
2. Results and discussions

2.1. Design of the FRET substrate

Fluorescence resonance energy transfer (FRET) is one of the most widely used techniques to evaluate kinetics of the cleavage reaction of a peptide.⁵ It was reported that a fluorescein-5-thiocarbamoyl (FTC)-dabsyl (Dab) based FRET system is compatible with a photo-removable *o*-nitrobenzyl (*o*-NB) group.⁶ Therefore, we designed FTC-Dab based FRET substrate **2** possessing a photo-responsive amino acid protected by an *o*-NB group (Scheme 2). If

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Scheme 2. FRET-based assay system for the kinetics of the processing reaction (*o*-NB: *o*-nitrobenzyl).

peptide **2** was irradiated by UV (>365 nm) for 3 min, it should be completely deprotected to generate intermediate **3** with weak fluorescence. Afterward, intermediate **3** would be converted to processing products **4** and **5** thereby emitting a strong fluorescence. Based on the time course of fluorescence intensity, kinetics of the processing reaction of intermediate **3** could be estimated.

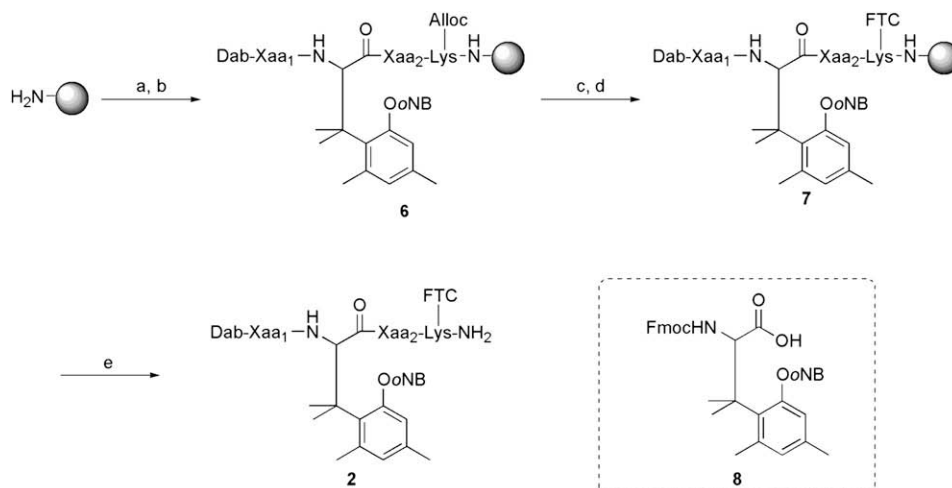
2.2. Synthesis of the FRET substrate

First, FRET substrates **2** were synthesized as shown in Scheme 3. The peptide was elongated on NovaSyn® TGR resin using Fmoc SPPS, and the peptide was capped with dabsyl chloride to yield peptide resin **6**. After removal of the Alloc group on side chain of Lys, the regenerated amine was reacted with fluorescein-5-isothiocyanate (FITC) to introduce a fluorophore. Finally, peptide resin **7** was treated with a cleavage cocktail (TFA/H₂O/thioanisole=95:2.5:2.5, v/v) to give FRET substrate **2** that was purified as a diastereomeric mixture before subsequent experiments.⁷ Because the coupling reaction of Fmoc protected photo-responsive amino acid **8**⁴ on sterically hindered

residues such as Asn(Trt), Cys(Trt), and Ser(*t*-Bu) did not proceed well, Cys(MPM) (MPM: *p*-methoxybenzyl) and side chain unprotected Asn, Ser, and Thr were used for the synthesis of peptide **2** (Xaa₁=Gly; Xaa₂=Asn, Cys, Ser and Thr), respectively. This result suggests that introduction of the photo-responsive amino acid on a sterically hindered residue should be avoided for facile synthesis.

2.3. Kinetics measurement of the processing reaction

First, we monitored the processing reaction of peptide **2** (Xaa₁=Gly; Xaa₂=Tyr) using HPLC, and processing products were characterized by ESI-MS. The UV-induced processing reaction of the FRET substrate is outlined in Scheme 2. Peptide **2** in phosphate buffer (pH 7.6) with 30% MeCN was irradiated with UV light (>365 nm) for 3 min and then incubated at 37 °C. After 3 min of UV irradiation, substrate **2** (Xaa₁=Gly; Xaa₂=Tyr) was completely deprotected to yield intermediate **3** (Xaa₁=Gly; Xaa₂=Tyr) (data not shown). As shown in Figure 1, intermediate **3** (Xaa₁=Gly; Xaa₂=Tyr) was converted to processing products **4** (Xaa₁=Gly) and **5** (Xaa₂=Tyr) after 3 h of incubation.



Scheme 3. Reagents and conditions: (a) Fmoc SPPS; (b) dabsyl chloride, Et₃N, DMF; (c) (Ph₃P)₄Pd, 1,3-dimethylbarbituric acid, 1,2-dichloroethane; (d) FITC, DIEA, DMF; (e) TFA/thioanisole/H₂O=95:2.5:2.5 (v/v) (*o*-NB: *o*-nitrobenzyl).

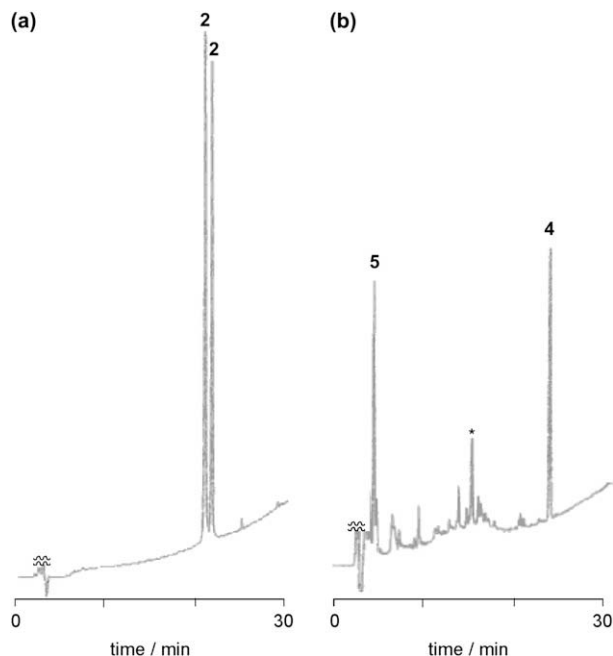


Figure 1. HPLC profiles (a) before UV irradiation (b) after 3 min of UV irradiation followed by 3 h of incubation at 37 °C (Xaa₁=Gly; Xaa₂=Tyr). Diastereomeric mixture of **2** was used. Peptides were detected by UV absorbance at 220 nm. Asterisked peak is an *o*-nitrobenzyl derived small molecule.

Next, we monitored the time course of the fluorescence intensity of the reaction mixture at 37 °C using a plate reader ($\lambda_{\text{ex}}=495$ nm; $\lambda_{\text{em}}=520$ nm). Before UV irradiation, fluorescence intensity of the reaction mixture was recorded as time=0 min. Then, the reaction mixture was irradiated by UV light (>365 nm) for 3 min, and the fluorescence intensities were recorded at 3 (just after UV irradiation), 5, 10, 15, 30, 60, 120, and 180 min. The percentage of remaining intermediate **3** was estimated based on relative fluorescence intensity. As shown in Figure 2, the processing reaction of intermediate **3** (Xaa₁=Gly; Xaa₂=Tyr) shows first-order dependence on the concentration of the intermediate, and the half life of **3** (Xaa₁=Gly; Xaa₂=Tyr) was determined as 18.0 min.⁸

These results encouraged us to determine half-lives of the stimulus-responsive peptides with various amino acids at a position adjacent to the stimulus-responsive residue. FRET substrates **2** possessing various amino acid sequences were synthesized as mentioned above. Their half-lives were estimated based on the FRET assay system, and results are summarized in Figure 3. When a sterically less-hindered amino acid had been introduced at Xaa₂

position, rapid processing reaction was observed. Interestingly, introduction of a polar residue at Xaa₂ position also accelerated the reaction regardless of its steric hindrance. The polar residue might assist the processing reaction by hydrogen bond-mediated activation of the carbonyl group of the peptide bond and/or deprotonation of the phenolic hydroxyl group. In the meantime, replacement of Xaa₁ with sterically less-hindered Gly or polar Glu or Lys also accelerated the reaction; however, the polarity effect of the side chain was smaller than that observed for Xaa₂.

3. Conclusion

In conclusion, the half-lives of the stimulus-responsive peptides were determined using a FRET-based assay system. Introduction of a sterically less-hindered or polar residue at position Xaa₂ significantly accelerated the reaction. Replacement of Xaa₁ with a sterically less-hindered Gly or polar Glu or Lys also accelerated the reaction whereas the polarity effect was smaller than that observed for Xaa₂. These results enable us to control the processing reaction rate for different purposes by choosing an appropriate residue at a position adjacent to the stimulus-responsive amino acid.

4. Experimental

4.1. General

Exact mass spectra were recorded on Bruker Esquire200T or Waters MICROMASS[®] LCT PREMIER[™]. For HPLC separations, a Cosmosil 5C₁₈-AR-II analytical column (Nacalai Tesque, 4.6×250 mm, flow rate 1 mL/min) or a 5C₁₈-AR-II semi-preparative column (Nacalai Tesque, 10×250 mm, flow rate 3.0 mL/min) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA in water (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution. Infinite M200 flexible microplate reader (TECAN Austria GmbH) was used for fluorescence measurements. Photolysis was performed using Moritex MUV-202U with the filtered output (>365 nm) of a 3000 mW/cm² Hg–Xe lamp.

4.2. General procedure for a preparation of FRET substrate **2**

Elongation of peptides on NovaSyn[®] TGR resin (0.25 mmol/g, 8.7 mg) was performed as described in previous report.⁴ For the synthesis of peptide **2** (Xaa₁=Gly; Xaa₂=Asn, Cys, Ser or Thr), Fmoc–Asn–OH, Fmoc–Cys(MPM)–OH, Fmoc–Ser–OH or Fmoc–Thr–OH was used as a building block, respectively. Obtained resin was reacted with dabsyl chloride (2.1 mg) and triethylamine

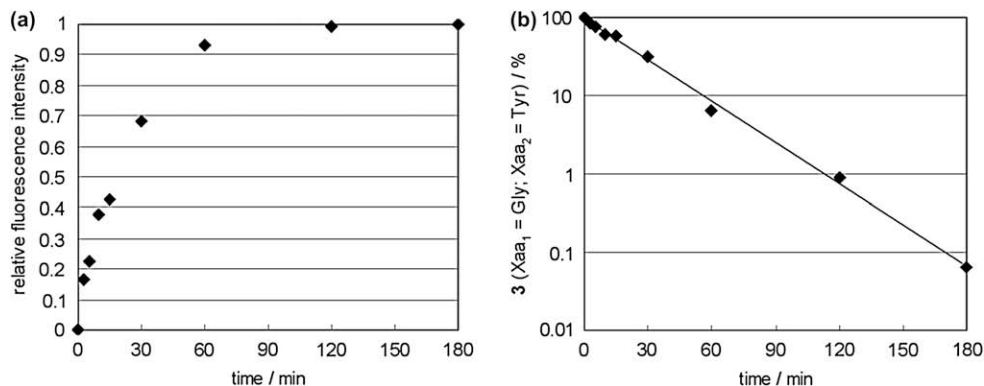


Figure 2. Kinetic measurement of the processing reaction of **3** (Xaa₁=Gly; Xaa₂=Tyr). (a) Change in fluorescence intensity in phosphate buffer (pH 7.6, 20 mM) with 30% MeCN at 37 °C after UV irradiation ($\lambda_{\text{ex}}=495$ nm; $\lambda_{\text{em}}=520$ nm). (b) First-order kinetic treatment of the data. Percentage of intermediate **3** was calculated as follow. **3** (%) = 100 × (1 – relative fluorescence intensity).

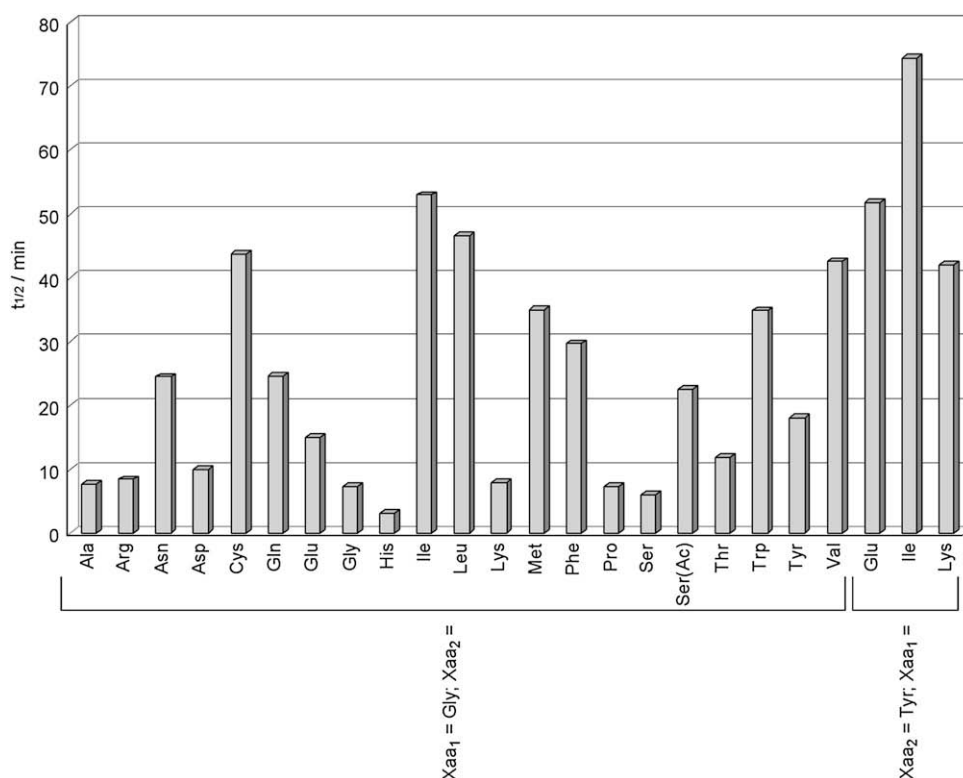


Figure 3. Half-lives of intermediates **3** in the processing reaction. Ser(Ac) is an O-acetyl serine.

(1.8 μ L) in DMF (70 μ L) for 3 h. After washing with DMF followed by dichloromethane, Alloc group was removed by a treatment with $(\text{Ph}_3\text{P})_4\text{Pd}$ (4.0 mg) and 1,3-dimethylbarbituric acid (3.4 mg) in 1,2-dichloroethane (172 μ L) overnight. Then the resin was washed with dichloromethane followed by DMF. To obtained resin were added fluorescein-5-isothiocyanate (FITC, 2.0 mg) and DIEA (10 μ L) in DMF (50 μ L), and the reaction was performed overnight. After filtration, the resin was treated with FITC (2.0 mg) and DIEA (0.88 μ L) in DMF (50 μ L) for additional 24 h. Finally, global deprotection of

the peptide with TFA/thioanisole/ H_2O (95:2.5:2.5, v/v) afforded a crude product. After trituration with diethyl ether, the precipitate was purified by HPLC to give FRET substrate **2** as a diastereomeric mixture in 10–20% yield. Analytical HPLC conditions: linear gradient of solvent B in solvent A, 30–90% over 30 min. Semi-preparative HPLC conditions: linear gradient of solvent B in solvent A, 40–70% over 30 min (Table 1).

4.3. Reaction monitoring of the processing reaction by using HPLC

The experiment was performed as described in previous report.⁴ Analytical HPLC conditions: linear gradient of solvent B in solvent A, 30–90% over 30 min. ESI-MS: Compound **4** ($\text{Xaa}_1=\text{Gly}$) calcd for $[\text{M}+\text{H}]^+$ 564.2, obsd 563.9. Compound **5** ($\text{Xaa}_2=\text{Tyr}$) calcd for $[\text{M}+\text{H}]^+$ 698.2, obsd 697.9. Retention time=23.5 or 5.4 min, respectively, for **4** ($\text{Xaa}_1=\text{Gly}$) or **5** ($\text{Xaa}_2=\text{Tyr}$).

4.4. Kinetics measurement based on FRET system

Fluorescence intensity of FRET substrate **2** (final concentration=8 μ M) in phosphate buffer (pH 7.6, 20 mM) with 30% MeCN was measured before photolysis and it was defined as time=0 min ($\lambda_{\text{ex}}=495$ nm; $\lambda_{\text{em}}=520$ nm). Then, the reaction mixture was irradiated by UV light (>365 nm) for 3 min and the fluorescence of the reaction mixture at 37 $^\circ\text{C}$ was recorded at 3, 5, 10, 15, 30, 60, 120, and 180 min. Percentage of remaining intermediate **3** was estimated based on relative fluorescence intensity. Remaining intermediate **3** (%) = $100 \times (1 - \text{relative fluorescence intensity})$.

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Table 1

2		ESI-MS ($[\text{M}+2\text{H}]^{2+}$)	
Xaa ₁	Xaa ₂	Calcd	Obsd
Gly	Ala	652.7	652.5
Gly	Arg	695.5	695.1
Gly	Asn	674.4	674.4
Gly	Asp	674.7	674.7
Gly	Cys	668.7	668.9
Gly	Gln	681.3	681.6
Gly	Glu	681.8	682.1
Gly	Gly	645.7	645.5
Gly	His	685.8	686.0
Gly	Ile	673.8	673.9
Gly	Leu	673.8	673.6
Gly	Lys	681.3	681.4
Gly	Met	682.8	682.9
Gly	Phe	690.8	690.9
Gly	Pro	665.8	666.0
Gly	Ser	660.7	660.9
Gly	Ser(Ac) ^a	681.8	681.9
Gly	Thr	667.8	667.9
Gly	Trp	710.3	710.4
Gly	Tyr	698.8	699.0
Gly	Val	666.8	666.7
Glu	Tyr	734.8	735.4
Ile	Tyr	726.5	727.0
Lys	Tyr	734.3	734.5

^a Ser(Ac)=O-acetyl serine.

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7. Racemic **8** was used for a synthesis of FRET substrates **2**.
8. Because pH dependence of the processing reaction kinetics was observed, kinetics of this reaction should be described by pseudo-first-order (half life of **3** (Xaa₁=Gly, Xaa₂=Tyr) in 30% MeCN/phosphate buffer (pH 5.5): 26.9 min).