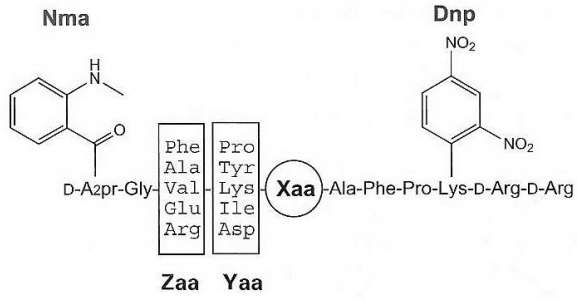
FRETS-25Xaa Peptide Library

FRETS: Fluorescence Resonance Energy Transfer Substrates

All library products have the general structure:
D-A2pr(Nma)-Gly-[Phe/Ala/Val/Glu/Arg]-[Pro/Tyr/Lys/Ile/Asp]-Gly-Ala-Phe-Pro-Lys(Dnp)-D-Arg-D-Arg
where A2pr(Nma) = Nβ-[2-(N-Methylamino)benzoyl]-2,3-Diaminopropionic Acid
All substrates are sold as trifluoroacetate salt and contain 1 µmol of stated library.



Principle

When an enzyme of interest cleaves any peptide bond between D-A2pr(Nma) and Lys(Dnp) in the substrate, the fluorescence at λex = 340 nm and λem = 440 nm increases in proportion to the release of the Nma fluorophore from the internal Dnp quencher.

Reagents

1) Each substrate stock solutions: each FRETS-25Xaa (***Code 3701-v - Code 3719-v***) in 1.0 ml of DMSO (1 mM, total of peptides)
2) Reference compounds stock solution: a 1:1 mixture of two solutions of ***Code 3720-v and Code 3721-v***, each of which is reconstituted by dissolving peptides in 0.5 ml of DMSO at the concentration of 2 mM (1 mM, each reference compound)
3) Enzyme solution: an enzyme of interest in an appropriate buffer
4) Buffer

Procedure for the deduction of the substrate specificity of an enzyme with unidentified cleavage specificity

Choose the proper conditions for the measurement, such as substrate concentration and sensitivity setting, depending on the purpose of the experiment and the instrument available. Described here is one of the recommended procedures for determining the enzymatic cleavage site by the combination of the fluorometric analysis and liquid chromatography-mass spectrometry (LC-MS) analysis.

i) Primary screening: selection of the favored Xaa

- Substrate solution for primary screening (PS solution): Dilute 20 µl of each of the above substrate stock solution with 1980 µl of an appropriate buffer (10 µM)
- Reference compounds solution for primary screening (PR solution): Dilute 20 µl of the above reference compounds stock solution with 1980 µl of an appropriate buffer (10 µl)

1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix one of the PS solution and the PR solution in ratios of 10/0, 9/1, 8/2, 5/5 and 0/10
3) Measure the fluorescence of the solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 µl each of all PS solutions into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity

8) Terminate of the enzymatic reaction by using a proper inhibitor (Leupeptin code 4041, E-64 code 4096, Pepstatin A code 4397) or changing the pH of the reaction medium (using TCA, AcOH, NaOH)
9) Choose the best Xaa-containing substrate for secondary screening

ii) Secondary screening: identification of the specificity of the enzyme (I)

- Substrate solution for secondary screening (SS solution): Dilute 200 µl of the stock solution of the best Xaa-containing substrate chosen by the above primary screening with 1800 µl of an appropriate buffer (100 uM)
- Reference compounds solution for secondary screening (SR solution): Dilute 200 µl of the above reference compounds stock solution with 1800 µl of an appropriate buffer (100 µM)

1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix the SS solution and the SR solution in ratios of 100/0, 95/5, 90/10, 80/20, 50/50 and 0/100
3) Measure the fluorescence of the solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 µl of the SS solution into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor or changing the pH of the reaction medium upon completion of the reaction at the points of 0%, 5%, 10% and 20% of the total Subject 100 µl aliquots to LC-MS

iii) LC-MS: identification of the specificity of the enzyme (II)

- Analytical conditions

column:	ODS
eluant:	A) H2O containing 0.05% TFA, B) CH3CN containing 0.05% TFA
gradient:	10% to 40% B) in A) over 50 min
detection:	UV at 220 nm and 400 nm or fluorescence

1) Inject 100 ul aliquots of each terminated solution at different stage of the reaction
2) Measure the MW of the cleaved product(s) in the peak(s) with the absorbance at 220 nm but not with 400 nm [identification of the N-terminal segment(s)]
3) Deduce their structure from the attached list of the theoretical MW for the cleaved products

Comment 1: If the N-terminal segment has the identical retention time to the C-terminal segment or one of the starting uncleaved substrates, detection of the products by fluorescence is recommended.

Comment 2: In the accidental case where the two products with the same MW (ex. Zaa-Yaa=Phe-Asp and Val-Tyr, Glu-Asp and Phe-Pro) are generated from one of the substrate, their analyses should be carried out by MS-MS sequencing and/or by Edman degradation.

Usefulness and limitation of FRETS-25Xaa series for screening of substrate specificities of proteases

We have confirmed that FRETS-25Xaa series are effectively used for the assay of numerous proteases such as trypsin, chymotyrpsin, elastase, thrombin, papain, calpain, pepsin and thermolysin. However, they did not work well for the assay of caspase-3, probably because they have only three changeable sites (Zaa-Yaa-Xaa) in each substrate (deficiency of P4 site). This fact implies that FRETS-25Xaa might not be applicable to the assay of an enzyme with wide range interacting sites with substrate.

1. K. Takada, M. Tsunemi, Y. Nishiuchi, and T. Kimura, A Fluorescence Resonance Energy Transfer Substrate (FRETS) Library for Determining Protease Specifi city. [Peptide Revolution: Genomic, Proteomics & Therapeutics] (Proceedings of the 18th American Peptide Symposium) In press.
2. S. Tanskul, K. Oda, H. Oyama, N. Noparatnaraporn, M. Tsunemi, and K. Takada, Substrate specifi city of alkaline serine proteinase isolated from photosynthetic bacterium, Rubrivivax gelatinosus KDDS1. ***Biochem. Biophys. Res. Commun.***, **309,** 547 (2003).