Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 358 (2006) 298–300
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Notes & Tips

Creation of a recombinant peptide substrate for fluorescence resonance energy transfer-based protease assays

Lin Zhang *, Heather L. Lawson, Vallathucherry C. Harish, Jason D. Huff, Mary Ann Knovich, John Owen

Section on Hematology and Oncology, Department of Medicine, Wake Forest University School of Medicine, Winston–Salem, NC 27157, USA

Received 13 June 2006
Available online 5 July 2006

The performance of protease assays has been revolutionized by the advent of fluorescence resonance energy transfer (FRET)[1]-based assays. Generation of pure, reliable, high-quality substrate is essential to such assays. Small FRET substrates can be easily prepared by chemical production. The preparation of fluorogenic substrate becomes cumbersome when a larger peptide substrate is required or when the amino acid residues that flank the protease cleavage site are critical to enzyme activity. We sought a novel method to generate a large FRET peptide substrate using facile DNA recombinant technology.

FRET is a phenomenon that describes the alteration in fluorescence emission that occurs with the interaction of two molecules in close proximity. Energy transferred from an excited donor molecule (fluor) to an acceptor molecule (quencher) is dependent on the Förster radius (R_0). This is the distance at which 50% efficient energy transfer occurs between these two molecules. Typical Förster radii are in the range of 30–50 Å [1]. The FRET rate is inversely proportional to the sixth power of the distance between the donor and the acceptor [2].

Two different approaches have been used to develop FRET protease assays based on minimal effective recognition sequence. Enzymes that have specificity defined by the residues amino to the cleavage site of the substrate (P residues) can be assayed using substrates containing a fluorescent leaving group at the carboxy terminus and a quenching group on the short peptide. Enzymes with critical residues carboxy to the cleavage site (P′ residues) require a second approach. Substrates are generated with a fluor attached to the amino terminus and a quencher attached to the carboxy terminus. The minimal peptide substrate typically is six residues: three on either side of the cleavage site. A number of fluor and quencher pairs have been described for these two approaches [3,4].

Recently, Kokame and coworkers showed that the metalloproteinase ADAMTS13 has a minimal substrate size of approximately 73 amino acid residues derived from the A2 domain of von Willebrand protein [5]. This group has succeeded in creating a suitable FRET substrate by chemical synthesis using Nma–Dnp as the donor–acceptor pair flanking the scissile bond [6]. We sought a facile recombinant method for generating a suitable FRET substrate, the sequence of which could be easily modified. The challenge was to devise a method of introducing a fluor–quencher pair.

A recombinant 73-amino acid peptide was generated using the M15 *Escherichia coli* expression system. The sequence coding for the critical 73-amino acid fragment of the A2 domain of von Willebrand protein, D1596–R1668 (VWF73), was amplified from genomic DNA. The PCR primers were designed to introduce a *Bam*HI site at the 5′ end (5′-AAG GCC GGA TCC GAC CGG GAG CAG GCG CCC-3′) and *Sal*I at the 3′ end (5′-AAG GCC GTC GAC CTA CCT CTG CAG CAC CAG GTC-3′) of the expected 246-bp product. The PCR product was cloned into the pQE-100-Double Tag plasmid (Qiagen, Valencia, CA, USA). The peptide amino acid sequence is shown below:

* Corresponding author.
E-mail address: lzhang789@yahoo.com (L. Zhang).

[1] *Abbreviations used:* FRET, fluorescence resonance energy transfer; IPTG, isopropyl β-D-1-thiogalactopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCEP, Tris(2-carboxyethyl)phosphine; TFA, trifluoroacetic acid; PNP, Pooled Normal Plasma.

0003-2697/$ - see front matter
doi:10.1016/j.ab.2006.06.022

```
  1   M R G S H H H H H H G S D R E C A P N L
  1   ATGAGAGGATCGCATCACCATCACCATCACGGATCCGACCGGGAGTGCGCGCCCAACCTG

 21   V Y M V T G N C A S D E I K R L P G D I
 61   GTCTACATGGTCACCGGAAATTGCGCCTCTGATGAGATCAAGAGGCTGCCTGGAGACATC

 41   Q V V P I G V G P N A N V Q E L E R I G
121   CAGGTGGTGCCCATTGGAGTGGGCCCTAATGCCAACGTGCAGGAGCTGGAGAGGATTGGC

 61   W P N A P I L I Q D F E T L P R E A P D
181   TGGCCCAATGCCCCTATCCTCATCCAGGACTTTGAGACGCTCCCCCGAGAGGCTCCTGAC

 81   L V L Q R -
241   CTGGTGCTGCAGAGGTAG
```

The native VWF sequence begins with DRE. The six histidines were introduced by the PQE-100 vector and confer affinity for nickel on the expressed peptide.

Qiagen's *E. coli* M15 (pREP4) was transformed with the pQE-100–VWF-73 vector using a MicroPulser electroporation apparatus (Bio-Rad, Hercules, CA, USA). Transformed M15 *E. coli* colonies were screened by PCR amplification of the plasmid insert. Primers used to amplify the insert were as follows: right, 5′-GGA AAC GTG CAG TCT CTT CA-3′; left, 5′-GAG AGG ATC GCA TCA CCA TC-3′. The sequence of the insert was confirmed using ABI Big Dye Terminator chemistry.

When two identical fluorescent molecules are in close proximity, emission is quenched due to reciprocal intermolecular interaction [7]. This gave us impetus to investigate whether introducing two fluorescein moieties, flanking the scissile bond, would give a functional FRET substrate.

Two cysteines flanking the tyrosine–methionine (Y1605–M1606) ADAMTS13 cleavage site were introduced by site-directed mutagenesis to serve as flourescein docking sites. The two cysteines are separated by 11 amino acids on the resulting VWF73 peptide. Using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), the first cysteine was introduced at P1611 (P28C), 5 amino acids away from the cleavage site on the carboxyl-terminal side of the peptide. The mutagenesis primer sequences are as follows: left, 5′-TCT ACA TGG TCA CCG GAA ATT GCG CCT CTG ATG AGA TCA AGA G-3′; right, 5′-CTC TTG ATC TCA TCA GAG GCG CAA TTT CCG GTG ACC ATG TAG A-3′. A subsequent mutagenesis reaction introduced a second cysteine at the Q1599 (Q16C) position on the amino side of the peptide. The mutagenesis primer sequences are as follows: left, 5′-GAT CCG ACC GGG AGT GCG CGC CCA ACC TGG T-3′; right, 5′-ACC AGG TTG GGC GCG CAC TCC CGG TCG GAT C-3′. Transformed *E. coli* colonies were screened using PCR amplification of the plasmid insert and digestion with *Ban*I. Positive colonies were identified by specific cleavage patterns and confirmed by DNA sequencing.

An expression culture from a clone shown to have the appropriate sequence was grown to $OD_{600} = 0.60$ in Luria broth, Miller formulation, with 25 μg/ml kanamycin and 100 μg/ml ampicillin. Peptide production was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h. The peptide, VWF73 with P28C and Q16C (VWF73–2Cys), was harvested using the CelLytic B Plus Kit (Sigma, St. Louis, MO, USA). Each liter of expression culture yielded an average of 2 g of bacterial paste. On extraction, the bulk of the peptide was found in the first lysis supernatant. Approximately 1 mg of fluorescein-labeled peptide was obtained from 2 g of bacterial paste. Peptide size (9.5 kDa) and specific fluorescent labeling as confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) are shown in Fig. 1.

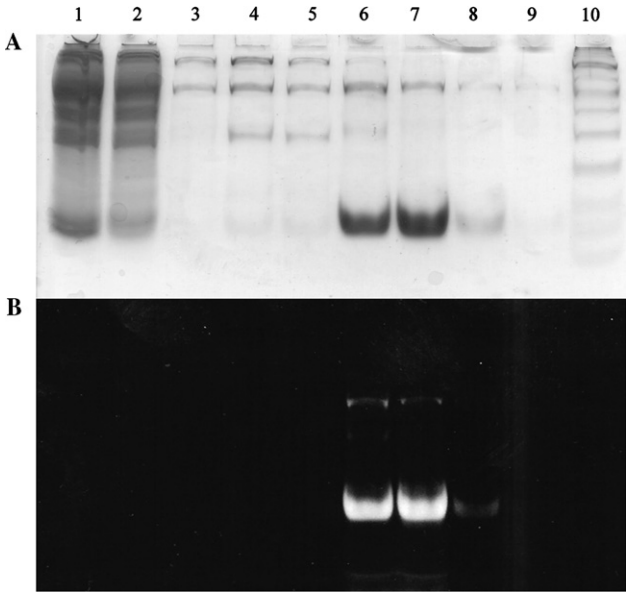

Fig. 1. (A) Coomassie blue-stained PAGE gel. Lanes 1–5 show nickel column flow-through and washes followed by fluorescein-labeled peptide elution in lanes 6 to 8. (B) UV stimulation–fluorescence image of the PAGE gel. Lanes 6 to 8 show fluorescein-labeled peptide present only in the elution fractions.

The culture extraction supernatant was loaded onto a 1-ml HisTrap HP Nickel Sepharose affinity column (Amersham Biosciences, Piscataway, NJ, USA) equilibrated with 50 mM sodium phosphate, 300 mm of 1 M NaCl, and 20 mM imidazole at pH 8.0. After loading and washing, 20 mM Bond-Breaker Tris(2-carboxyethyl)phosphine (TCEP, Pierce Biotechnology, Rockford, IL, USA) was used to reduce the peptide on the column. The reduced cysteines on the peptide were labeled with two identical fluorescein moieties, whereas the peptide was still on the affinity column. Fluorescein-5-maleimide (2 mg in 1 ml equilibration buffer, Pierce) was loaded onto the column and allowed to react for 15 min. The column was washed with equilibration buffer, and then fluorescein-labeled peptide was eluted from the column in 50 mM sodium phosphate, 500 mM NaCl, and 250 mM imidazole. For our particular assay purposes, the labeled peptide was desalted into 1% trifluoroacetic acid (TFA) using a Bio-Rad 10 DG column and dried to a pellet using high-speed vacuum concentration.

Free sulfhydryl groups were measured in the eluted and labeled peptide to test the labeling efficiency, and there were no detectable free sulfhydryl groups. The peptide was adequately labeled with fluorescein.

The fluorescence and quenching properties of the peptide initially were analyzed with chymotrypsin, a nonspecific protease that cleaves the Y–M bond at the

ADAMTS13 cleavage site in addition to any tryptophan (W) and phenylalanine (F) amino acid bonds. The labeled VWF73–2Cys was digested with serial dilutions of chymotrypsin. Conditions were as follows: pH 7.4 at room temperature for 15 min. A dose-dependent increase in fluorescence release from the peptide was observed. The maximum increase was approximately 2.5-fold after complete digestion (Supplementary Fig. 2A), demonstrating that separation of self-quenching fluors was sufficient to generate a measurable fluorescence increase.

An assay specific for ADAMTS13 was then performed with the rate of fluorescence increase being measured using standard dilutions of Pooled Normal Plasma (PNP, Precision Biologics, Dartmouth, Nova Scotia, Canada) in heat-inactivated PNP. All plasma samples were diluted 1:8 with assay buffer (5 mM Bis–Tris, 25 mM $CaCl_2$, and 0.005% Tween 20 at pH 6.0) before being mixed 1:1 with a working peptide dilution (0.043 mg/ml, 4.55 μM). Final molarity of the peptide in the assay was 2.28 μM. All reactions were performed on Corning solid white 96-well assay plates (Corning, NY, USA) in a final reaction volume of 200 μl. Fluorescence increase was measured kinetically using a Cytofluor series 4000 (PerSeptive Biosystems, Foster City, CA, USA) with the wavelengths of the excitation filter at 485 nm and the emission filter at 530 nm. Fluorescence readings were taken every 1 min for 60 min. The reactions were performed in triplicate, and mean values were used to establish a standard curve and assess unknowns. Heat-inactivated PNP and known ADAMTS13-deficient patient plasmas were used as negative controls.

A specific ADAMTS13 enzyme kinetic study was performed by incubating the peptide with dilutions of PNP in heat-inactivated PNP. A nearly linear standard curve of rates of fluorescence increase versus ADAMTS13 activity is shown in Supplementary Fig. 2B. The correlation between the ADAMTS13 activity and the rate of fluorescence increase (even at very low enzyme concentrations) makes it a sensitive and specific assay.

In this study, we have provided proof of principle that large peptide substrates suitable for FRET-based protease assays can be generated by recombinant technology. In addition, the FRET effect produced by the incorporation of two identical proximate self-quenching fluors is adequate for fluorogenic assay. The modified substrate can be effectively produced in large quantities in bacteria, and the use of a poly-histidine tag simplifies the purification. Thus, the efficiency of production is high and could be enhanced by further optimization of expression conditions and/or host system. In addition, peptide labeling is achieved in one step during the purification because the use of identical fluorophores obviates the need for a second quencher molecule.

In our peptide, both fluorescein molecules will fluoresce on separation, resulting in a greater fluorescence as compared with a peptide labeled with one fluorescein molecule and one quencher molecule. In theory, a recombinant dual-labeled fluorogenic peptide substrate may prove to be a better alternative for FRET-based protease assays.

The locations of the docking cysteines are critical for an optimal FRET effect. Labeling with a fluorophore requires an optimal intercysteine distance as defined by the Förster radius of the chosen fluorophore [2]. Fluors with larger Förster radii would produce more efficient self-quenching. This also would allow greater separation of the labeled cysteines. Thus, optimization of each enzyme–substrate system may be achieved by systematic examination of cysteine–fluorophore placement.

Using the methods of protein modification we have described, it would be simple to test variations in amino acid sequence or to modify larger substrates to enhance the performance of the FRET substrate. The major limitation of our method is that the substrate cannot contain an essential cysteine because the reduction of disulfide bonds is required for fluor labeling. On the other hand, to avoid unwanted labeling, nonessential cysteines could be mutated so that only the cysteines flanking a cleavage site remain. Again, systematic examination of the cysteine placement might identify substrates with even more fluorescence increase after enzymatic cleavage.

In summary, large peptide substrates suitable for use in FRET-based protease assays can be generated by recombinant technology and subsequently labeled with a self-quenching fluorophore. This method will significantly expand the application of FRET-based assays to proteases that require relatively large minimal substrates.

Acknowledgment

This work was supported, in part, by an unrestricted grant from the MacKay Foundation for Cancer Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.06.022.

References

[1] M. Dahan, A.A. Deniz, T.J. Ha, D.S. Chemla, P.G. Schultz, S. Weiss, Ratiometric measurement and identification of single diffusing molecules, Chem. Phys. 247 (1999) 85–106.

[2] L. Stryer, Fluorescence energy transfer as a spectroscopic ruler, Annu. Rev. Biochem. 47 (1978) 819–846.

[3] J.E. Sheppeck, H. Kar, L. Gosink, J.B. Wheatley, E. Gjerstad, S.M. Loftus, A.R. Zubiria, J.W. Janc, Synthesis of a statistically exhaustive fluorescent peptide substrate library for profiling protease specificity, Bioorg. Med. Chem. Lett. 10 (2000) 2639–2642.

[4] B.F. Le Bonniec, T. Myles, T. Johnson, C.G. Knight, C. Tapparelli, S.R. Stone, Characterization of the P2′ and P3′ specificities of thrombin using fluorescence-quenched substrates and mapping of the subsites by mutagenesis, Biochemistry 35 (1996) 7114–7122.

[5] K. Kokame, M. Matsumoto, Y. Fujimura, T. Miyata, VWF73, a region from D1596 to R1668 of von Willebrand factor, provides a minimal substrate for ADAMTS-13, Blood 103 (2004) 607–612.

[6] K. Kokame, Y. Nobe, Y. Kokubo, A. Okayama, T. Miyata, FRETS–VWF73, a first fluorogenic substrate for ADAMTS13 assay, Br. J. Haematol. 129 (2005) 93–100.

[7] X. Zhuang, T. Ha, H.D. Kim, T. Centner, S. Labeit, S. Chu, Fluorescence quenching: a tool for single-molecule protein-folding study, Proc. Natl. Acad. Sci. USA 97 (2000) 14241–14244.