Received 4 September 1998; accepted 19 April 1999.

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Use of GFP as a Reporter for the Facile Analysis of Sequence-Specific Proteases

BioTechniques 27:28-32 (July 1999)

A large number of sequence- and structure-specific peptidases are recognized to play important roles in cell biology, although they are relatively difficult to characterize (1,3). A major factor limiting the characterization of sequence- and structure-specific peptidases is the time-consuming nature of their assay. The requirement for specific amino acid sequences or peptide structures generally precludes the use of the small chromogenic peptide analog substrates, which have proven so useful with catalytic peptidases. The most common assay for sequence- and structure-specific proteases requires in vitro synthesis of radiolabeled protein substrates, incubation of the substrate with an enzyme, separating the reaction products by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and quantitating the amount of proteolytic product with a phosphor imager or by densitometry of fluorographs. The time and labor-intensive nature of this assay represents a major impediment to the purification and characterization of these interesting enzymes.

Development of a rapid and convenient assay with general applicability to structure- and sequence-specific proteases will greatly assist in the purification and analyses of these important enzymes. Figure 1 portrays a simple and rapid fluorescent assay that can be

Figure 1. Schematic representation of the fluorescent assay for site-specific proteases.

We thank Michael Wessels and Dennis Kasper for helpful discussion and for reagents used in the assays of group B polysaccharide. This work was supported by Public Health Service (PHS) Grant Nos. AI38424 and AI33963 and PHS Contract Nos. AI25152 and AI732600. Address correspondence to Dr. David E. Kling, Department of Pediatric Surgery, WRN-1024, Massachusetts General Hospital, 32 Fruit Street, Boston, MA 02114, USA. Internet: kling@helix.mgh.harvard.edu
adapted for the assay of most site- and sequence-specific proteases. The basis of this assay is a protease substrate composed of an N-terminal His tag and a polypeptide containing the peptidase cleavage site fused to green fluorescent protein (GFP). Proteolysis releases GFP from the fusion protein, and batch metal-chelate affinity chromatography is used to separate the fluorescent uncleaved fusion protein (pellet) from GFP liberated by proteolysis (Figure 1). The amount of liberated GFP in the supernatant is then measured in a spectrofluorometer.

A plasmid, pETEGFP, encoding a protein containing an N-terminal His tag and a 23-amino acid peptide containing a thrombin cleavage site and EGFP (CLONTECH Laboratories, Palo Alto, CA, USA), was constructed by excising the EGFP coding region from the pEGFP vector as a BamHI/NorI fragment and cloning it into the BamHI/NorI site of the bacterial expression vector pET28b (Novagen, Madison, WI, USA). The fluorescent thrombin substrate was produced by growing Escherichia coli BL-21 (DE-3) cells transformed with the pETEGFP plasmid in 3 L of LB media containing 50 µg/mL kanamycin at 37°C. Protein expression was induced at a culture density of 1.8 A600 by addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 6 h. Cells were harvested by centrifugation at 6000 × g for 10 min, resuspended in TALON Binding Buffer (CLONTECH) and lysed by sonication. The soluble cell fraction obtained after centrifugation at 32 500× g for 15 min was incubated at 70°C for 5 min. Under these conditions, most cellular proteins were denatured with no loss of EGFP fluorescence. Denatured proteins were removed by centrifugation at 32 500× g for 15 min, and the EGFP fusion product in the supernatant was purified using metal-chelate chromatography on commercially available TALON Metal Affinity Resin (CLONTECH) as described by the manufacturer. In the case of heat-labile fusion proteins, the soluble cell fraction can be applied directly to the TALON Column (CLONTECH). Omission of the heat treatment has no effect on the subsequent purification of the fusion protein substrates, but this does result in longer column chromatographic times due to increased protein load. After elution from the metal-chelate column, the purified fusion protein substrate was buffer-exchanged into thrombin buffer (20 mM Tris-HCl, 150 mM NaCl, 2.5 mM CaCl2, pH 8.4; Novagen) and adjusted to a fluorescence of approximately 46 000 fluorescence U (Flu)/mL (Ex = 484 nm; Em = 509 nm). Thrombin was assayed by incubation at 37°C in thrombin buffer containing varying or fixed amounts of the purified EGFP fusion protein. Aliquots of 200 µL were removed from the reaction mixtures at specific time points (during the linear phase of the assay) and heat-treated at 70°C for 5 min to stop the reaction by denaturation of thrombin. The heat-treated sample was mixed with 200 µL of 5x binding buffer, 200 µL of a 1:1 aqueous slurry of TALON Beads (CLONTECH) and 400 µL of water and shaken at 200 rpm for 30 min at room temperature. After a brief centrifugation at 13 000× g for 30 s to sediment the beads containing unreacted substrate and His-tag fusion peptides, the supernatant was analyzed for released GFP fluorescence in a Model LS 50 Luminescence Spectrofluorimeter (Perkin-Elmer, Norwalk, CT, USA). TALON was the most effective resin tested, removing greater than 97% of the fluorescent protein from solution in the absence of thrombin.

Preliminary experiments indicated that the crude bacterial cell-free extract containing the EGFP fusion protein substrate contained inhibitors of throm-
bin. Purification of the substrate by metal-chelate chromatography removed the inhibitory materials producing a substrate suitable for protease assays. At saturating substrate concentrations, the rate of thrombin-dependent release of EGFP from purified EGFP fusion protein substrate was linear for at least 60-min over a thrombin concentration range of 0.57–4.6 U/µL reaction (Figure 2A). At the highest thrombin concentration tested, approximately 23% of the EGFP added to the reaction was released from the fusion protein. By sampling at multiple time points over a 60-min period and using a least-squares linear regression analysis to fit a line to the experimental points, differences in background levels do not interfere with the accurate measurement of reaction rates. Lines fitted to the experimental points (Figure 2A) by least-squares linear regression analysis had correlation coefficients >0.95, indicative of the accuracy of the rate determinations. Over approximately a tenfold concentration difference of thrombin, the reaction rates, as determined by linear regression analysis, were directly proportional to the amount of thrombin added to the assay (Figure 2B).

A useful enzyme assay requires a simple method for measuring the amount of substrate converted to product. Conversion must also be linear with time and proportional to the amount of enzyme added, and sufficient purified substrate must be readily available to perform reactions at saturating substrate concentrations. Using thrombin as a model protease, release of EGFP from an EGFP fusion protein was shown to be proportional to time and the amount of thrombin present in the assay. The assay was sensitive and had a large dynamic range capable of measuring differences of at least an order of magnitude in thrombin concentration. The assay is also rapid, requiring a minimum number of steps, thus making it suitable for assaying the large number of samples generated during enzyme purification.

The major limitation to the general use of this assay to measure sequence-specific proteases will be the ability to produce large quantities of a soluble His-tagged EGFP fusion protein having the necessary protease recognition sequence. EGFP has been fused to a large number of proteins producing soluble biologically active fluorescent proteins (5). Conditions for soluble expression of foreign proteins in bacterial cells is becoming better understood. The ability to easily engineer EGFP fusions with exotic protease recognition sequences and inexpensively express them as soluble proteins in bacterial cells makes this assay ideally suited for the characterization of a large variety of sequence- and domain-specific peptidases.

An assay for site-specific proteases has been published based on measuring fluorescent resonance energy transfer (FRET) between two GFP variants linked by the protease target sequence (2). Cleavage of the substrate separates the two GFPs, thus preventing resonance energy transfer. Although very elegant, FRET is a very complex function of the distance between the chromophores and the angle formed by their dipole moments (4), requires specialized instrumentation and is limited to assaying one sample at a time. Measuring the direct release of EGFP from a fusion protein as described in this paper is simpler, a more suitable method for the rapid analysis of many samples for enzyme kinetics and provides a good complementation to other fluorescence-based proteolytic assays.

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This work was supported in part by a grant from the Center for Biotechnology, University of Nebraska-Lincoln funded through the Nebraska Research Initiative (G.S., J.M. and S.D.S.), The National Science Foundation Grant No. MCB-9630817 (S.D.S.) and by the Howard Hughes Medical Institute for Undergraduate Research Support. This paper is No. 12481 in a journal series from the Nebraska Agricultural Research Division. Address correspondence to Dr. Steven D. Schwartzbach, E-207 The Beadle Center, School of Biological Science, University of Nebraska-Lincoln, Lincoln, NE 68588, USA. Internet: schwartz@biocomp.unl.edu

Received 15 December 1998; accepted 23 April 1999.

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