Reports

A bioluminescent assay for the sensitive detection of proteases

Donna M. Leippe[1], Duy Nguyen[2], Min Zhou[3], Troy Good[3], Thomas A. Kirkland[3], Mike Scurria[3], Laurent Bernad[3], Tim Ugo[3], Jolanta Vidugiriene[1], James J. Cali[1], Dieter H. Klaubert[3], and Martha A. O'Brien[1]

[1]Research and Development, Promega Corporation, Madison, WI, USA, [2]Protein Purification, Promega Corporation, Madison, WI, USA, and [3]Promega Biosciences, Inc., San Luis Obispo, CA, USA

BioTechniques 50:105-110 (August 2011) doi 10.2144/000113716

Keywords: protease substrates; peptide substrates; protease contamination; general protease assays; trypsin assay; bioluminescence; bioluminescent protease assays; luciferase

Supplementary material for this article is available at www.BioTechniques.com/article/113716.

A bioluminescent general protease assay was developed using a combination of five luminogenic peptide substrates. The peptide-conjugated luciferin substrates were combined with luciferase to form a homogeneous, coupled-enzyme assay. This single-reagent format minimized backgrounds, gave stable signals, and reached peak sensitivity within 30 min. The bioluminescent assay was used to detect multiple proteases representing serine, cysteine, and metalloproteinase classes. The range of proteases detected was broader and the sensitivity greater, when compared with a standard fluorescent assay based on cleavage of the whole protein substrate casein. Fifteen of twenty proteases tested had signal-to-background ratios >10 with the bioluminescent method, compared with only seven proteases with the fluorescent approach. The bioluminescent assay also achieved lower detection limits (≤100 pg) than fluorescent methods. During protein purification processes, especially for therapeutic proteins, even trace levels of contamination can impact the protein's stability and activity. This sensitive, bioluminescent, protease assay should be useful for applications in which contaminating proteases are detrimental and protein purity is essential.

Proteases are enzymes that catalyze the hydrolysis of protein peptide bonds. Ubiquitous in nature, proteases are present in all living organisms and have multiple and essential functions in cellular processes (1). They form a large, diverse group that is classified into six classes based on catalytic mechanism: serine, cysteine, aspartic, metallo, threonine, and glutamic (1). They are referred to as endoproteinases, responsible for cleaving internal peptide bonds, or as exopeptidases, which cleave terminal amino acids from either the N or C terminus (aminopeptidases and carboxypeptidases, respectively). A comprehensive listing and classification of proteases can be found in the *MEROPS* database (http://merops.sanger.ac.uk) (2). Due to their widespread and abundant nature, proteases are unavoidable and occur as contaminants during protein purification processes, negatively impacting the purified protein's yield, purity, activity, and stability. It is necessary to remove these deleterious activities either through the protein purification process itself or by inhibition. Broad-spectrum inhibitors or mixtures of inhibitors are typically used to inactivate proteases, but there are many downstream applications in which the presence of inhibitors is detrimental. In particular, the presence of neither proteases nor inhibitors is acceptable when producing biologics and biosimilars for the therapeutic market. A sensitive method for detecting trace amounts of proteolytic activity is important for validating the effectiveness of protein purification methods during process development stages, as well as confirming the absence of protease in final purified proteins.

Activity assays for a specific protease typically use a single synthetic substrate consisting of a peptide of known sequence conjugated to a fluorophore (3). However, when assaying multiple proteases, proteases of unknown identities, or proteases with unknown or different sequence requirements, a single peptide substrate is not suitable or sufficient. These applications, including protein purification, have used whole proteins, such as gelatin and casein, as universal protease substrates (4–6). Several colorimetric and fluorometric general protease assays are based on the cleavage of casein in solution (7,8). Using fluorophore-labeled casein, caseinolytic activity can be measured by increased fluorescence due to either the release of small fragments that remain in the supernatant after trichloroacetic acid (TCA) precipitation (7) or the release of fluorescent small fragments from heavily conjugated, and therefore quenched, undigested protein (8). Drawbacks of the casein-based assays include multiple manipulations such as TCA precipitation steps, long incubations (e.g., overnight), and poor sensitivity.

Bioluminescent protease assays employing peptide-conjugated aminoluciferins were developed as an alternative to peptide-conjugated fluorophore assays to capitalize on the sensitivity of bioluminescence and to avoid fluorescence interference issues (9–11). The aminoluciferin derivatives, or "pro-luciferins," are not utilized by firefly luciferase until first processed by a protease that can release the peptide and free aminoluciferin. The first reported bioluminescent protease assays based on peptide-conjugated aminoluciferins were two-step assays, whereby the protease cleavage was allowed to occur prior to the addition of luciferase (9,10). Subsequently, homogeneous bioluminescent protease assays were developed that coupled the protease and luciferase activ-

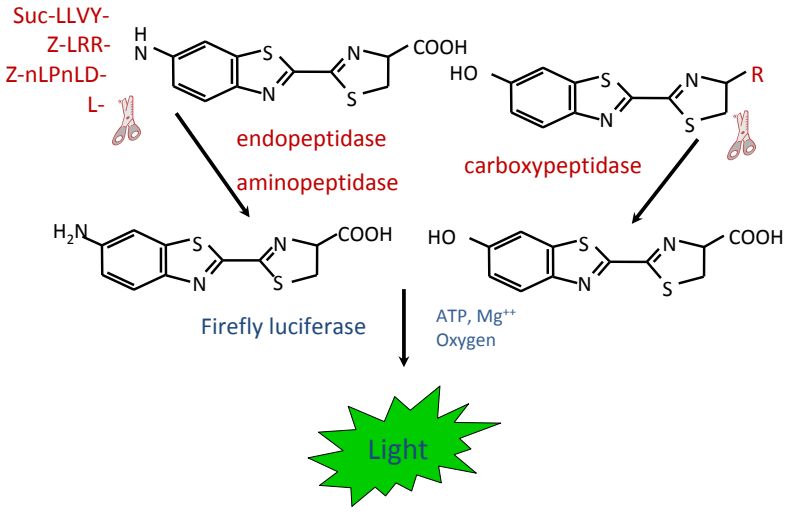

Figure 1. Concept for a bioluminescent protease assay. Protease substrates in this assay are luciferins modified with single amino acids, tripeptides, or tetrapeptides. Five such modified luciferins are depicted. These derivatized luciferins are not substrates for firefly luciferase. However, in the presence of proteolytic activities that can cleave the bond between the amino acid and luciferin, free aminoluciferin and/or luciferin are released and utilized in the bioluminescent reaction. The generated light signal is proportional to the amount of protease activity and released aminoluciferin and/or luciferin. Amino acids are represented by single-letter code; nL, nor-leucine; Suc, succinyl blocking group; Z, carboxybenzyl blocking group.

ities into a single step (12). The coupled system generated a continuous, stable signal that was proportional to the amount of protease. Using this technology, assays have been developed for several proteases including caspases, calpain, dipeptidyl peptidase IV, and the three catalytic activities of the proteasome (12–15). In all cases, the bioluminescent protease assays have been demonstrated to be substantially more sensitive than fluorescent assays using comparable peptide-conjugated substrates (15).

The improved sensitivity of bioluminescent protease assays led to the idea of creating a general protease assay by combining luminogenic peptide substrates. The mixture needed to contain substrates that could detect a broad range of proteases and retain good sensitivity. This meant optimizing the concentrations of, and limiting the number of, substrates to keep the additive endogenous background to a minimum. The substrates for the detection of the three catalytic activities of the proteasome were considered to be an excellent starting point, because the proteasome hydrolyzes hundreds to thousands of proteins using three distinct catalytic activities that cleave peptide bonds following basic, hydrophobic, or acidic residues (16,17). Substrates for these three catalytic activities would likewise be expected to be cleaved by many endoproteinases that recognize basic, hydrophobic, or acidic residues. We combined the three proteasome substrates with two other single amino acid-conjugated aminoluciferins designed for the detection of exopeptidases. In this report, we demonstrate the detection of a broad range of proteases from multiple classes using this mixture of five luminogenic substrates. Comparison of this bioluminescent system to fluorescent, casein-based methods shows that the range of detection is broader, the sensitivity is greater, and the assay is simpler and faster to use. Also, an additional advantage of the substrate mixture strategy is the ability to use the substrates individually to confirm and further characterize any proteolytic activity.

Materials and methods

Proteases

Proteases were obtained from Sigma-Aldrich (St. Louis, MO, USA), Promega (Madison, WI, USA), and EMD Chemicals (Gibbstown, NJ, USA). Details can be found in the Supplementary material.

Luminescent protease substrates

Luminogenic protease substrates, Succinyl-Leu-Leu-Val-Tyr-aminoluciferin (Suc-LLVY-aminoluciferin), Z-Leu-Arg-Arg-aminoluciferin (Z-LRR-aminoluciferin), and Z-Nle-Pro-Nle-Asp-aminoluciferin (Z-nLPnLD-aminoluciferin), were synthesized using standard Fmoc chemistry (12) and have been previously described (13,14). The synthesis and analytical characterization of Leu-aminoluciferin (H-L-aminoluciferin) and luciferin-Arg (Luc-R-OH) are described in the Supplementary material.

Luminescent protease assay

Modified luciferin substrates were added to reconstituted Luciferin Detection Reagent (LDR; Promega), which contains a thermostable, recombinant form of firefly luciferase (18) and ATP required for the luciferase reaction. The LDR reconstitution buffer was 100 mM HEPES, pH 7.1, and 10 mM $MgSO_4$. The substrate/LDR mixture was preincubated at room temperature for 1 h to allow the luciferase to consume any trace amount of free luciferin present in the substrates, which can contribute to the background signal. The protease assay was then performed in a total volume of 100 µL, composed of 50 µL substrate/LDR mixture and the indicated amount of protease in 50 µL 10 mM HEPES, pH 8.0. Peptide-luciferin substrates were each present at a concentration of 15 µM in the final reaction. All incubations were carried out directly in the wells of a 96-well white luminometer plate at room temperature for the indicated time (typically 10–60 min), after which luminescence was monitored using a GloMax 96 Microplate Luminometer (Promega).

Fluorescent protease assays

Three fluorescent assays were used and conducted according to the manufacturer's instructions. The Protease Fluorescent Detection kit (Sigma-Aldrich) utilizes the fluorescein isothiocyanate (FITC)–casein substrate (7) and requires TCA precipitation to separate cleaved fragments from undigested protein. The casein cleavage reactions contained the indicated amount of protease in 50 µL 500 mM Tris buffer, pH 8.5, and were incubated in 1.5-mL microcentrifuge tubes at 37°C for 60 min in the dark. TCA was then added to precipitate undigested casein. The supernatant was neutralized, and a sample corresponding to 0.5 µL initial protease reaction was transferred to the wells of a 96-well black plate. The EnzChek Protease Assay kit (Life Technologies, Carlsbad, CA, USA) consists of casein protein that is labeled with green-fluorescent BODIPY FL dye (8). The conjugation density is such that the fluorophore is quenched until smaller fragments are released into solution upon cleavage by proteases; this approach eliminates the need for a TCA precipitation step (8). Protease was added to 100 µL reconstituted BODIPY FL-casein (10 µg/mL) and 100 µL 1× digestion buffer (10 mM Tris-HCl, pH 7.8) in wells of a 96-well black plate and incubated in the dark at room temperature. Fluorescence was measured after an overnight incubation (20 h) to obtain maximal sensitivity as recommended by the manufacturer. The third fluorescent protease assay was a peptide-conjugated rhodamine 110 fluorophore [Bis-(CBZ-Arg)-R110, BZAR; Life

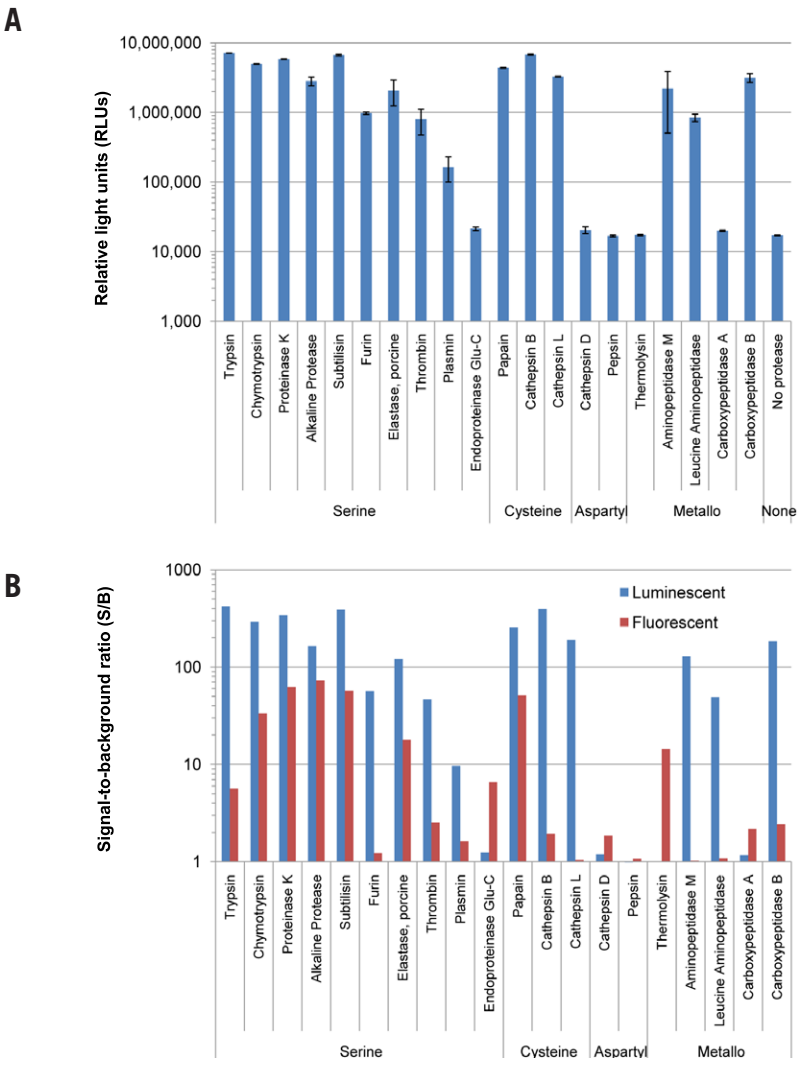

Figure 2. Broad detection coverage and high S/B ratios using the bioluminescent protease assay. (A) The mixture of five peptide-luciferins was reacted with a panel of 20 proteases, each at a final concentration of 1 µg/mL (i.e., 100 ng/100 µL reaction), except for alkaline protease and Furin, for which no concentration was provided. These two proteases were used at final concentrations of 5 and 20 U/mL, respectively. Each reaction was conducted for 30 min in triplicate, and the average RLUs were calculated. Error bars represent ±1 SD. (B) To compare the sensitivity of the bioluminescent and fluorescent methods, proteases at a final concentration of 1 µg/mL (except as noted above) were also incubated with the BODIPY FL-casein substrate overnight for 20 h. Each reaction was performed in triplicate. The average signals were used to calculate S/B ratios for both methods. The absolute RLUs and FLUs can be found in Supplementary Table S1 in the Supplementary material. Additional data concerning intra- and interexperiment variation of the luminescent assay can be found in Supplementary Table S2 and Supplementary Figure S1.

Technologies] used as a substrate for many serine proteases. Reactions with the peptide substrate (final concentration 20 µM) were conducted in 100 µL 10 mM HEPES, pH 8.0, in wells of a 96-well black plate for 3 h in the dark at room temperature. For all three assays, fluorescence intensity was measured at excitation and emission wavelengths of 485 and 527 nm, respectively, using a fluorescent plate reader (Thermo Scientific Fluoroskan Ascent; Thermo Scientific, Waltham, MA, USA).

Results and discussion

Bioluminescent general protease assay concept

A general assay for monitoring contaminating proteases needs to be sensitive and capable of detecting many proteases from multiple classes. We developed a set of peptide-conjugated luciferin substrates that, when combined, were likely to detect a wide variety of proteases from multiple classes. The mixture included five substrates: Z-LRR-aminoluciferin,

Suc-LLVY-aminoluciferin, Z-nLPnLD-aminoluciferin, H-L-aminoluciferin, and luciferin-R-OH. The first three were optimized for detecting the catalytic activities of the proteasome and were included to detect proteases selective for basic, hydrophobic, and acidic residues, respectively. The last two were included to detect aminopeptidases and carboxypeptidases, respectively. The assay principle (Figure 1) is based on a coupled-enzyme system. All five of the protease substrates and all components for the luciferase detection reaction were combined into one reagent. A brief incubation step allowed luciferase to utilize trace amounts of contaminating unconjugated luciferin or aminoluciferin. This reduced the background and increased assay sensitivity. Then, upon adding protease, both the proteolytic and luminescent reactions occurred simultaneously (12). This single reagent addition simplified the assay protocol and allowed for a homogeneous, continuous-read assay.

Bioluminescent protease assay detects a broad range of proteases

To define its breadth of utility, the bioluminescent protease assay was tested with a selection of 20 purified proteases. Members of the serine, cysteine, aspartyl, and metalloproteinase classes were represented in this initial screen (Figure 2A and Supplementary Table S1). In this coupled-enzyme system, maximum sensitivity is rapidly reached in 20–30 min (data not shown), and therefore, luminescent readings were taken at 30 min. The background signal of the buffer control without protease was ~17,000 relative light units (RLUs) and is attributed to the cumulative inherent background of the five substrates. Of the 20 proteases tested, 15 proteases gave signals significantly above background, with RLUs >100,000 in all cases. For 11 of the 15 proteases, RLUs were >1,000,000 (Figure 2A). These high signals and low background resulted in signal-to-background (S/B) ratios that were at least 10 for the 15 detected proteases and over 100 for 11 of these proteases (Figure 2B).

In parallel with the bioluminescent protease assay, the panel of 20 proteases was tested with a fluorescent casein method using the BODIPY FL-casein substrate. This particular fluorescent method was used because it has fewer manipulations than other casein methods. However, the reaction was incubated for 20 h to get maximum signal as recommended in the manufacturer's instructions, compared with the 30-min incubation for the luminescent assay. The signals ranged from ~350 fluorescent light units (FLUs) for the negative control lacking protease to the highest signal of ~26,000 FLUs (Supplementary Table S1). In comparison to the luminescent assay, none of the proteases resulted in S/B ratios >100 (Figure 2B). Seven had S/B ratios between 10 and 100, and 13 had S/B ratios ≤10, with S/B ratios ≤2 for 8 of those proteases (Figure 2B). We also calculated signal-to-noise ratios (S/N; net signal divided by the standard deviation of the background) (Supplementary Table S1). The S/N ratios were ≥1000 for 14 of the proteases detected using the luminescent assay and ≥10,000 for 11 of these. In contrast, all S/N ratios were below 10,000 with the fluorescent assay, and only five proteases had S/N ratios ≥1000.

We expected that some proteases would not be detected with the bioluminescent protease assay. For the luciferin to become a substrate for luciferase, all amino acids must be removed from the peptide-conjugated luciferin. Therefore, the bioluminescent protease assay will not detect proteases that require a peptide bond and have absolute requirements for amino acids in P′ sites (i.e., amino acids in positions on the carboxyl side of the cleaved bond) (19). Examples of such endopeptidases are thermolysin and the aspartyl proteases, which were tested in this assay, and the matrix metalloproteinases, which were not included in this study. The bioluminescent protease assay also does not detect proteases with specific amino acid requirements not present in the substrate mixture. An example is endoproteinase Glu-C, which cleaves only after glutamate residues. Also, the low signal from carboxypeptidase A (Figure 2B) was not unexpected, given its lack of preference for arginine residues (http://merops.sanger.ac.uk). The luciferin-R-OH substrate was optimized for B-type carboxypeptidases, but was also recognized by carboxypeptidases E, P, and Y (data not shown). This bioluminescent protease assay is performed at neutral pH, an unfavorable condition for proteases that are most active in acidic environments. Two examples are pepsin and cathepsin D, which had low activity at the neutral pH of either assay. Despite these limitations, only two of the 20 tested proteases, thermolysin and

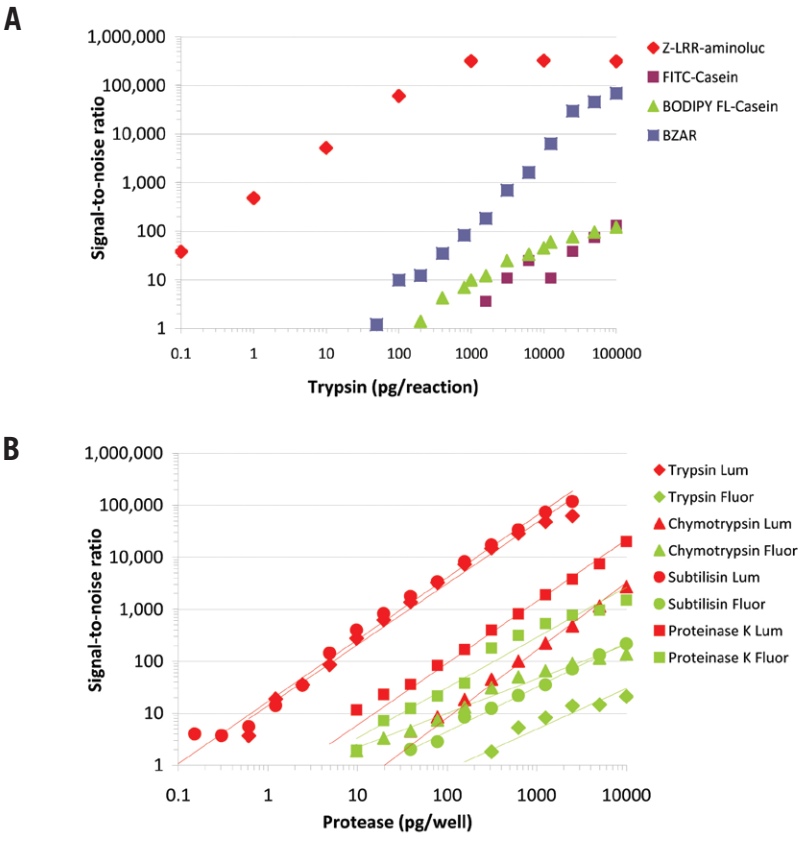

Figure 3. Sensitivity of the bioluminescent protease assay. (A) Titration of trypsin using the Z-LRR-aminoluciferin and three fluorescent substrates. Reactions were done in triplicate and were read after incubation times of 30 min (Z-LRR-aminoluciferin), 1 h (FITC-casein), 20 h (BODIPY FL-casein), and 3 h (BZAR). The S/N ratios were calculated using the formula: (mean of the signal - mean of the background)/standard deviation of the background. A value of S/N = 3 was defined as the limit of detection. (B) Four proteases were titrated and assayed with either the mixture of five peptide-luciferins or the BODIPY FL-casein substrate. All reactions were done in triplicate for 1 h (luminescent) or 20 h (fluorescent). The S/N ratios were calculated as described above. R^2 values for the trend lines are 0.98, 0.97, 0.99, and 0.97 for the luminescent assay and 0.91, 0.97, 0.99, and 0.97 for the fluorescent assay of trypsin, chymotrypsin, subtilisin and proteinase K, respectively.

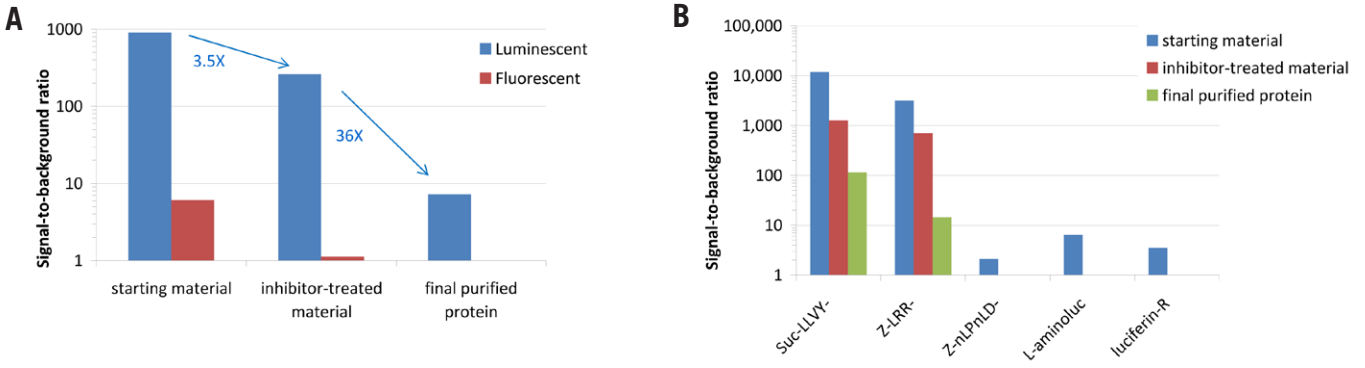

Figure 4. Monitoring protease contamination during a protein purification process. (A) Material collected from different steps in a purification protocol was tested using the mixture of five peptide-luciferins and the BODIPY FL-casein assay. All reactions were done in triplicate. Incubation times were 10 min for the luminescent assay and 20 h for the fluorescent assay. S/B ratios were calculated and normalized to the mass of protein present. The 3.5- and 36-fold reductions in signal, indicated by the arrows, were calculated using the luminescent assay. (B) The purification samples were also tested with each individual peptide-luciferin. Duplicate reactions were incubated for 10 min.

endoproteinase Glu-C, were readily detected by the fluorescent casein method but not the luminescent peptide method.

Although the casein protein is considered a universal protease substrate, the fluorescent casein assay did not efficiently detect exopeptidases, nor did it detect other proteases that were detected by the bioluminescent assay. Surprisingly, all aminopeptidases and carboxypeptidases had low signals with the BODIPY FL-casein substrate (Supplementary Table S1). This may be due to the inaccessibility of the termini in the whole protein. Furin, plasmin, and cathepsins B and L, resulted in S/B ratios of 10 to 400 in the bioluminescent assay, but ≤2 in the fluorescent assay (Figure 2B). These poor results in the casein assay were not easily predicted. Despite the theoretical universality of the whole-protein fluorescent assay and the known limitations of the peptide-based luminescent assay, the fluorescent assay did not achieve greater breadth of detection in our sample of 20 proteases. These results indicated that sensitivity, rather than universality, can be important for greater detection capabilities of a broader array of proteases and supported the hypothesis that a mixture of individual luminogenic substrates can provide broad detection of a variety of protease classes.

Bioluminescent protease assay has greater sensitivity and better linearity

Results of the initial screen of 20 proteases indicated that the luminescent general protease assay was more sensitive than the fluorescent casein assay. We investigated this by titrating select proteases to determine limits of detection. First, trypsin was titrated to compare the luminogenic, trypsin-like

substrate, Z-LRR-aminoluciferin, to three different fluorescent assays, including two casein-based assays and a peptide-based serine protease substrate, BZAR. The luminescent method was more sensitive than any of the three fluorescent assays (Figure 3A). The limit of detection of trypsin was estimated to be <0.1 pg for the Z-LRR-aminoluciferin substrate, 50 pg for the fluorescent peptide substrate, 200 pg for the BODIPY FL-casein, and 2 ng for the FITC-casein substrate (Figure 3A). Next, we compared the luminescent protease assay containing the complete mixture of five substrates to the BODIPY FL-casein assay using four proteases that had high S/B ratios (Figure 2B) in both assays: trypsin, chymotrypsin, subtilisin, and proteinase K. The luminescent assay demonstrated limits of detection of <1 pg for trypsin and subtilisin, approximately 100 times more sensitive than the fluorescent casein method (Figure 3B). The limit of detection for proteinase K at <10 pg was approximately three times lower than with the fluorescent assay. For chymotrypsin, the two methods demonstrated comparable limits of detection (~20–50 pg), although the linear range was greater for the luminescent assay. The linear range was greater for the luminescent assay with the other three proteases as well (Figure 3B).

Applications for protein purification

Production-scale protein purification, especially of commercial and therapeutic proteins, requires careful protocol optimization and the removal of proteases. The sensitivity, simplicity, and short incubation time of the peptide-luciferin approach suggested it might be useful for this application. Therefore, the luminescent assay was evaluated using a production-scale protein purification protocol for which the casein method had been found to be insensitive, laborious, and time-consuming. The two approaches were compared by testing three samples from this protocol: (*i*) the starting material, which was a protein partially purified from bovine pancreas; (*ii*) the starting material after the addition of PMSF serine protease inhibitor; and (*iii*) the final purified protein obtained following column purification. The fluorescent method detected contaminating protease activities in the first two samples, but not in the final material (Figure 4A). The luminescent method detected contaminating protease activities in all three samples and had the sensitivity necessary to detect trace contaminants in the final material. Higher signals of the luminescent assay also permitted quicker and more accurate measurements of contaminating proteases, and their removal and/or inactivation, at each step (Figure 4A).

An advantage of using a peptide mixture strategy over a whole protein approach is the ability to use the same technology to further characterize a positive signal by subsequently assaying with the individual substrates. Figure 4B shows the results of incubating all three samples with each of the five peptide-luciferins individually. The contaminating proteolytic activity was found to react with the Z-LRR- and Suc-LLVY-aminoluciferins, but not the other three. This information is useful for the optimization of the protocol and the addition of the appropriate inhibitors. The luminescent system has also been used to confirm the presence of suspected contaminating protease in other purified proteins and to assess the activity of specific protease inhibitors (data not shown).

Results presented here suggest that a set of well-defined peptide substrates in combination with a bioluminescent detection method can function as a "general protease assay" and serve as an alternative to whole protein universal substrates. The luminescent assay sensitively detected proteases from multiple classes and required fewer steps and shorter incubation times, allowing for faster analysis of contaminating protease activities. The assay is designed to have utility in cases when a single peptide substrate of well-defined sequence may not be adequate, such as when multiple proteases are present, the identities of proteases or their specific target sequences are not known, or when substrates specific for proteases are not available. Possible applications include detection of trace levels of protease contaminating purified proteins, including protein therapeutics, development of effective protein purification protocols, inhibitor testing, or profiling newly identified putative proteases.

Acknowledgments

The authors would like to thank Jean Osterman for technical assistance and Brian McNamara for helpful discussions.

Competing interests

The authors are employees of Promega Corporation.

References

1. **López-Otín, C. and J.S. Bond.** 2008. Proteases: multifunctional enzymes in life and disease. J. Biol. Chem. *283*:30433-30437.
2. **Rawlings, N.D., A.J. Barrett, and A. Bateman.** 2010. MEROPS: the peptidase database. Nucleic Acids Res. *38*:D227-D233.
3. **Harris, J.L., B.J. Backes, F. Leonetti, S. Mahrus, J.A. Ellman, and C.S. Craik.** 2000. Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc. Natl. Acad. Sci. USA *97*:7754-7759.
4. **Liz, M.A., C.J. Faro, M.J. Saraiva, and M.M. Sousa.** 2004. Transthyretin, a new cryptic protease. J. Biol. Chem. *279*:21431-21438.
5. **Thwaite, J.E., S. Hibbs, R.W. Titball, and T.P. Atkins.** 2006. Proteolytic degradation of human antimicrobial peptide LL-37 by *Bacillus anthracis* may contribute to virulence. Antimicrob. Agents Chemother. *50*:2316-2322.
6. **Caputo, E., G. Manco, L. Mandrich, and J. Guardiola.** 2000. A novel aspartyl proteinase from apocrine epithelia and breast tumors. J. Biol. Chem. *275*:7935-7941.
7. **Twining, S.S.** 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. *143*:30-34.
8. **Jones, L.J., R.H. Upson, R.P. Haugland, N. Panchuk-Voloshina, M. Zhou, and R.P. Haugland.** 1997. Quenched BODIPY dye-labeled casein substrates for the assay of protease activity by direct fluorescent measurement. Anal. Biochem. *251*:144-152.
9. **Monsees, T., W. Miska, and R. Geiger.** 1994. Synthesis and characterization of a bioluminogenic substrate for alpha-chymotrypsin. Anal. Biochem. *221*:329-334.
10. **Monsees, T., R. Geiger, and W. Miska.** 1995. A novel bioluminogenic assay for alpha-chymotrypsin. J. Biolumin. Chemilumin. *10*:213-218.
11. **Fan, F. and K.V. Wood.** 2007. Bioluminescent assays for high-throughput screening. Assay Drug Dev. Technol. *5*:127-136.
12. **O'Brien, M.A., W.J. Daily, P.E. Hesselberth, R.A. Moravec, M.A. Scurria, D.H. Klaubert, R.F. Bulleit, and K.V. Wood.** 2005. Homogeneous, bioluminescent protease assays: caspase-3 as a model. J. Biomol. Screen. *10*:137-148.
13. **O'Brien, M.A., R.A. Moravec, T.L. Riss, and R.F. Bulleit.** 2008. Homogeneous, bioluminescent proteasome assays. Methods Mol. Biol. *414*:163-181.
14. **Moravec, R.A., M.A. O'Brien, W.J. Daily, M.A. Scurria, L. Bernad, and T.L. Riss.** 2009. Cell-based bioluminescent assays for all three proteasome activities in a homogeneous format. Anal. Biochem. *387*:294-302.
15. **O'Brien, M.A.** 2006. A comparison of homogeneous bioluminescent and fluorescent methods for protease assays, p.125-139. *In* L.K. Minor (Ed.), Handbook of Assay Development in Drug Discovery. CRC Press, Boca Raton, FL.
16. **Glickman, M.H. and A. Ciechanover.** 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. *82*:373-428.
17. **Kisselev, A.F. and A.L. Goldberg.** 2005. Monitoring activity and inhibition of 26S proteasomes with fluorogenic peptide substrates. Methods Enzymol. *398*:364-378.
18. **Hall, M.P., M.G. Gruber, R.R. Hannah, M.L. Jennens-Cluogh, and K.V. Wood.** 1998. Stabilization of firefly luciferase using directed evolution, p. 392-395. *In* A. Roda, M. Pazzagli, L.J. Kricka, and P.E. Stanley (Eds.), Bioluminescence and Chemiluminescence: Perspectives for the 21st Century. Wiley, Chichester, UK.
19. **Schechter, I. and A. Berger.** 1967. On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. *27*:157-162.

Received 22 February 2011; accepted 26 May 2011.

Address correspondence to Donna M. Leippe, Research and Development Department, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711, USA. e-mail: donna.leippe@promega.com