Light Up Your Calpain Activity

A Bioluminescent Assay for Calpain Activity

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Abstract

We have developed an exceptionally sensitive assay for the proteases calpain I and II. The Calpain-Glo™ Protease Assay is a rapid, homogeneous assay with a luminescent signal that is proportional to the amount of calpain present in the sample. The Calpain-Glo™ Assay uses a recombinant thermostable luciferase, Ultra-Glo™ Luciferase, that dramatically improves sensitivity and detects calpain over a broad range of activity.

This homogeneous, coupled-enzyme format is especially well suited for a rapidly autolysed enzyme like calpain; maximum sensitivity is reached while the enzyme is fully active.

Introduction

Calpains are a family of calcium-activated cysteine proteases involved in cleaving a wide variety of proteins. They include the ubiquitous calpains, I(μ) and II(m), as well as numerous tissue-specific calpains. Calpains modulate the biological activities of their substrates through limited proteolysis and have been implicated in numerous calcium-regulated cellular processes including: cell motility, cell cycle progression, cell proliferation, apoptosis, necrosis, differentiation, membrane fusion and platelet activation (1–7). Calpains are also thought to be involved in numerous pathological conditions, making them important therapeutic targets (8–10). Deregulated calpain activity following loss of calcium homeostasis results in tissue damage in response to events such as myocardial infarcts, stroke and brain trauma (3,8). Defining physiological substrates for calpains has been challenging; their rapid autolysis and inactivation upon calcium activation makes calpain enzymes difficult to work with and limits the sensitivity and usefulness of traditional fluorometric and colorimetric assays (3,11).

Calpain-Glo™ Assay Principle

The Calpain-Glo™ Protease Assay[a,b] is a homogeneous, luminescent assay that measures calpain I and II activities. The Calpain-Glo™ Assay provides a luminogenic succinyl calpain substrate, Suc-LLVY-aminoluciferin, in a buffer system optimized for calpain activity and luciferase activity. The addition of a single Calpain-Glo™ Reagent in an "add-mix-measure" format results in calpain cleavage of the substrate and generation of a luminescent signal produced by the luciferase reaction (Figure 1). The Calpain-Glo™ Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) that is formulated to generate a "glow-type" luminescent signal and improve performance across a wide range of assay conditions. This coupled-enzyme system, with simultaneous calpain cleavage of substrate and luciferase consumption of the released aminoluciferin, results in a luminescent signal that is proportional to the amount of calpain activity present. The assay has a broad dynamic range of calpain concentration, resulting in exceptional sensitivity (Figure 2).

Calcium requirements for half-maximal activity in vitro are 2–80μM for calpain I and 0.2–0.8mM for calpain II (8). When calcium titrations were performed with the Calpain-Glo™ Protease Assay, tests showed EC_{50} values of 7μM for calpain I (Figure 3) and 0.6mM for calpain II (data not shown).

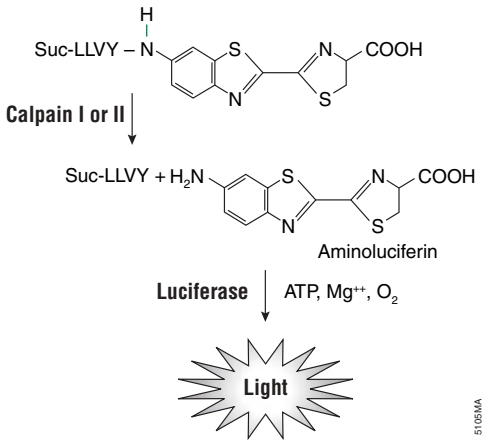

Figure 1. The luminogenic substrate containing the Suc-LLVY sequence recognized by calpain. Following calpain cleavage, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to produce light.

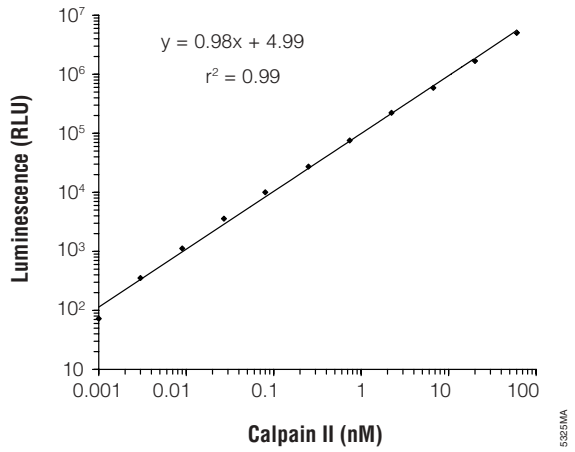

Figure 2. Luminescence is proportional to calpain activity. A titration assay of calpain was performed in 96-well plates using the Calpain-Glo™ Protease Assay. Rat recombinant calpain II was serially diluted in 10mM HEPES (pH 7.2), 10mM DTT, 1mM EDTA + 0.1% Prionex® as a carrier. Five minutes after addition of the Calpain-Glo™ Reagent, luminescence was recorded as relative light units (RLU) on a Veritas™ Microplate Luminometer. The results were linear over 4 logs of calpain concentration (r^2 = 0.99, slope = 0.98). Each point represents the average of 4 wells. The background (blank without calpain) was subtracted from each (average blank RLU = 995). r^2 and slope were calculated after transforming the data to a $\log_{10}$-$\log_{10}$ plot.

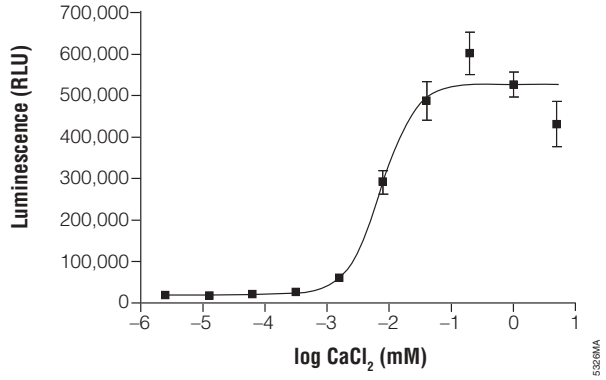

Figure 3. Calcium titration gives the expected EC_{50}. A titration of calcium was performed using the Calpain-Glo™ Protease Assay. $CaCl_2$ was serially diluted in 96-well plates and human calpain I (10nM) was added in buffer (10mM HEPES [pH 7.2], 10mM DTT, 1mM EDTA + 0.1% Prionex®). Five minutes after addition of the Calpain-Glo™ Reagent, luminescence was recorded as relative light units (RLU) on a Veritas™ Microplate Luminometer. GraphPad Prism® software was used to calculate the EC_{50} of 7µM.

Ideal for a Rapidly Inactivating Enzyme

In this homogeneous, coupled-enzyme format, the calpain and luciferase enzyme activities reach a steady state so that the luminescent signal peaks rapidly, achieving maximum sensitivity in 5–10 minutes (Figure 4). As the calpain enzyme becomes autolysed and inactivated after calcium activation, the luminescent signal decreases because the signal is directly proportional to the calpain activity. Figure 4, Panel A, shows a typical time course for the signal decay over a broad concentration of human calpain I. The half-life for the signal at room temperature is typically 30–70 minutes for calpain I (Figure 4, Panel B) and 10–20 minutes for calpain II. There are many factors that affect the inactivation rate of calpains I and II including temperature, ionic strength, pH, calcium concentration and the source of the enzyme preparation (11). These factors will determine the half-life of the signal. The shorter half-life of the luminescent signal with calpain II is consistent with differences in the autolysis of the two enzymes (11). This homogeneous, coupled-enzyme format is especially well suited for a rapidly autolysed enzyme like calpain; maximum sensitivity is reached rapidly while the enzyme is fully active.

More Sensitive than Comparable Fluorescent Assays

We compared the luminescent Calpain-Glo™ Protease Assay to fluorescent calpain assays using the Suc-LLVY-AMC substrate and a fluorescence resonance energy transfer (FRET)-based substrate, [H-Lys (FAM)-EVYGMMK(Dabcyl)-OH] (12). The Calpain-Glo™ Protease Assay was significantly more sensitive at all time points (Figure 5). Signal-to-noise ratios [(mean signal–mean background)/standard deviation of the background] (13) were used to compare the three formats. Because the fluorescence assays rely on the accumulation of cleaved fluorophore, the assays require a longer time to achieve optimum sensitivity. The signal-to-noise ratio for the Calpain-Glo™ Protease Assay after 12 minutes was greater than tenfold better than the FRET-based assay after 30 minutes and a thousandfold better than the Suc-LLVY-AMC assay after 60 minutes. Likewise, the limit of detection is much lower for the luminescent assay (Figure 5). The Suc-LLVY-AMC substrate gives a very poor dynamic range because the calpain is inactivated before there is enough time to accumulate sufficient cleaved AMC. The sensitivity of the luminescent assay drops over time due to the inactivation of calpain, but even after 1 hour, the signal-to-noise ratio is dramatically improved over the fluorescent calpain assays.

Ideal for High-Throughput Calpain Inhibitor Screens

The sensitivity and speed of the assay makes the Calpain-Glo™ Assay a useful tool for inhibitor screening. Since compound libraries are frequently stored in DMSO, we determined the effect of DMSO on the assay. Concentrations up to 2.5% DMSO had no effect on the assay. At concentrations as high as 5% DMSO, the signal decreased by just 26%, and with 10% DMSO, the signal decreased by 54% (Figure 6).

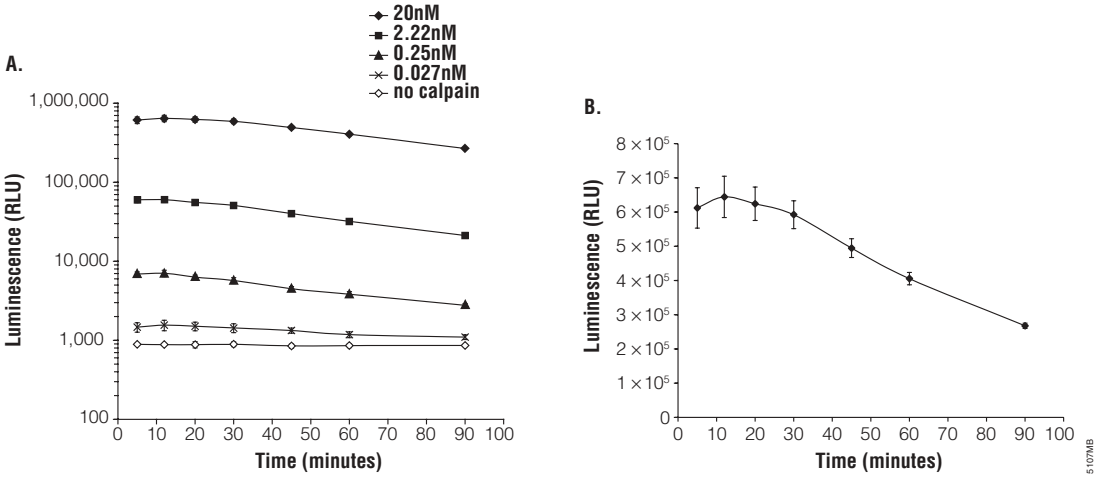

Figure 4. Signal half-life of the Calpain-Glo™ Protease Assay. Human calpain I from plasma was titrated and assayed in 96-well plates using the Calpain-Glo™ Assay. Luminescence was recorded at variouspoints over 1.5 hours on a Veritas™ Microplate Luminometer. **Panel A.** The signal peaks rapidly and then decreases uniformly over a broad calpain concentration. **Panel B.** The luminescent signal shows a half-life of >60 minutes (20nM calpain). The signal decrease is primarily a result of inactivation of calpain upon calcium activation.

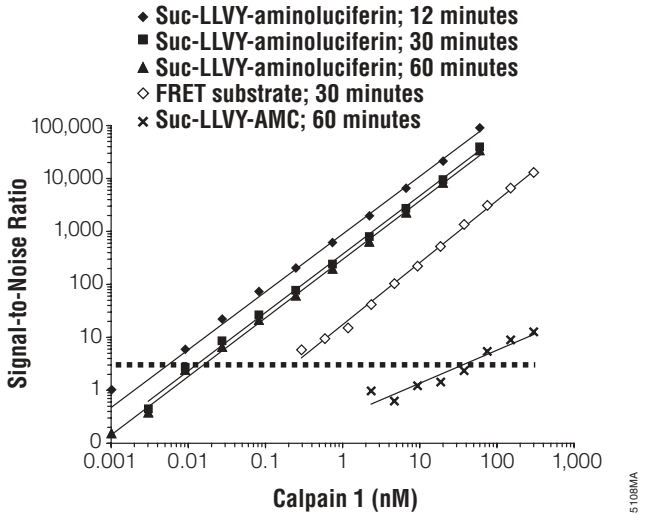

Figure 5. Superior sensitivity of the Calpain-Glo™ Protease Assay compared to fluorescent calpain assays. Human calpain I was titrated and assayed in 96-well plates using the Calpain-Glo™ Assay, a Suc-LLVY-AMC fluorescent substrate, or the FRET-based substrate, [H-Lys (FAM)-EVYGMMK(Dabcyl)-OH]. Luminescence was recorded at various times after reagent addition on a Veritas™ Microplate Luminometer. Fluorescence was monitored on a LabSystems Ascent fluorometer. The results were plotted as signal-to-noise ratios (7). The limit of detection was defined as the amount of calpain giving a signal-to-noise ratio = 3 (dashed line). The bioluminescent assay demonstrated a limit of detection of 5pM in 12 minutes, whereas the FRET-based fluorescent assay demonstrated a limit of detection of only 200pM after 30 minutes, and the Suc-LLVY-AMC substrate gave a limit of detection of 30nM after 60 minutes (earlier time points did not result in fluorescence above background).

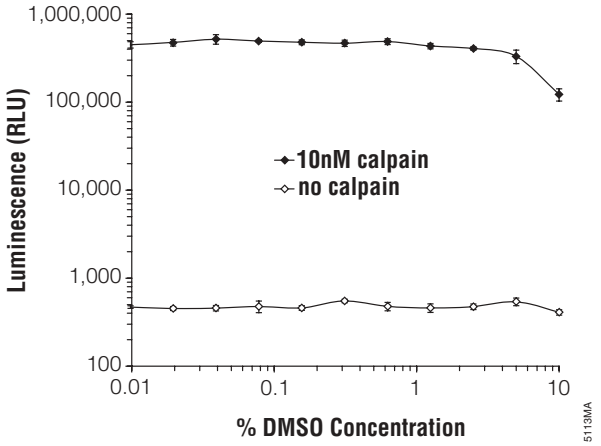

Figure 6. DMSO effects on Calpain-Glo™ Assay. DMSO was titrated and combined with human calpain I (10mM HEPES (pH 7.2), 10mM DTT, 1mM EDTA, 1mM EGTA + 0.1% Prionex®) in 96-well plates (50µl/well). The Calpain-Glo™ Reagent + 2mM $CaCl_2$ was added, and luminescence was recorded after 10 minutes on a Veritas™ Microplate Luminometer.

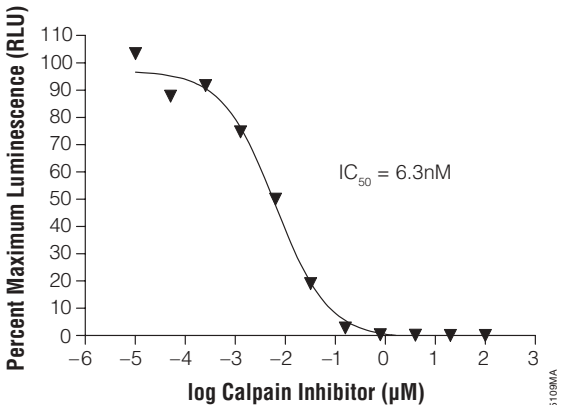

Figure 7. Determination of IC_{50} values. The concentration of inhibitor that results in 50% inhibition (IC_{50}) was determined for the calpain competitive inhibitor, Z-Val-Phe-CHO (inhibitor III), using the Calpain-Glo™ Protease Assay. The inhibitor was resuspended in DMSO, serially diluted and combined with human calpain I (20nM) in 10mM HEPES (pH 7.2), 10mM DTT, 1mM EDTA, 1mM EGTA + 0.1% Prionex® in 96-well plates. The Suc-LLVY-Glo™ Substrate was used at the apparent K_m and 2mM $CaCl_2$ was added to the Calpain-Glo™ Reagent. Luminescence was recorded 10 minutes after reagent addition, and GraphPad Prism® software was used to calculate the IC_{50} of 6.3nM. This corresponds closely to the K_i provided by Calbiochem (8nM; Cat.# 208722).

We also used the Calpain-Glo™ Assay to test a known calpain inhibitor (Figure 7). Calpain inhibitor III (Z-Val-Phe-CHO) was resuspended in DMSO and serially diluted in buffer. The maximum DMSO concentration was 0.1%. Z-Val-Phe-CHO exhibited potent inhibition of calpain in the Calpain-Glo™ Protease Assay. The assay can be used for accurate determination of half-maximal inhibition constants (IC_{50}).

Conclusions

The new Calpain-Glo™ Protease Assay is the first bioluminescent calpain assay. This homogeneous, coupled-enzyme format is uniquely suited for rapidly autolysed and inactivated enzymes such as calpain. Assay sensitivity does not depend on the accumulation of cleaved product; instead, calpain-cleaved substrate is immediately converted by luciferase, resulting in maximum light output after 5–10 minutes of incubation with calpain. The assay demonstrates dramatically improved sensitivity compared to current fluorescent-based calpain assays (12,14) and a very broad linear range for calpain activity, allowing the researcher to use less enzyme while still achieving accurate results. The Calpain-Glo™ Protease Assay provides a rapid, sensitive and accurate calpain activity assay.

References

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Protocols

- *Calpain-Glo™ Protease Assay Technical Bulletin* #TB344, Promega Corporation.
  www.promega.com/tbs/tb344/tb344.html

Ordering Information

Product	Size	Cat.#
Calpain-Glo™ Protease Assay	10ml	G8501
	50ml	G8502

For Laboratory Use.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

(a) U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

(b) The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.