Supplementary Material For:

A bioluminescent assay for the sensitive detection of proteases

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Materials and methods

Proteases
Preparations of proteases were obtained from commercial sources. The following proteases were from Sigma-Aldrich (St. Louis, MO, USA): carboxypeptidase A (Cat. no C9268; 71 U/mg), carboxypeptidase B (Cat. no C9584; 140 U/mg), cathepsin B (Cat. no C6286; 20 U/mg), cathepsin D (Cat. no C3138; 13 U/mg), cathepsin L (Cat. no C6854; 2.3 U/mg), α-chymotrypsin (Cat. no C1429; 59.3 U/mg), elastase (porcine; Cat. no E0258; 7.4 U/mg), endopeptidase Glu-C (Cat. no P2922; specific activity not provided), furin (Cat. no F2677; 2000 U/mL), leucine aminopeptidase (Cat. no L0632; 30 U/mg), papain (Cat. no P4762; 23 U/mg), pepsin (Cat. no P6887; 4220 U/mg), plasmin (Cat. no P1867; 47.6 U/mg), subtilisin A (Cat. no P5380; 10 U/mg), thrombin (Cat. no T6884; 3143.6 U/mg), and thermolysin (Cat. no P1512; 64 U/mg). Trypsin (Cat. no V5280; ≥15,000 U/mg), alkaline protease (Cat. no A1441; 500 U/mL), and proteinase K (Cat. no V3021; >30 U/mg) were from Promega (Madison, WI, USA). Amino- peptidase M (Cat. no 164598; 17.3 U/mg) was from EMD Chemicals (Gibbstown, NJ, USA). Specific activity is included as units/milligram protein when provided by the manufacturer. For furin and alkaline protease, concentrations were not provided, but activities were available in terms of units/milliliter. Unit definitions should be obtained from the manufacturer.

Synthesis of luminescent protease substrates
To synthesize Leu-aminoluciferin, Fmoc-Leu-OH (0.79 g, 2.2 mmol) was dissolved in tetrahydrofuran (THF; 6 mL) cooled to -20°C. N-methyl morpholine (0.5 mL, 2.5 eq) was added, followed by isobutylchloroformate (0.3 mL, 1.1 eq). This was stirred for 20 min, and a solution of 6-amino-2-cyanobenzothiazole (0.32 g, 1 eq) was added. This reaction was allowed to come to room temperature as it was stirred for 16 h. The reaction was then concentrated, and the resulting crude material was purified by flash chromatography with 3/7 EtOAc/heptanes to provide Fmoc-Leu-aminocyanobenzothiazole as a white solid (0.91 g). This material was dissolved in CHCl3 (20 mL), and DBU (1.8-Diazabicyclo[5.4.0]undec-7-ene; 0.27 mL, 1 eq) was added. After stirring for 1.5 h at room temperature, the reaction was concentrated, and the crude material was purified by flash chromatography with 97/3 CHCl3/MeOH to provide H-Leu-aminocyanobenzothiazole as a white solid (0.33 g). This material was dissolved in CH3CN (10 mL) and water (1 mL), and HPLC was used to monitor the reaction. The final product was purified with prep-HPLC with a gradient of acetonitrile in 10 mM NH4OAc, and 52 mg Luciferin-Arg-OH were obtained as a white solid after lyophilization: mass spectroscopy (ESI) calculated: 436.5; found: 437.2 (M+1).

dichloromethane. The solution was washed with water, dried with Na2SO4, and concentrated. The residue was purified by flash chromatography eluting with a gradient of EtOAc in heptane, and Boc-Cys(Trt)-Arg(Pbf)-OtBu (250 mg) was obtained as a white solid. The dipeptide was protected in a solution of dichloromethane/trifluoroacetic acid (TFA) (1:1) for 2.5 h, and the solvent was removed. The resulting H-Cys-Arg-OH was dissolved in THF (6 mL) and water (1 mL). 6-Hydroxy-2-cyanobenzothiazole (0.9 eq) and TCEP (1.1 eq) were added, and the pH was adjusted to 8.0 with triethylamine. The solution was kept stirring at room temperature for about 30 min, and HPLC was used to monitor the reaction. The final product was purified with prep-HPLC with a gradient of acetonitrile in 10 mM NH4OAc, and 52 mg Luciferin-Arg-OH were obtained as a white solid after lyophilization: mass spectroscopy (ESI) calculated: 436.5; found: 437.2 (M+1).
Supplementary Table S1. Comparison of luminescent and fluorescent general protease assays.

<table>
<thead>
<tr>
<th>Protease</th>
<th>Luminescent assay</th>
<th>Fluorescent assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RLU</td>
<td>S/B</td>
</tr>
<tr>
<td>Trypsin</td>
<td>7,149,483 (0.3%)</td>
<td>417</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>5,012,434 (1.4%)</td>
<td>293</td>
</tr>
<tr>
<td>Trypsinase K</td>
<td>5,848,099 (0.9%)</td>
<td>341</td>
</tr>
<tr>
<td>Alkaline protease</td>
<td>2,825,936 (14.7%)</td>
<td>165</td>
</tr>
<tr>
<td>Subtilisin</td>
<td>6,685,593 (2.6%)</td>
<td>390</td>
</tr>
<tr>
<td>Furin</td>
<td>975,302 (3.8%)</td>
<td>57</td>
</tr>
<tr>
<td>Elastase</td>
<td>2,079,887/40.9%</td>
<td>121</td>
</tr>
<tr>
<td>Thrombin</td>
<td>795,127 (40.3%)</td>
<td>46</td>
</tr>
<tr>
<td>Plasma</td>
<td>164,431 (39.3%)</td>
<td>10</td>
</tr>
<tr>
<td>Endoproteinase Glu-C</td>
<td>21,308 (5.7%)</td>
<td>1</td>
</tr>
<tr>
<td>Papain</td>
<td>4,380,592 (1.5%)</td>
<td>256</td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>6,774,213 (2.1%)</td>
<td>395</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>3,276,891 (0.9%)</td>
<td>191</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>20,402 (11.4%)</td>
<td>1</td>
</tr>
<tr>
<td>Pepsin</td>
<td>16,822 (2.6%)</td>
<td>1</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>17,324 (1.8%)</td>
<td>1</td>
</tr>
<tr>
<td>Aminopeptidase M</td>
<td>2,203,635 (7.7%)</td>
<td>129</td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>842,598 (12.3%)</td>
<td>49</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>10,986 (2.0%)</td>
<td>1</td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>3,164,520 (14.2%)</td>
<td>185</td>
</tr>
<tr>
<td>No protease (background)</td>
<td>17,134 (0.9%)</td>
<td>1</td>
</tr>
</tbody>
</table>

Twenty proteases were individually tested in two different protease assays. Each protease was assayed in triplicate at a final concentration of 1 µg/mL, except for alkaline protease and furin, which were used at final concentrations of 5 and 20 U/mL, respectively. Incubation times were for 30 min (luminescent assay) or 20 h (fluorescent assay). Average signals and the percent coefficient of variation (100 × sd/average) are listed. The average RLUs were used to calculate S/B and S/N ratios. These data are also depicted in Figure 2, A and B, of the main text. RLU, relative light unit; FLU, fluorescent light unit; S/B, signal-to-background ratio; S/N, signal-to-noise ratio; NA, not applicable.
Supplementary Table S2. Interexperiment variation of the luminescent general protease assay.

<table>
<thead>
<tr>
<th>Protease</th>
<th>RLU Experiment A</th>
<th>RLU Experiment B</th>
<th>RLU Experiment C</th>
<th>Interexperiment %CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>7,518,552 (5.6%)</td>
<td>7,724,362 (3.0%)</td>
<td>8,223,886 (1.4%)</td>
<td>4.6</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>4,046,506 (3.7%)</td>
<td>4,200,755 (3.4%)</td>
<td>4,996,307 (2.5%)</td>
<td>11.5</td>
</tr>
<tr>
<td>Elastase</td>
<td>922,022 (8.4%)</td>
<td>993,693 (8.4%)</td>
<td>1,025,651 (10.7%)</td>
<td>5.4</td>
</tr>
<tr>
<td>Thrombin</td>
<td>340,548 (9.0%)</td>
<td>208,294 (5.1%)</td>
<td>234,498 (8.1%)</td>
<td>26.8</td>
</tr>
<tr>
<td>Plasmin</td>
<td>33,378 (4.0%)</td>
<td>37,065 (2.6%)</td>
<td>38,186 (5.0%)</td>
<td>6.9</td>
</tr>
<tr>
<td>Aminopeptidase M</td>
<td>2,733,144 (3.1%)</td>
<td>3,217,846 (1.8%)</td>
<td>2,939,681 (6.6%)</td>
<td>8.2</td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>5,081,895 (3.1%)</td>
<td>5,092,573 (3.8%)</td>
<td>4,756,367 (9.1%)</td>
<td>3.8</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>11,622 (3.4%)</td>
<td>11,393 (2.8%)</td>
<td>11,238 (2.5%)</td>
<td>1.7</td>
</tr>
<tr>
<td>No protease</td>
<td>11,241 (2.4%)</td>
<td>10,869 (1.4%)</td>
<td>11,052 (1.1%)</td>
<td>1.7</td>
</tr>
</tbody>
</table>

A subset of proteases from the initial screen of 20 proteases depicted in Figure 2, A and B, from the main text, were further tested in three separate experiments to determine the variation within and between experiments. Each protease was assayed in six wells at a final concentration of 1 µg/mL for 30 min. Average signals and the percent coefficient of variation (%CV; 100 x s.d/average) are listed for each protease. Interexperiment variation was also calculated. This data are represented graphically in Supplementary Figure S1. RLU, relative light unit.

Supplementary Figure S1. Interexperiment variation of the luminescent general protease assay. A subset of proteases from the initial screen of 20 proteases depicted in Figure 2, A and B, from the main text, were further tested in three separate experiments to determine the variation within and between experiments. The data from Figure 2A and the three additional experiments are graphed. Error bars represent ±1 s.d. For experimental details, see Supplementary Table S2. RLU, relative light unit.