Program
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Simple Protease Assays Using Whole-Protein Substrates

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1 Abstract

Protein and peptide products resulting from the cleavage of proteins by intracellular or extracellular proteases are known biomarkers or effectors for disease, such as Alzheimer's and hypertension. Indeed, the identification of specific modulators for proteases makes up a large fraction of the actual screening pipeline. Here, we describe the development of a high throughput assay for monitoring protease cleavage activity of whole protein substrates. Using two protease models – angiotensin-converting enzyme (ACE) and β-secretase (BACE) – we show that protein substrates can be cleaved into products that can be directly detected and quantitated. Inhibitors of these enzymes resulted in IC_{50} values in agreement with the literature. The use of whole-proteins in these cleavage assays offers the advantage of using physiological enzymatic substrates and could eventually be transferred to cell-based assays.

2 Materials and Methods

BACE

Reaction (reagents are diluted in 10 mM Sodium Acetate buffer, 1% Triton X-100, pH 4.0):
- 5 µL of Amyloid Precursor Protein α; APPα (Sigma)
- 5 µL of Beta-Amyloid Cleaving Enzyme; BACE (R&D Systems)

Incubate 60 minutes at 23ºC

Detection (beads mixes are diluted in 100 mM Hepes buffer, 10% Pierce Casein Block, 0.5% Triton X-100, 1 mg/mL Dextran T-500, pH 8.0):
- 20 µL of biotinylated-anti-APP (Covance) [2.5 nM] / anti-APPβ acceptor beads (PerkinElmer) [25 µg/mL] mix

Incubate 60 minutes at 23ºC
- 20 µL of Streptavidin donor beads (PerkinElmer) [100 µg/mL]

Incubate 30 minutes at 23ºC

Samples were read from a 384-well Optiplate using the EnVision® Multilabel Plate Reader (PerkinElmer).

ACE

Reaction (reagents are diluted in 25 mM Hepes buffer, 300 mM NaCl, 0,1% Tween-20, pH 7,4):
- 5 µL of biotinylated-Angiotensin I (Bachem)
- 5 µL of buffer or ACE inhibitor
- 5 µL of Angiotensin-Converting Enzyme; ACE (Sigma)

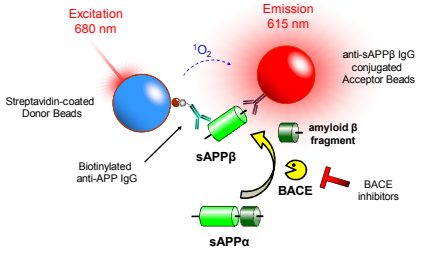
Incubate 60 minutes at 23ºC

Detection (beads mixes are diluted in reaction buffer supplemented with 12.5 mM EDTA):
- 5 µL of Streptavidin donor beads [20 µg/mL]
- 5 µL of anti-angiotensin II (Phoenix Pharmaceuticals) / Protein A acceptor beads (PerkinElmer) [20 µg/mL] mix

Incubate 1 hour at 23ºC

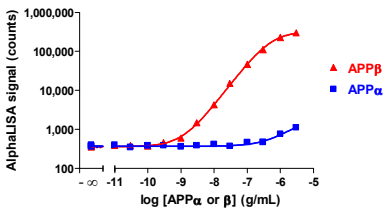
Samples were read from a 384-well Optiplate using the EnVision Multilabel Plate Reader (PerkinElmer).

3 BACE Assay Format



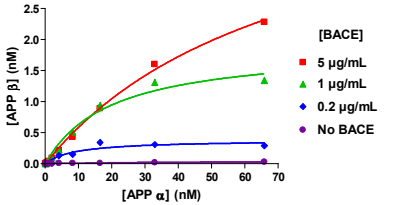
In this assay fomat, sAPPα is cleaved to sAPPβ by the action of BACE. The anti-sAPPβ binds specifically a neo-epitope present on the cleaved protein. The biotinylated anti-APP brings the streptavidin Donor beads and the antibody-conjugated Acceptor beads into proximity to generate an AlphaScreen® signal. The presence of an enzyme inhibitor blocks its activity and results in a signal decrease.

4 Assay Selectivity for APPβ



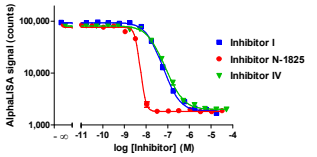
To test specificity we performed titration curves for the substrate and product. Since the affinity of the antibodies for the two proteins differs over three logs, we confirmed that the antibody pair was selective for APPβ over APPα.

5 Cleavage by BACE



Substrate APPα titrations were performed using three different BACE concentrations. The cleavage reaction was incubated for 60 minutes, followed by addition of AlphaLISA® reagents. Results were converted to quantities of APPβ by using a standard curve produced in parallel under the same assay conditions.

6 Inhibition of BACE Activity

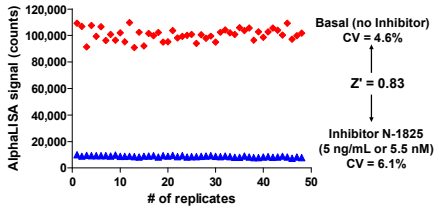


Statistics for Inhibitors	Inhibitor I	Inhibitor N-1825	Inhibitor IV
IC_{50} (nM)	23.5	3.2	10.4
Literature IC_{50} (nM)	30 [1]	0.3 [2]	15 [3]

References
1 - Sinha, S., et al. 1999. *Nature* **402**, 537.
2 - Hong, L. et al. 2002 *Biochemistry* **41**, 10963.
3 - Stachel, S.J., et al. 2004. *J. Med. Chem.* **47**, 6447.

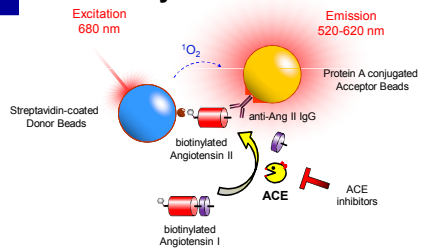
BACE inhibitor I, N-1825, and IV were tested on enzyme activity using 1.0 µg/mL BACE and 3.0 µg/mL of APPα. Different concentrations of each inhibitor were incubated with BACE at 23°C for 30 minutes, before adding the BACE – inhibitor mixes to the APPα for the cleavage reaction.

7 BACE Assay Reproducibility



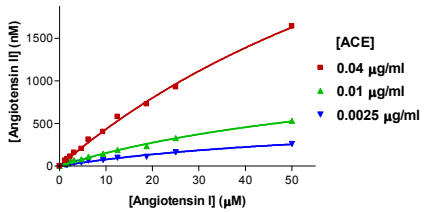
Plates were loaded with 3 µg/mL of APPα, and 1 µg/mL BACE in the presence or the absence (basal) of inhibitor. The enzymatic reaction was incubated for 60 minutes at 23°C. The addition of the antibody mix at pH 8.0 stopped the reaction. The Z'-factor values obtained were > 0.5 indicating the suitability of the platform for subsequent HTS application. This is a representative result of over three independent experiments .

8 ACE Assay Format



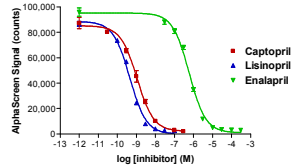
In this assay fomat, the ACE substrate is a biotinylated derivative of angiotensin I (bio-Ang I), which is converted to biotin-angiotensin II by the action of ACE. The bio-Ang II produced brings the streptavidin Donor beads and the antibody-protein A-conjugated Acceptor beads into proximity to generate an AlphaScreen signal. The presence of an enzyme inhibitor blocks its activity and results in a signal decrease.

9 Cleavage by ACE



Substrate Ang I titrations were performed and the reactions were incubated for 30 minutes at 23 C, followed by the addition of detection reagents. To determine the quantity of Ang II produced by ACE, an Ang II standard curve was produced in parallel using the same assay conditions.

10 Inhibition of ACE Activity

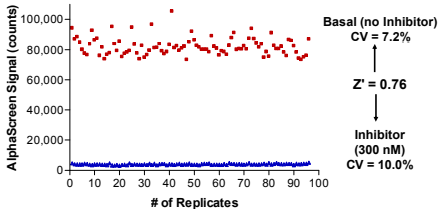


Statistics for Inhibitors	Captopril	Lisinopril	Enalapril
IC_{50} (nM)	1.1	0.45	590
Literature IC_{50} (nM)	0.498 [1]	0.099 [1]	1 560 [2]

References
1 - Bull, H.G, et al. 1985. *J. Biol. Chem.* **260**, 5, 2952.
2 - Vermeirssen, V., et al. 2002. *J. Biochem. Biophys. Methods* **51**, 75.

Captopril, lisinopril and enalapril ACE inhibitors were tested on enzyme activity using 2.5 µg/ml ACE and 6 nM of biotinylated-Ang I. The results are in agreement with previously reported order of potency.

11 ACE Assay Reproducibility



Plates were loaded with 6 nM of biotinylated-Ang I in the presence or absence of 300 nM of captopril. 2.5 µg/ml of ACE were added and incubated for 60 minutes at 23°C. The addition of the antibody/beads mix in the presence of 12.5 mM EDTA stopped the reaction. The Z'-factor obtained were > 0.5, indicating suitability of the platform for subsequent HTS assays. This is a representative result of three independent experiments.

12 Conclusions

- We developed high throughput assays for monitoring protease cleavage activity on **whole protein substrates** using AlphaScreen and AlphaLISA technologies.
- Inhibitors of these enzymes resulted in IC_{50} values in agreement with the literature.
- The Z'-factor obtained were > 0.5, indicating suitability and robustness of the platforms for HTS applications.
- The use of whole proteins as substrates in protease assays provide a novel tool that will allow compound characterization in a context closer to the physiological environment.