A Fluorimetric Assay for Detection of Aggrecanase-1 (ADAMTS-4) Activity Using a Long Wavelength FRET Peptide Substrate

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Introduction

Aggrecanase-1, or ADAMTS-4, is a member of the ADAMTS (A disintegrin and metalloprotease with thrombospondin motif) family of proteases. Aggrecanase cleaves specific peptide bonds in aggrecan, the main proteoglycan of articular cartilage. The high activity of aggrecanase results in cartilage degradation in diseases such as arthritis. To develop a homogeneous high throughput screening (HTS) of ADAMTS-4 inhibitors, we synthesized a novel internally quenched peptide substrate for aggrecanase-1 consisting of 5-FAM/TAMRA as the FRET (Fluorescence or Förster resonance energy transfer) pair. Active ADAMTS-4 cleaves this FRET substrate into two separate fragments resulting in an increase of 5-FAM fluorescence, which is monitored at excitation/emission = 490 nm/520 nm. The new FRET substrate is more sensitive to aggrecanase-1 cleavage than the previously described Abz/Dnp substrate and can detect subnanogram amounts of ADAMTS-4. Its cleavage by other members of aggrecanase family, such as ADAMTS-1 and ADAMTS-5, is negligible. Inhibitor screening assays with several known metalloprotease inhibitors were validated using this substrate.

This novel FRET substrate provides a convenient homogeneous format for aggrecanase-1 activity assay. The signal is less interfered by autofluorescence of test compounds due to the long wavelength excitation and emission of 5-FAM, resulting in an overall increase of signal to background ratio.

Assay Principle

The Aggrecanase-1 Activity Assay is based on FRET principle, with 5-FAM and TAMRA as the donor - acceptor pair.

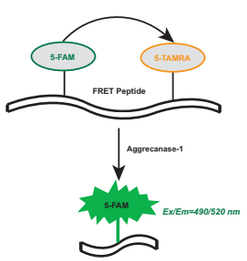

Figure 1. Proteolytic cleavage of 5-FAM/TAMRA FRET peptide by Aggrecanase-1. TAMRA quenches the fluorescence of 5-FAM. Upon cleavage of the FRET substrate by protease, the fluorescence of 5-FAM is recovered and can be monitored at Ex/Em = 490 /520 nm.

Materials and Methods

- SensoLyte® 520 Aggrecanase-1 Activity Assay Kit (Cat# 72114)
  - √ Aggrecanase-1 5-FAM/TAMRA FRET Substrate - designed and synthesized by Fmoc solid phase peptide synthesis method.
  - √ Assay Buffer
- Matrix Metalloproteinases Inhibitors
  - √ TAPI-0 is a patented product of Research Corp.Technologies
  - √ Galardin is a broad-spectrum inhibitor of matrix metalloproteinases (Calbiochem, San Diego, CA)
- Recombinant Human ADAMTS-4, ADAMTS-5, ADAMTS-1 - expressed in a baculovirus system and purified from insect cells.
- Aggrecanase-1 Abz/Dnp (Abz-TEGEARGSVI-Dap(Dnp)-KK-NH_2) FRET substrate - synthesized by Fmoc solid phase peptide synthesis method.

SensoLyte® 520 Aggrecanase-1 Assay Kit was used as recommended by the protocol. The reaction volumes were 40 µl of enzyme, 10 µl of test compound/buffer, 50 µl of substrate. Assays were done in 96-well black opaque plates.

Results

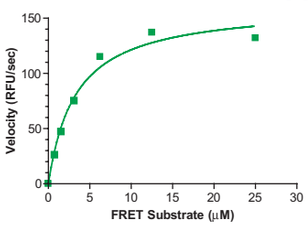

Figure 2. Michaelis-Menten plot of the 5-FAM/TAMRA Aggrecanase-1 FRET substrate. Initial velocities (V_0) of hydrolysis by enzyme were determined after incubation of 10 ng enzyme with a range of concentrations of a substrate. The resulting data were analyzed by non-linear regression. The Km value obtained was 3.3 µM.

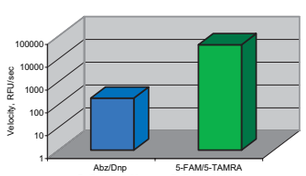

Figure 3. Comparison of ADAMTS-4 substrates. ADAMTS-4 substrates (Abz/Dnp and 5-FAM/TAMRA FRET pairs) at final concentration 5 µM were incubated with 10 ng of enzyme. Fluorescent signal was continuously monitored for 60 min.

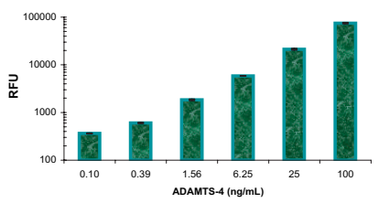

Figure 4. Sensitivity of the enzyme assay for 5FAM/TAMRA ADAMTS-4 FRET substrate. Fluorescence was measured after 1 hour incubation of FRET substrate with serial dilutions of Aggrecanase-1. Sensitivity of assay at these conditions was 0.1 ng/mL of enzyme.

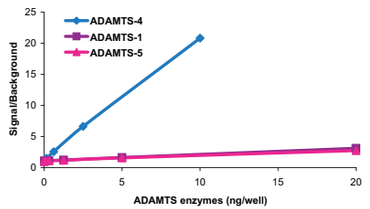

Figure 5. Analysis of substrate specificity. Various ADAMTS enzymes were incubated with ADAMTS-4 FRET substrate. We confirmed that 5-FAM/TAMRA ADAMTS-4 FRET substrate can discriminate between members of ADAMTS family.

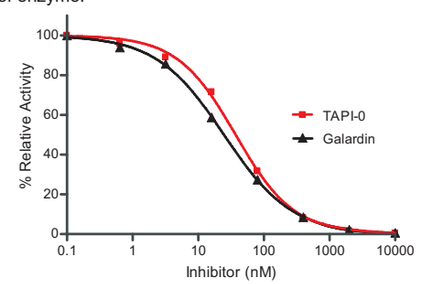

Figure 6. Inhibitor studies. To validate assay for inhibitor screening, 5-FAM/TAMRA substrate was incubated with 10 ng of ADAMTS-4 enzyme in the presence of matrix metalloproteinases inhibitors. Kinetic readings were taken every 5 min for 60 min. The calculated IC50 were 37 nM for TAPI-0 and 24.7 nM for Galardin.

Conclusions

- We have developed the SensoLyte® 520 Aggrecanase-1 Activity Assay Kit, based on a novel 5-FAM/TAMRA FRET substrate. The assay detects subnanogram concentration of enzyme.
- The longer excitation and emission wavelengths of 5-FAM minimize interference from autofluorescence of test compounds.
- The new 5-FAM/TAMRA FRET substrate is able to discriminate between ADAMTS enzymes. Its cleavage by ADAMTS-4 is better compared to cleavage by ADAMTS-1 and ADAMTS-5.
- The new assay was validated for inhibitor screening using broad-spectrum matrix metalloproteinase inhibitors.