A Fluorescent Assay for FAAH1 Activity Suitable for High-throughput Screening of Lipophilic Compounds

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INTRODUCTION

Endocannabinoids such as anandamide are synthesized from precursor components in the cell membrane in an on-demand fashion as a response to injury or stress. Endocannabinoid signaling is terminated by the enzyme fatty acid amide hydrolase (FAAH1). FAAH1 represents a promising pharmacologic target because its inactivation produces analgesic, anti-inflammatory, anxiolytic, and antidepressant effects without the undesirable psychoactive effects of direct cannabinoid receptor agonists. Traditional FAAH1 activity assays utilize radiolabeled anandamide substrate followed by phase separation and extraction of the ethanolamine 1-^{3}H product, making it unsuitable for high-throughput screening. While a fluorescent assay has recently been developed, it utilizes microsomal preparations of FAAH1. This poses a problem because the lipophilic nature of FAAH1 substrates and inhibitors may cause them to partition within the microsomal membrane. The goal of this project was to purify a recombinant, soluble form of FAAH1 and determine its suitability for use in plate reader-based assays.

METHODS

FAAH1 was produced recombinantly in *E.coli* using an expression construct lacking the trans-membrane domain to increase solubility. Immobilized metal ion affinity chromatography was used to purify the enzyme. To assess the purity and verify the identity of the recombinant protein, SDS-PAGE and Western blotting were employed. Activity of purified FAAH1 was characterized using the substrate analog aminomethyl coumarin (AMC) arachidonoyl amide, which is cleaved by FAAH to release the fluorescent AMC moiety.

RESULTS

Purification of Recombinant FAAH1

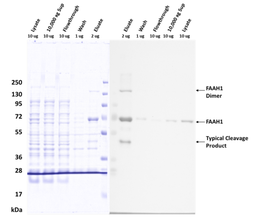

Figure 1: Purification of recombinant FAAH1. *E.coli* cell pellets were resuspended in 10x pellet volumes of lysis buffer (50 mM Tris pH 8.0, 100mM NaCl, and 1% Triton X-100). Protease inhibitors were added to cell suspensions immediately prior to lysozyme lysis. The lysate was centrifuged at 4 °C for 45 minutes at 10,000 xg to remove unbroken cells and debris. Clarified lysate was loaded on to a 1 ml cobalt metal affinity column (Clontech Laboratories) equilibrated with lysis buffer. After washing (50 mM Tris pH 8.0, 1000 mM NaCl, 1% Triton X-100, and 10 mM imidazole) with 15 column volumes, FAAH1 was eluted with buffer containing 50 mM Tris pH 8.0, 1000 mM NaCl, 1% Triton X-100, and 200 mM imidazole. Protein was concentrated and imidazole removed by buffer exchange in centrifugal ultrafiltration device. Enzyme yield was 2.11 mg at 80% purity from 1 liter of TB induced for 4 hours.

Fluorescent Assay for FAAH1 Activity

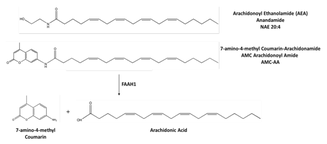

Figure 2: Chemical structures FAAH substrate, substrate analog, and reaction products. FAAH1 is the principal enzyme responsible for termination of signaling by the endocannabinoid anandamide (top). However, FAAH1 is a relatively unspecific enzyme and will hydrolyze a variety of fatty acid amides. FAAH1 hydrolysis of the anandamide analog 7-amino-4-methyl Coumarin-Arachidonamide (AAMC; middle) yields the fluorescent 7-amino-4-methyl Coumarin (AMC; bottom left) and arachidonic acid (bottom right).[4]

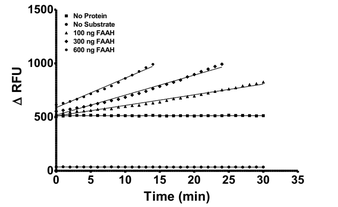

Figure 3: AMC fluorescence increases linearly with protein concentration and time. Reactions were carried out in black-walled 96-well plates. Indicated concentrations of purified recombinant FAAH1 were incubated in reaction buffer (125 mM Tris HCl pH 9.0, 1 mM EDTA) for 10 minutes at room temperature. Reactions were initiated by the addition of an excess (20 µM) of AMC arachidonoyl amide. Final reaction conditions were: 113 mM Tris HCl, 0.9 mM EDTA, 5% ethanol, 0.05% Triton X-100, pH 9.0 in a volume of 200 µl. Fluorescence was measured four times per minute at 340 nm excitation/455 nm emission for 30 min at 37 °C.

FAAH1 Activity in Fluorescent Assay is Consistent with Traditional Assay Methods

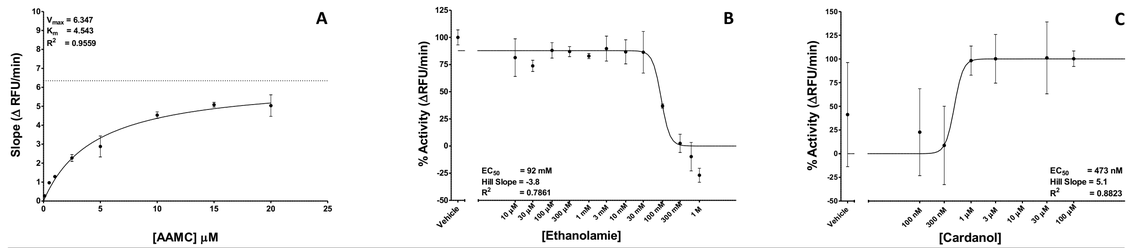

Figure 4: Characterization of recombinant FAAH1 activity with the anandamide substrate analog concentrations 7-amino-4-methyl Coumarin-Arachidonamide (AAMC). Reactions were carried out in black-walled 96-well plates. 100 ng of purified recombinant FAAH1 was incubated in reaction buffer (125 mM Tris HCl pH 9.0, 1 mM EDTA) for 10 minutes at room temperature. Reactions were initiated by the addition of AAMC. Final reaction conditions were: 113 mM Tris HCl, 0.9 mM EDTA, 5% ethanol, 0.05% Triton X-100, pH 9.0 in a volume of 200 µl. Fluorescence was measured four times per minute at 340 nm excitation/455 nm emission for 30 min at 37 °C. Initial rates were determined from the linear portion of the kinetic curve. Data represents mean ± SEM of 3 experiments. **(A) Michaelis constant determination** The Km for AAMC was determined to be 4.5 µM which is similar to the value reported for microsomal hamster FAAH1 (0.48 µM).[3] **(B) Product inhibition by ethanolamine.** FAAH1 was incubated for 10 min with the indicated concentration of ethanolamine prior to initiation of the reaction with 5 µM AAMC. Inhibition of FAAH1 at high concentrations of ethanolamine has previously been described using NAE 12:0 as the substrate however, the EC_{50} was not determined.[7] We found that the EC_{50} for ethanolamine was 92 mM which is consistent previous observations. **(3) Activation by cardanol.** FAAH1 was incubated for 10 min with the indicated concentration of the phenoxyacyl-ethanolamide cardanol prior to initiation of the reaction with 5 µM AAMC. Activation of FAAH1 by cardanol has previously been described however, the EC_{50} was not determined.[5] We found that the EC_{50} for cardanol was 473 nM which is consistent previous observations.

The Assay is Suitable for High-throughput Screening

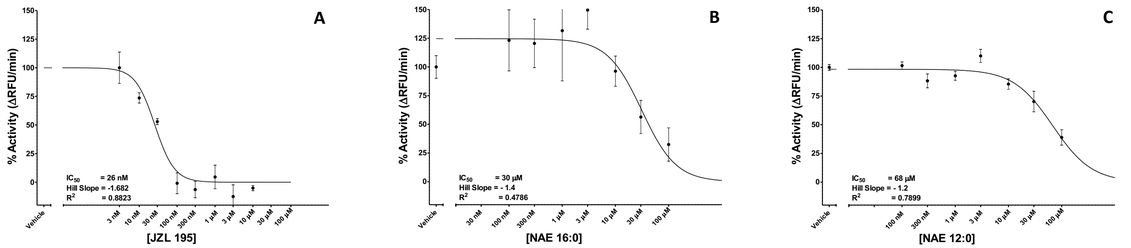

Figure 5: Demonstration of assay suitability for use in high-throughput screening. Previous fluorescent assays for FAAH1 activity utilized microsomal protein preparations which may sequester lipophilic substrates and/or inhibitors. To demonstrate the suitability of purified recombinant FAAH1 for use in the plate-based fluorescent assay, IC_{50}s of known inhibitors were determined and compared to previously published values. Reactions were carried out as described for figure 4 and the substrate concentration was maintained at K_m (figure 4A). Data represents mean ± SEM of 3 experiments. **(A)** The IC_{50} for the known inhibitor JZL 195 was determined to be 26 nM which is comparable to the previously reported value of 2 nM.[6] **(B)** The IC_{50} for the known inhibitor NAE 16:0 (palmitoylethanolamide) was measured at 30 µM which is consistent with the reported value of 5.3 µM.[7] **(C)** The IC_{50} for the known inhibitor NAE 12:0 (lauroylethanolamide) was found to be 68 µM, a value that is comparable to the published value of 4.8 µM.[7]

SUMMARY

- Truncated recombinant FAAH1 was purified to about 80%. FAAH1 activity increases linearly with protein concentration and time.
- The K_m for AMC arachidonoyl amide is 4.5 µM, which is similar to values reported for the hamster microsomal protein.
- While product inhibition by ethanolamine and activation by the phenoxyacyl-ethanolamide cardanol have been reported, the EC_{50} values were not measured. We found these values to be 92 mM and 473 nM respectively.
- IC_{50} values for the known inhibitors JZL 195, NAE 16:0, and NAE 12:0 were measured at 26 nM, 31 µM, and 68 µM respectively.

CONCLUSION

When AMC arachidonoyl amide is used as a substrate for purified, truncated recombinant FAAH1, the enzyme behaves in a manner that is consistent with previous reports for the natural substrate, anandamide. IC50 values measured with our assay are consistent with values previously reported. This system is rapid, inexpensive, and suitable for high-throughput screening of FAAH1 inhibitors.

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