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## Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes

The field of biochemistry is currently faced with the enormous challenge of assigning functional significance to more than thirty thousand predicted protein products encoded by the human genome. In order to accomplish this daunting task, methods will be required that facilitate the global analysis of proteins in complex biological systems. Recently, methods have been described for simultaneously monitoring the activity of multiple enzymes in crude proteomes based on their reactivity with tagged chemical probes. These activity based probes (ABPs) have used either radiochemical or biotin/avidin-based detection methods to allow consolidated visualization of numerous enzyme activities. Here we report the synthesis and evaluation of fluorescent activity based probes for the serine hydrolase super-family of enzymes. The fluorescent methods detailed herein provide superior throughput, sensitivity, and quantitative accuracy when compared to previously described ABPs, and provide a straight-forward platform for high-throughput proteome analysis.

**Keywords:** Fluorescence / Hydrolase / Fluorophosphonate / Protease

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### 1 Introduction

Although standard genomics and proteomics methods provide significant insights into changes in the abundance of transcripts and proteins, respectively, these methods are less effective at recording variations in protein activity. Considering that the catalytic activities of many enzyme classes, including kinases, phosphatases, and proteases are subject to post-translational regulation [1], the need for proteomics methods that can record dynamics in enzyme activity is apparent. Recently, chemical approaches have emerged that permit the consolidated detection and identification of collections of enzyme activities in complex proteomes [2]. Activity-based probes (ABPs) have been generated that profile the serine hydrolase superfamily [3, 4], as well as subclasses of the cysteine hydrolase superfamily, including cathepsins [5, 6] and caspases [7]. More recently, combinatorial libraries of ABPs were used in a proteome-wide screen to identify novel active site-directed affinity agents for the aldehyde dehydrogenase class of enzymes [8]. Continuing efforts to develop novel ABPs should allow a

significant fraction of the proteome to be analyzed in an activity-based manner, permitting the distinction of active enzymes from their inhibitor-bound and/or inactive precursor forms [4].

Previous ABP reagents have utilized either direct radiochemical ( $^{125}\text{I}$ ) detection [5, 6] or indirect biotin/avidin based methods [3, 4, 7] to assess the extent of labeling of active proteins. While radiochemical detection benefits from a direct readout, the safety issues and disposal costs associated with the use of  $^{125}\text{I}$  limit its use to lab scale operations. Biotinylated ABPs allow for avidin-based purification of target proteins, however the indirect and time consuming nature of the detection process limits the throughput and robustness of this method. Here, we report the synthesis and evaluation of fluorescent activity-based probes (fABPs) for the serine hydrolase super-family of enzymes. These probes permit the direct in-gel analysis of target protein activities and provide superior sensitivity, quantifiability, and throughput to previous ABPs.

### 2 Materials and methods

#### 2.1 Synthesis of fABPs

Fluorophosphonate-tetraethyleneglycol-tetramethyl rhodamine (FP-Peg-TMR) and FP-Peg-Fluorescein were synthesized using methodology similar to that described for the synthesis of FP-Peg-Biotin [4] with the following mod-

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**Abbreviations:** ABP, activity-based probe; fABP, fluorescent activity-based probe; FP, fluorophosphonate; FAAH, fatty acid amide hydrolase; Peg, tetraethyleneglycol; TMR, tetramethyl rhodamine

ifications. A solution of tetramethylrhodamine cadaverine (0.005 g, 0.010 mmol) or fluorescein cadaverine (0.005 g, 0.008 mmol) (Molecular Probes, Eugene, OR, USA) in DMF (0.5 mL) was added to compound 1 (neat, 0.007 g, 0.016 mmol) and the reaction mixture was stirred for 30 min at room temperature. The solvent was removed under vacuum and the products were resuspended in a 0.35 mL of a water-acetonitrile mixture (1:1 v/v) containing 0.1% (v/v) TFA. An aliquot of this solution (0.30 mL) was injected on a preparative reverse phase HPLC column (Haisil 100 C8, Higgins Analytical, Mountain View, CA, USA, 20 mm × 150 mm), separated using a 0–100% acetonitrile gradient in 30 min at 10 mL/min. The retention time under these conditions was 19.95 min for FP-Peg-TMR and 19.8 min for FP-Peg-Fluorescein. The solvent was removed under vacuum using a rotary evaporator, and afforded FP-Peg-TMR and FP-Peg-Fluorescein as judged by nuclear magnetic resonance and electrospray mass spectrometry. Detailed characterization of these and other fABPs will be published elsewhere.

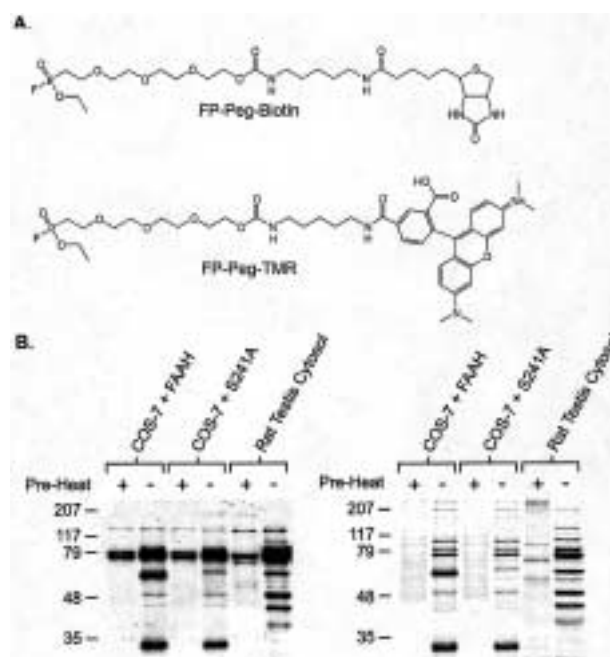
## 2.2 Preparation of proteomic samples

COS-7 cells were grown in a 10 cm round petri plate in RPMI-1640 media containing 10% fetal calf serum. Fatty acid amide hydrolase (FAAH) transfections were performed as described [9]. Cells were grown to confluence, the media was removed, and the cells were harvested by treatment with trypsin. The cells were pelleted by centrifugation for 5 min at 800 × *g* and resuspended in 0.6 mL PBS. The suspension was then sonicated, and protein concentrations were determined using the D<sub>c</sub> Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The proteome was used immediately, or stored at –80°C. Prior to use, the concentration of the final supernatants were adjusted to 1.5 mg/mL.

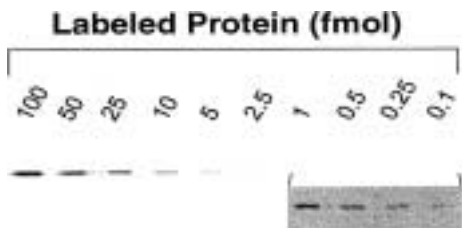
Frozen rat tissues were purchased from Pel-Freeze Biologicals (Rogers, AR, USA). The tissues were diced into cubes (1 mm<sup>3</sup>) and placed in Tris buffer (50 mM, pH 8) containing sucrose (320 mM). The tissues were then disrupted using a Tissue Tearor (BioSpec Products Bartlesville, OR, USA) and filtered through cheesecloth. The tissue extracts were Dounce homogenized and clarified by sequential centrifugation (spin 11100 × *g*, 5 min; spin 2, 22000 × *g*, 30 min; and spin 3, 105 000 × *g*, 60 min). The supernatant from the final spin constituted the soluble protein fraction of the proteome. Protein concentrations were determined using the D<sub>c</sub> Protein Assay Kit (Bio-Rad). The concentration of final supernatants were adjusted to 1.5 mg/mL prior to storage at –80°C. FAAH was expressed in *E. coli* with a (His)<sub>6</sub> tag and purified as described [10].

## 2.3 ABP labeling and analysis of proteomes

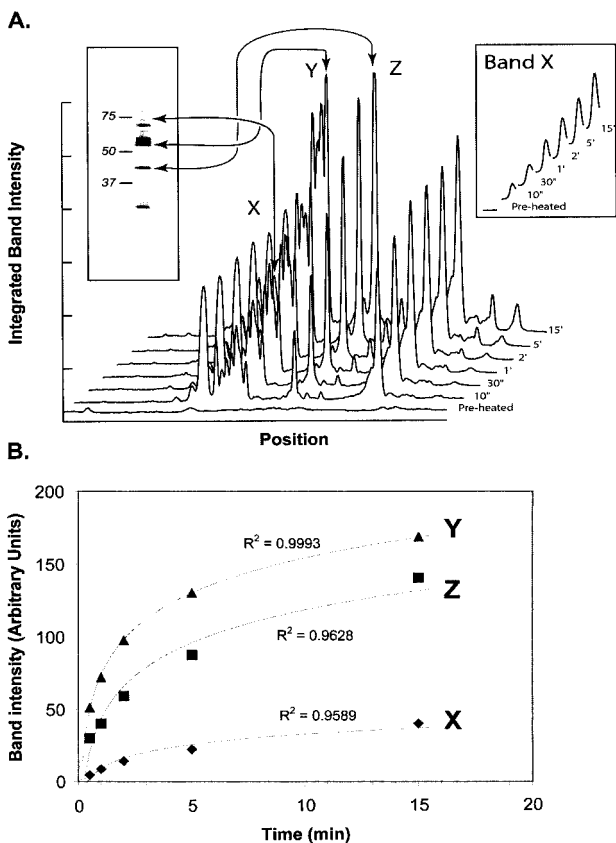
A solution of the selected ABP (100 μM in DMSO) was added to an aliquot (0.050 mL) of the chosen proteome in 50 mM Tris (pH 8.0 (Fig. 1), pH 7.0 (Figs. 2–4)) or, 100 mM NaCl to a final concentration of 2 μM (Figs. 2–4) or 5 μM (Fig. 1). The mixture was allowed to stand at room temperature for 1 h, prior to quenching with an equal volume of 2X SDS loading buffer (125 mM Tris (pH 6.75), 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.005% bromo-



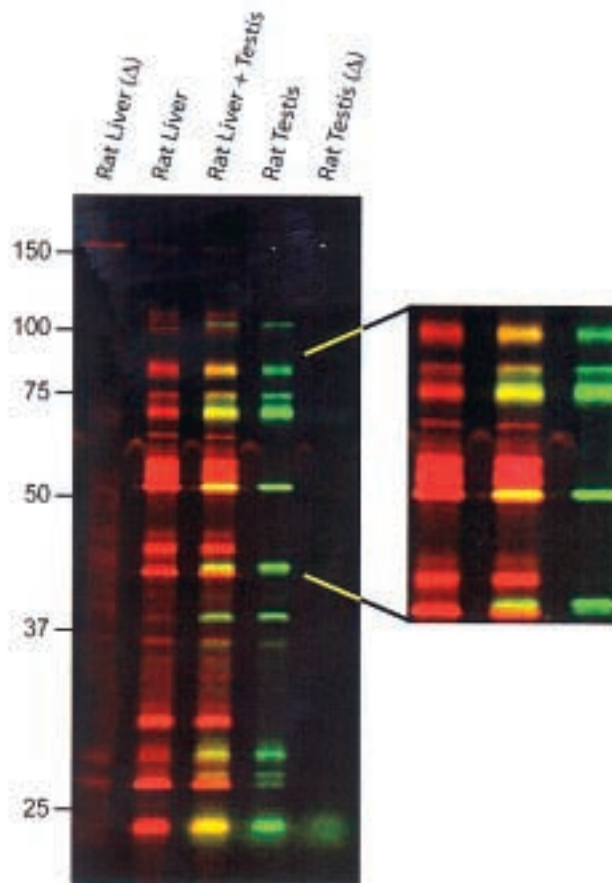
**Figure 1.** Comparison of fluorescence and biotin-avidin detection modes for analyzing proteomes with ABPs. The structures of the fluorophosphonate (FP) inhibitors used in this study are shown in (A). The reactivities of FP-Peg-Biotin and FP-Peg-TMR were compared against three proteomic samples. (B): (1) COS-7 cells transfected with fatty acid amide hydrolase [COS-7 + FAAH]; (2) COS-7 cells transfected with an inactive FAAH mutant [COS-7 + S241A]; (3) Rat testis soluble proteins (rat testis cytosol). A heat-inactivated sample of each proteome was profiled as a control. All samples were treated with the respective ABP under identical conditions which were optimized for FP-Biotin (5 μM ABP, 50 mM Tris pH 8.0, 100 mM NaCl, 1 h reaction). Reactivity of FP-biotin was assessed using a western blot as described [3, 4], while fABP reactivity was monitored in-gel using a Hitachi FMBio Ile flatbed fluorescence scanner. Both probes reacted with wild type FAAH (band at 65 kDa) but not the inactive S241A mutant. Additionally similar reactivity profiles in each proteomic sample were observed for FP-Peg-Biotin and FP-Peg-TMR. Note the presence of bands resulting from endogenously biotinylated proteins in the lanes analyzed by avidin blotting that are not observed with FP-Peg-TMR. Additionally, proteins larger than 200 kDa are observed using FP-Peg-TMR, but not FP-Peg-Biotin.



**Figure 2.** Detection limits of tetramethylrhodamine-based fABPs. A sample of purified FAAH labeled with FP-Peg-TMR was diluted serially and separated on a 1-D SDS-PAGE gel. The FAAH sample was labeled to completion as judged by kinetic analysis (as in Fig. 3). The resulting gel was scanned using the Hitachi FMBio IIe scanner. A band at the lowest dilution, 0.1 fmol (equivalent to 0.1 fmol rhodamine) could be detected (inset). The inset image was obtained by adjusting the brightness and contrast of the original image to maximize the visibility of the lower dilutions.

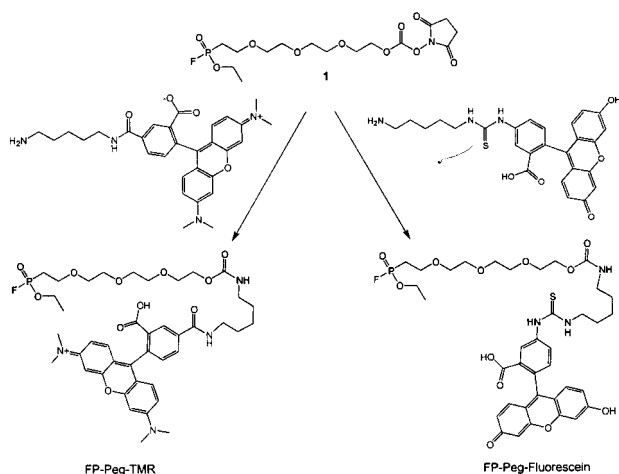


**Figure 3.** Determination of protein labeling rates in crude proteomes. A rat liver soluble proteome was labeled with FP-Peg-TMR, then quenched at 10 s, 30 s, 1, 2, 5, and 15 min with SDS loading buffer, prior to electrophoretic separation. Lanes from each time point were analyzed and plotted as pixel intensity versus position (A). Three individual bands (labeled X, Y, and Z) were chosen for quantitation. The increases in integrated band area over time for the three bands analyzed were fit to first order exponentials in (B).



**Figure 4.** Multiplexing fluorescent probes in 1-D analysis of proteomic samples. Soluble proteome samples from rat liver and testis were labeled with FP-Peg-TMR and FP-Peg-Fluorescein respectively, under identical conditions (2 μM probe, 50 mM Tris pH 7.0, 1 h labeling). The samples were run either separately or in the same lane on a 25 cm long SDS-PAGE gel. The gel was scanned directly in the glass plate sandwich with simultaneous monitoring through a 505 nm bandpass filter for fluorescein and a 605 nm bandpass filter for TMR. Very little spectral overlap was observed with two fluorescent probes allowing for the signals from each sample to be readily distinguished.

phenol blue). A sample (0.025 mL; 0.018 mg protein) of the solution was removed and separated on a 10% SDS gel. For the FP-Peg-Biotin treated proteomes, labeling was visualized using a Western blot as described [3, 4]. fABP labeled samples were visualized in-gel on a Hitachi FMBio IIe flatbed fluorescence scanner (MiraiBio, Alameda, CA, USA), with excitation provided by the 532 nm line of a 50 mW neodymium-doped yttrium-aluminum-garnet (Nd: YAG) laser. A 605 nm bandpass filter was used to detect FP-Peg-TMR, while a 505 nm bandpass filter was used for FP-Peg-Fluorescein.



**Figure 5.** Route for the synthesis of FP-Peg-TMR and FP-Peg-Fluorescein. Compound 1 was synthesized according to Kidd *et al.* [4]. The two fABPs were synthesized from compound 1 and the corresponding amine terminated fluorophore as shown.

### 3 Results and discussion

The fABPs used in this study were synthesized using amine terminated fluorophores as shown in Fig. 5. Compound 1 was synthesized as described previously [4]. For comparison, the structures of a serine hydrolase-directed fluorophosphonate fABP (FP-Peg-TMR) and a biotinylated fluorophosphonate probe [4] are shown in Fig. 1A. Both probes contain a reactive fluorophosphonate (FP) group that has been shown to react with most serine hydrolases including proteases, esterases and lipases [11, 12]. The FP functionality is tethered to a tag for subsequent detection (biotin or tetramethylrhodamine) through a tetraethylene glycol spacer. We compared the serine hydrolase activity profiles generated by both probes against three distinct proteomic samples: (1) COS-7 cells transfected with fatty acid amide hydrolase (FAAH), a known serine hydrolase; (2) COS-7 cells transfected with an inactive mutant of FAAH lacking a serine nucleophile (S241A); and (3) rat testis soluble proteome. As a control in all cases, heat-inactivated samples of the proteomes were reacted with the probes under identical conditions. The FP-Peg-Biotin probe was analyzed using avidin-based blotting methods as described [3, 4]. The reactivity of the FP-Peg-TMR probe was assessed directly in-gel using a Hitachi FM-Bio IIe flatbed laser induced fluorescence scanner. The results are shown in Fig. 1B. In both cases, the probes reacted with numerous proteins selectively in the unheated proteomes. Additionally both probes labeled FAAH in transfected cells, but not the inactive S241A FAAH mutant. Although both probes appeared to react with similar

sets of proteins in the three samples, several qualitative differences between the two probes are apparent. First, fluorescence detection eliminates the interference caused by endogenously biotinylated proteins (Fig. 1B; heated controls). Second, because the detection of biotin requires an electrophoretic transfer step, some very large proteins exhibit weak signals likely due to inefficient transfer. Third, it is evident that biotin detection is lower resolution (Fig. 1B: note the broader band patterns for biotinylated detection throughout). Finally, the nonspecific reactivity of the FP-Peg-TMR in the preheated control was, in this experiment, slightly higher than FP-Peg-Biotin under conditions that were optimized for FP-Peg-Biotin (5  $\mu\text{M}$  probe, pH 8.0, 1 h reaction). Further optimization of the reaction conditions for the FP-Peg-TMR probe (2  $\mu\text{M}$ , pH 7.0, 1 h) significantly reduced the non-specific reactivity of this probe as shown in later experiments (see Figs. 3 and 4).

In order to evaluate the absolute detection limit of the in-gel fluorescence detection method we labeled a known quantity of purified FAAH, reacted it to completion with FP-Peg-TMR and evaluated the ability to detect serial dilutions of the labeled protein. The results of this experiment are shown in Fig. 2A. FP-Peg-TMR labeled FAAH is clearly detected below 1 fmol (Fig. 2A, inset), and a reasonable limit of detection for this method is on the order of 100 amol. This sensitivity limit is more than 100-fold better than the reported detection limit of 15 fmol for biotin-based ABPs [3]. Plotting the integrated band intensities from 0.25 fmol to 100 fmol of FP-Peg-TMR labeled FAAH yielded a straight line with an  $R^2$  value of 0.98. These results suggest that the accurate quantifiable range of the fABP method is 2–3 orders of magnitude. It should be noted that the photomultiplier voltage of the laser scanner can be adjusted, thus allowing for tuning of the effective dynamic range to the range of band intensities observed in a particular experiment. Overall, the sensitivity limits and quantifiability of the fABP method are superior to the biotin/avidin based ABP detection schemes.

One important feature of ABPs is an ability to measure the changes in the relative activity of proteins in samples of interest that may take place independent of changes in protein abundance [3]. For example, ABPs can detect activity-altering post-translational modifications [4], and/or the interaction of an enzyme with an exogenously applied or endogenously produced inhibitor [3–6]. In order to ensure that such changes in activity will be detected using ABPs, the kinetics of labeling must be such that the proteins are not labeled to completion in a single time point experiment. The extent of protein labeling can be determined by monitoring the reactivity

of proteins with ABPs over multiple time points [3, 4]. A kinetic view of labeling of rat liver soluble proteins with probe incubation times between 10 s and 15 min is shown in Fig. 3. It can be seen that most proteins are incompletely labeled at the earliest time point used in this series (10 s), allowing their activities to be monitored and compared among samples. A few proteins were observed to exhibit maximal labeling at the shortest time point measured. Observation of activity changes for these proteins would require either shorter time points or lower ABP concentrations. Importantly, the increased throughput and greater quantitative accuracy of the fABPs when compared to the biotin/avidin based ABPs should facilitate the detection of even subtle changes in protein activity.

The fABPs examined in this study have exhibited several additional advantages over both biotin and radiochemical type ABPs. First, the ability to measure fluorescence directly in-gel without removing the gel from the glass plates allows for very large SDS-PAGE gels to be utilized. Biotin/avidin based detection suffers from nonuniform transfer efficiency that increases with gel size. Second, many fluorophores with distinguishable spectral properties are commercially available and can be used for multiplexing applications as demonstrated in Fig. 4. Several spectrally distinct, fluorescent ABPs targeting distinct enzyme classes could allow for the simultaneous analysis of multiple enzyme families in a single experiment. The spectral properties of the fluorescent tags could effectively provide a second dimension of resolution without decreasing sample throughput. Finally, the ability to purify proteins that have been labeled with fABPs is an important requirement to allow for the identification of labeled proteins. In the case of fABPs, our preliminary results suggest that affinity columns bearing antibodies to the fluorophore of the probe are highly effective at capturing and purifying labeled proteins (data not shown).

## 4 Concluding remarks

Incorporating fluorescence detection into activity-based probes enhances the ease and versatility of proteomic analysis. Further, the use of fluorescent labels has greatly increased the sensitivity, throughput, quantitative accuracy, and the range of applicability of activity-based protein profiling. Together, these features are beginning to point the way toward robust, fluorescence-based profiling of numerous protein activities in diverse proteomic samples, and promise to deliver a straightforward platform for high-throughput analysis.

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