



SINGLE ENZYME KINETICS OF CHYMOTRYPSIN

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ABSTRACT

We have developed an assay where we can study the activity of single molecules of bovine alpha-chymotrypsin without posing any extraneous constraints on the molecule such as immobilization. The assay is based on the synthetic substrate (bis(suc-AAPF)-rhodamine 110), which is non-fluorescent. When the enzyme cleaves this substrate, the product is intensely fluorescent (Ex. 500 nm/Em. 520 nm), due to the unbound rhodamine 110. To study the activity of single enzymes, the enzyme and substrate are encapsulated in microdroplets of water surrounded by oil. We have developed a protocol that produces micron-sized droplets of water in a water-in-oil emulsion. On average, each micro-droplet contains less than one enzyme. The fluorescence of these micro-droplets of water is monitored over time using a fluorescence microscope and a cooled CCD camera to give a measure of the kinetic activity of individual enzymes. We are able to monitor multiple enzymes simultaneously using this technique.

Background

The goal of single molecule experiments is to determine mechanisms of action for a protein. Bulk measurements on ensembles of proteins can sometimes give an insight into mechanisms of action, but this information can also be lost in the averaging across the ensemble. Also, single molecule experiments have consistently shown that a population of proteins is almost always heterogeneous in its activity. [2-5] We chose to study chymotrypsin because (in bulk) it is the best characterized serine protease. Proteases are a key component of many physiological processes. We plan on extending this work to single molecule studies of the proteasome complex.

Materials and Experimental Methods

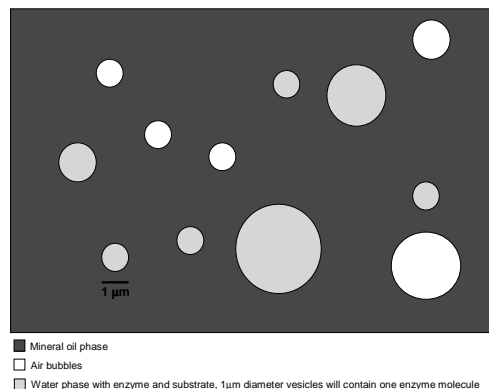
α -chymotrypsin (C-7762) is purchased from Sigma Chemical and the fluorescent substrate for chymotrypsin is rhodamine 110 bis-(succinoyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl amide) purchased from Molecular Probes. Rhodamine 110 has an extinction coefficient of $81,000 \text{ cm}^{-1} \text{ M}^{-1}$ at 498nm with a quantum yield of 0.91. [1] α -chymotrypsin is dissolved in 10mM HEPES buffer pH 7.5 for a 1unit/ μL (1mM) stock solution and the substrate is dissolved in DMSO for 15mM stock.

One unit of α -chymotrypsin (25 ng) contains 6×10^{14} molecules. α -chymotrypsin and the R110-AAPF substrate are first diluted to 100nM (or 6×10^{10} molecules/100 μL) and 150uM (or 9×10^{15} molecules/100 μL) and then mixed in an Eppendorf tube. The mixture is divided between a fluorescence microplate reader (Fluorskan Ascent FL from Labsystems) and the emulsion.

The emulsion is created using a Waring blender with a mini-blade assembly design to blend small volumes. The mixture of mineral oil (~15 mL) and reaction mixture (200 μL) is blended for ten seconds in the blender. The resulting emulsion is then pipetted (~10 μL) onto a microscope slide for observation under a fluorescent microscope (Nikon TE 200). The fluorescent microscope uses a 100W mercury arc lamp with the Chroma FITC HQ series filter set (Ex 480/40 nm, Em 535/50 nm) and the Nikon 40x/NA 1.3 oil immersion objective. Images are recorded using an Apogee 7P CCD camera containing the SITE 512x512 back illuminated scientific grade CCD, which has a quantum efficiency of 75% at 520 nm and 24 μm square pixels.

Both the microplate reader and the microscope are programmed to take a reading every 5 minutes with a 1 second integration time. Images taken by the CCD camera are subsequently processed to remove dark noise and correct for flat field distortions. Images are filtered to select spots approximately 1 μm in diameter and the fluorescence intensity is quantified from each of these spots in the series of images. The results are shown in Figure 3.

Concept of Experiment



Realization of Experiment

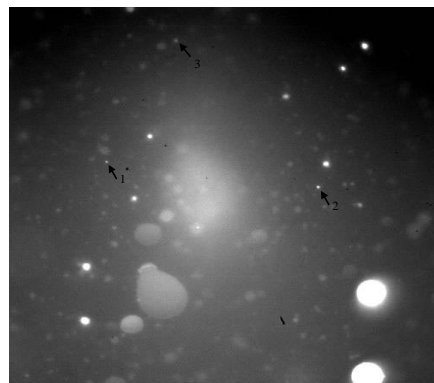


Figure 2. Fluorescent image of water-in-oil emulsion where the water phase contains 100nM α -chymotrypsin and 150 μM of R110-AAPF. A one micron diameter reaction droplet would contain approximately 1 molecule of α -chymotrypsin and $\sim 2 \times 10^5$ molecules of R110-AAPF. Three reaction droplets are identified and quantified in Figure 3.

Conclusion

- The emulsion method is a simple yet effective method for isolating and observing single enzyme molecules.
- We are able to observe multiple single enzyme reaction simultaneously under the microscope and record the progress of these individual enzymes using a CCD camera.
- Isolated single enzyme molecules exhibit heterogeneity, turning over the substrate at different individual kinetic rates.

Future Directions

- Conjugate the enzyme molecules with another fluorophore and confirm that the emulsion created contains only one enzyme per water droplet.
- Use laser induced fluorescence to increase the fluorescence signal and to measure the amount of substrate turned over by individual enzyme molecules.

Microplate Reader Fluorescence Signal through Time

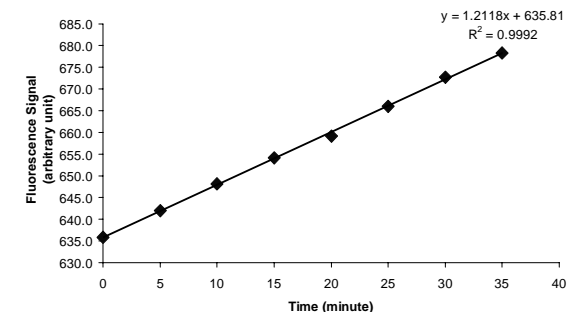


Figure 1. Bulk fluorescence over time as measured in a microplate reader, α -chymotrypsin 100nM or $\sim 6 \times 10^{10}$ molecules/100 μL , R110-AAPF 150 μM or $\sim 9 \times 10^{15}$ molecules/100 μL .

Individual Fluorescence Signal through Time

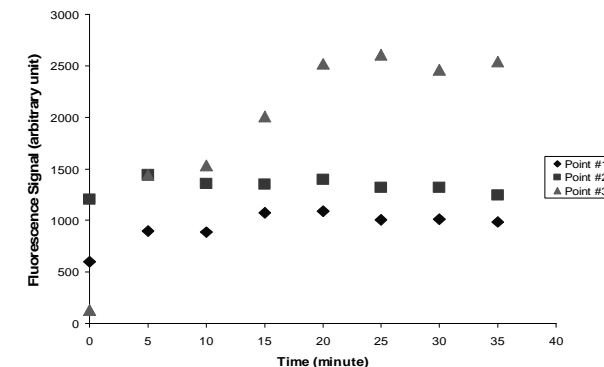


Figure 3. Fluorescence signal over time as measured from three individual water droplets each droplet contains 1 molecule of α -chymotrypsin and $\sim 2 \times 10^5$ molecules of R110-AAPF.

References

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