PROTEASE DIFFUSION AND REACTION ON IMMOBILIZED SUBSTRATE SURFACES

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Abstract

Enzymatic reactions with surface bound substrates are important in such applications as detergent enzyme additives and food processing. Here, we study an important surface property of the enzyme: lateral diffusivity. Microfluidic patterning is used to decorate a region of the bovine serum albumin substrate surface with a subtilisin protease. Any spreading of the enzyme from this initial region indicates surface diffusion. By varying the ionic strength of the system and studying subtilisin having single point mutations, we investigate the influence of electrostatic interactions on the surface mobility and surface reactivity of the subtilisin enzyme.

Keywords: Subtilisin, surface diffusion, adsorption, bovine serum albumin (BSA)

1. Introduction

A common practice in the manufacture of laundry detergents is to package enzyme molecules with detergent solutions to enhance cleaning power. We study the interaction of a subtilisin protease with model protein coated surfaces to understand the kinetics of stain removal at fabric surfaces. Earlier work in our laboratory [1] has shown that the cleavage of surface bound substrates is a multi-step adsorption/surface diffusion/reaction process in which any of the three processes can be reaction rate-limiting. However, to date, the surface diffusivity of these enzymes has never been effectively quantified . We present a microfluidic patterning technique for the label-free measurement of enzyme lateral diffusivity on a substrate surface.

2. Theory

In a microfluidic cell with a three inlet channel geometry similar to that used by Takayama *et al.* [2], a solution containing subtilisin enzyme is flowed down the middle lane over a glass surface coated with a monolayer of covalently bound bovine serum

0-9743611-0-0/μTAS2003/$15.00©2003TRF

albumin (BSA). Because of the small dimensions of the channels, all fluids are in creeping flow and thus the lanes are formed in the absence of physical barriers. The BSA serves as a model substrate for the enzyme and it is cleaved from the surface in the areas exposed to the subtilisin. Thus, a trench forms down the middle of the substrate surface. The principle of the surface diffusion measurement is that although enzyme molecules are confined to the middle lane in the bulk, they are free to diffuse laterally once they become adsorbed to the surface. Adsorbed subtilisin molecules can thus cleave BSA outside this original trench and trench widening can be correlated to surface diffusion.

There are two important considerations to note regarding this measurement. The first is that there is a small degree of blurring of the lane boundaries due to diffusion in the bulk fluid. Any enzyme molecule that crosses a lane boundary by bulk diffusion is promptly swept downstream by the flowing buffers in the side lanes. As a result, bulk diffusion reaches a steady state while diffusion on the surface is time-dependent. The two processes are thus separable.

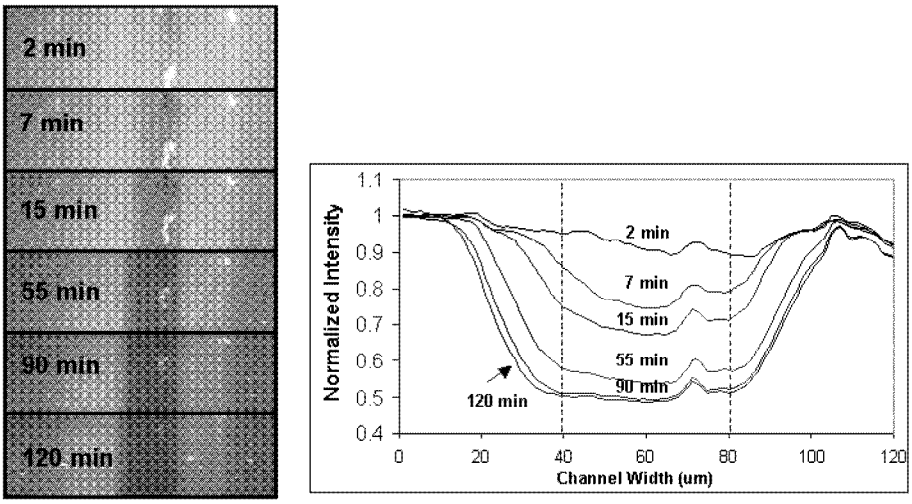
The second important consideration is that we earlier showed [1] that single amino acid substitutions result in substantial changes to the enzyme's surface properties. It is therefore imperative that the experiment be run with no extrinsic labels coupled to the subtilisin enzyme. Because in our experiment the presence of subtilisin is indicated by a loss of substrate, we can measure enzyme surface diffusion without actually labeling the enzyme; rather we gain the option of marking the substrate with a label. Here, the BSA substrate is fluorescently labeled with Texas Red and the loss of substrate visualized using fluorescence microscopy.

3. Three Lane Results

Fluorescence micrographs depicting a basic three lane experiment are shown in Figure 1. BLSv1 subtilisin [1] is flowed down the middle lane over a fluorescent BSA surface. The beginnings of trench formation are first seen after about 2 minutes. As time progresses the intensity in the middle region drops and the trench deepens and widens providing an indication of reaction and diffusion. In Figure 1b, we see the intensity profiles across the channel. The dotted lines indicate the original enzyme lane width. It is apparent that after almost two hours of enzyme flow, the trench has widened considerably beyond the original lane. This is a clear indication that the enzyme is diffusing along the surface as it cleaves the substrate.

4. Subtilisin Mutant Experiments

In our earlier work with subtilisin [1], we studied the concurrent adsorption and reactivity characteristics of several mutant forms of BLSv1 as they interacted with a surface bound BSA substrate. One mutant in particular, BLSv1-Q109R, displayed very strong adsorption to the BSA surface. Interestingly, despite the abundance of enzyme on



(a) (b)

Figure 1: (a) Enzyme flow down middle lane results in trench that deepens and widens with the passage of time. (b) Corresponding fluorescence intensity profiles taken across the channel

the surface, the mutant was relatively slow in catalyzing surface hydrolysis. At the time, we hypothesized that this negative correlation between enzyme adsorption and reactivity could be due to surface mobility limitations. That is, BLSv1 molecules become so strongly adsorbed to the surface during the reaction that they are unable to move along the surface as efficiently and their ability to access substrate molecules is adversely affected.

We now wish to confirm this hypothesis using our microfluidic patterning surface diffusion assay. As the low-Reynolds number condition places no limitations on the number of inlets that can be used, we chose to work with a five inlet channel geometry. This allowed us to assay two enzyme variants simultaneously, keeping BLSv1 as a reference in each case.

The results of this experiments are shown in Figure 2. Because the interaction between the negatively charged surface and the enzyme is primarily electrostatic, we found that we could limit the strength of adsorption by increasing the ionic strength. Both low (Figure 2a) and high (Figure 2b) ionic strength cases are shown. In Figure 2a,

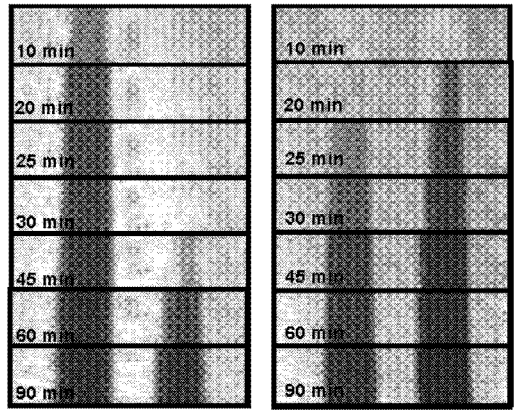


Figure 2: Reaction/diffusion data for BLSv1 flowed alongside BLSv1-Q109R under conditions of (a) low and (b) high ionic strength. In either case, BLSv1 is in the left lane.

the strongly adsorbing BLSv1-Q109R appears to be much more slowly reacting and slowly diffusing than the wildtype BLSv1, in accordance with our hypothesis. Figure 2b shows a dramatic ionic strength effect, as the BLSv1-Q109R lane actually appears before the corresponding wildtype lane. The two lanes spread at similar rates. Again, this result fits with our hypothesis, as the adsorption of BLSv1 at higher ionic strength is much less strong and it is therefore reasonable that a surface mobility limitation is no longer evident. Quantitative analysis of these results still remains, but qualitatively, the impact of surface diffusion on surface reactivity can be observed.

References

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