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Immobilized protein films for assessing surface proteolysis kinetics

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Abstract

Enzymatic cleavage of protein substrates at solid surfaces is important in the food and detergent industries, and in biomedical applications. Creation of a reproducible protein substrate to study surface proteolysis is difficult as protein monolayers may not necessarily provide complete coverage of the surface, and protein multilayer systems are often unstable and nonuniform. We present a method to form a reproducible, immobilized, multilayer protein substrate. A 100-nm ovalbumin protein film is spin-cast onto an amine-functionalized silicon wafer and chemically cross-linked using glutaraldehyde to create a multilayer film. This protein film is stable in the presence of non-protease components such as detergents, and can be tailored to include different proteins and their mixtures, and varying degrees of susceptibility to proteolysis. Ellipsometry was used to measure the protein-film thickness as the substrate is cleaved by the protease subtilisin Carlsberg. The decrease in film thickness over time was found to be linear, indicating the depth-homogeneity of the model substrates. Lateral-homogeneity of the substrates was corroborated by atomic force microscopy (AFM) and by the reproducibility of the ellipsometric film thickness measured across different spots on the sample substrates. AFM of the multilayer protein surface before and after exposure to enzyme suggests uniform areal surface cleavage by the protease.

Keywords: Immobilized protein; Cross-linking; Surface enzyme catalysis; Biological films; Detergency

1. Introduction

Catalytic action of enzymes at solid surfaces is important in a broad range of applications including paper processing, food processing, automatic dishwashing and laundry detergents, enzymatic stonewashing of textiles, as well as removal of biological films from contact lenses and from teeth and dental instruments (Berg et al., 2001; Bhat, 2000; Ito et al., 1998; Levy et al., 2002; Villeneuve et al., 2000). Consequently, there is increasing interest in understanding the mechanism of enzyme action at solid surfaces (Eriksson et al., 2005; Karajanagi et al., 2004; Rabinovich et al., 2002; Tsung and Tilton, 1999). Control and optimization of enzymatic activity requires detailed understanding of the sorption and kinetic processes at the interface. Though much is known about the cleavage of substrates in solution by enzymes, previous studies have shown that bulk aqueous rates of proteolysis are not good indicators of protease performance on substrates attached to solid surfaces, as interfacial cleavage is affected by the surface activity of the enzyme (Brode and Rauch, 1992; Kim et al., 2002), the nature of the substrate (Brode et al., 1994; Esker et al., 2000), the distance between substrate molecules at the surface (Gaspers et al., 1995), as well as protease conformational changes at the surface (Karajanagi et al., 2004). Understanding of interfacial cleavage can only be obtained by studying the mechanisms of proteolysis on a surface that is reproducibly coated with a relevant protein film.

Industrial approaches to study enzymatic degradation of proteins at solid surfaces often employ protein substrates that are stamped or wiped onto a solid support as a wet stain and allowed to dry (Cutler and Kissa, 1987). Application of the protein, the mixtures of proteins studied, as well as the surface properties and available surface area of the support (e.g., textiles), all introduce significant variation in the sample, since some of the protein at the surface is reversibly bound and is immediately removed upon rewetting. Typically, the lack of lateral and depth uniformity of such protein substrates is insufficient to permit quantitative or mechanistic studies of enzyme behavior; such protein substrates only allow coarse evaluation of total mass removal by gravimetric or colorimetric analytical methods. To date, studies on interfacial proteolysis have relied on immobilization of protein substrates to glass beads (Brode et al., 1994; Esker et

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al., 2000) and to gold-coated glass slides (Kim et al., 2002; Roy et al., 2002). As a consequence of the immobilization schemes, monolayers and sub-monolayers of protein are formed at the surface. The difficulty with sub-monolayer surface coverage is its non-homogeneous nature. The enzyme can interact with both protein-occupied and unoccupied sites on the surface, making interpretation of adsorption and proteolysis kinetics difficult. Another deficiency with both monolayer and sub-monolayer substrate coverage is that lateral inhomogeneity of the surface is a function of time. As the enzyme cleaves the protein bound to the surface, there is a decreasing number of occupied sites for enzyme–protein interaction and binding. Moreover, actual protein stains are not likely to be sub-monolayers.

In the present study, we design and characterize a well-defined, multilayer protein film that can serve as a model substrate for surface proteolysis. The protein film is built on an amine-functionalized silicon support and protein multilayers are bound to the support using chemical vapor deposition of a bifunctional aldehyde. The smooth, reflective silicon support permits studies of film surface morphology using atomic force microscopy (AFM) and optical thickness of the film using ellipsometry. Success of the model substrate is examined in the context of reproducibility, lateral and depth uniformity, stability against solvent removal, and extent of multilayers present. Stability of the protein films in non-enzymatic cleaning solutions is investigated, as well as the potential of the method to study possible synergy of surfactant cleaning-components with proteases. The proposed film-formation method provides controlled variations in the extent of protein cross-linking, and hence, in the rate of proteolytic cleavage.

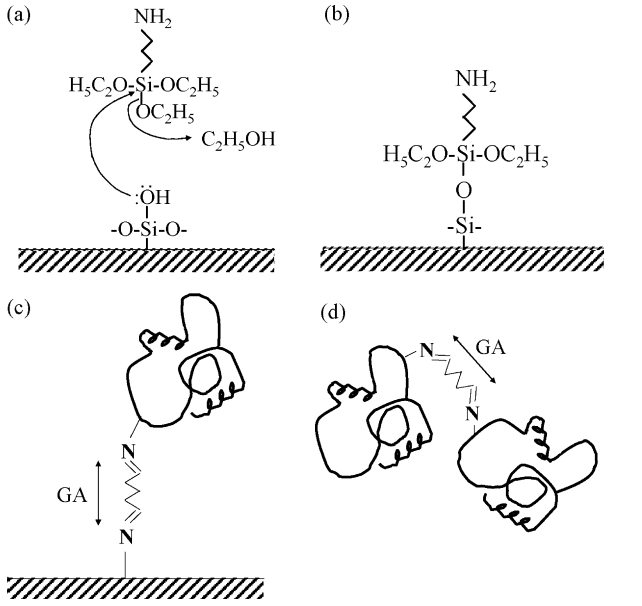

Fig. 1. Schematic of surface functionalization and protein immobilization. (a) 3-Aminopropyltriethoxysilane (APTES) is deposited by CVD onto a plasma-oxidized silicon-wafer surface. (b) Reaction of the APTES at the surface creates an amine-functionalized silicon support, here represented by one APTES monomer. In practice, the APTES polymerizes on the silicon during the CVD procedure, creating a 2–3 nm film. (c) Glutaraldehyde (GA) is introduced in the vapor phase after a protein solution is spin-cast onto the amine-functionalized silicon support. GA tethers the lysines on the proteins to the APTES on the support surface by the Schiff base chemistry mechanism. (d) Analogous reactions in the bulk of the film bind lysines on neighboring proteins to one another to create inter-protein cross-links that hold the protein multilayers immobilized to the support. Figure is not to scale.

2. Materials and methods

2.1. Functionalization of the support

Silicon wafers (International Wafer Service, Inc.) were cut into 1-cm^2 pieces and cleaned with a plasma oxidizer (Harrick Plasma Cleaner/Sterilizer PDC-32G) for 2 min on a medium setting, corresponding to a 60 W radiofrequency excitation. Plasma cleaning removes hydrocarbons adsorbed to the silicon-wafer surface, and also activates surface silanols for subsequent reaction with the surface-functionalizing agent, 3-aminopropyltriethoxysilane (APTES). After plasma oxidation, the silicon-oxide thickness on the wafers is measured by ellipsometry (Sentech SE400) as approximately 2 nm.

After cleaning, bare silicon-wafer supports are functionalized by chemical vapor deposition (CVD) of APTES (Fluka purum ≥ 98.0% by GC). Vapor-phase deposition creates a uniform APTES film compared to solution deposition in which polymerized forms of APTES may deposit in mounds, as observed by atomic force microscopy in our laboratory and elsewhere (Haller, 1978). CVD was performed at room temperature with 1 ml of liquid APTES, pipetted into an annular well in a closed 71-cm^3 vessel holding the silicon-wafer samples. After functionalization, the samples were placed in an oven at 110 °C for 15 min to remove unbound APTES. As measured by ellipsometry, a deposition time of 1 h results in an APTES layer of 2–3 nm on top of the silicon wafers with a standard deviation of approximately 0.5 nm. This functionalized-layer thickness is sufficient to ensure complete coverage of the silicon support, and serves as a stable platform for tethering of the protein film. Fig. 1(a) and (b) displays the chemical steps involved in APTES-coating of the silicon supports.

2.2. Protein-film attachment

APTES-functionalized silicon supports were coated with a concentrated aqueous (distilled/deionized water, Millipore MilliQ Filter Unit, 18.2 MΩ cm resistivity) protein solution (41 mg ml^{-1}) at 3000 rpm using a spin coater (Cookson P-6000). Ovalbumin (Calbiochem, 5× crystalline, ≥98.0% by SDS-PAGE), a protein in hen egg white, served as the substrate protein as it is relevant to automatic-dishwashing detergency applications (i.e. it is one component found in egg stains). Resulting ovalbumin multilayer films were approximately 100 nm thick (determined by ellipsometry), corresponding roughly to 17 layers of protein based on the 6-nm Stokes diameter of ovalbumin (Johnson et al., 1996). Both the spin-coat protein-solution concentration and the rotational speed may be altered to create films with differing initial thicknesses.

CVD of glutaraldehyde (Sigma–Aldrich, 25 wt% in water) accomplished both covalent attachment of the protein film to the APTES-functionalized silicon support and inter-protein cross-