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Savinase action on bovine serum albumin (BSA) monolayers demonstrated with measurements at the air–water interface and liquid Atomic Force Microscopy (AFM) imaging

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ABSTRACT

We studied the enzymatic action of Savinase on bovine serum albumin (BSA) organized in a monolayer spread at the air/water interface or adsorbed at the mica surface. We carried out two types of experiments. In the first one we followed the degradation of the protein monolayer by measuring the surface pressure and surface area decrease versus time. In the second approach we applied AFM imaging of the supported BSA monolayers adsorbed on mica solid supports and extracted information for the enzyme action by analyzing the obtained images of the surface topography in the course of enzyme action. In both cases we obtained an estimate for the turnover number (TON) of the enzyme reaction.

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1. Introduction

Langmuir monolayers represent well defined molecular systems for studying the enzyme reactions that occur at the air–water interface [1]. The strictly determined molecular organization by means of occupied molecular area, surface concentration, dipole moments, etc., allows various parameters (e.g., surface pressure and surface potential) to be measured as a function of precisely established experimental conditions and further to be employed for enzyme kinetics studies. In the beginning of 1970s the pioneer work of Verger and deHaas [2] attracted the scientific attention towards the interfacial lipolytic catalysis in monolayers and in the following three decades many research groups systematically studied the hydrolysis of lipids and phospholipids organized at the air–water interface [3,4]. The method was utilized for studying different *enzyme–substrate* (*monolayer*) systems at the air–water interface i.e. Cutinase (*enzyme*)–polyethylene glycol/poly(D,L-lactide-co-glycolide) polymers (*monolayer*) [5], *humicola lanuginosa lipase* (HLL) (*enzyme*)–polycaprolactone (*monolayer*) [6], and Savinase (*enzyme*)–alpha gliadin (*monolayer*) [7]. In the core of the method lays the physicochemical characterization of the properties of substrate–enzyme systems by means of surface parameters – surface pressure, surface potential and molecular area, measured in the course of the enzyme reactions. The experimentally obtained mechanical and the electrical constants of the monolayers were further employed in different kinetic models [5–7].

One of the aims of this article is to utilize the monolayer technique for the hydrolysis of protein monolayers of bovine serum albumin (BSA) undergoing proteolytic action of *Savinase* (EC 3.4.21.14). It is well experimentally established that proteins and particularly BSA can be assembled as monomolecular layers at the air–water interface [8]. Once formed at the interface protein monolayers represent well define organized substrates for the enzyme action of certain proteases. Savinase is a protease secreted by the alkalophilic bacterium *Bacillus lentus* and belongs to the subgroup of subtilisin-like enzymes. The crystal structure of the native form of *Savinase* has been obtained using X-ray diffraction data [9] and the enzyme is reported as widely used industrial applicant in detergents [10].

In this report we present the experimental data for the change of the surface parameters – surface pressure and surface area of spread BSA monolayers at the air–water interface, recorded before and after the injection of Savinase into the liquid subphase. On the basis of the experimental data – surface area versus time we estimated the turnover number (TON) for the enzyme reaction at the interface.

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In this article we also demonstrate the application of Atomic Force Microscopy (AFM) for the visualization of BSA films adsorbed on mica surfaces and further exposed to the action of the Savinase. AFM proved to be very reliable tool for biological applications and for the investigation of enzyme structure and enzyme behavior in organized molecular media that mimics in vivo systems [11,12]. AFM data for the single enzyme activity [13], hydrolysis of a supported phospholipid bilayer by Phospholipase A_2 (PLA_2) [14–16], *humicola lanuginosa* lipase (HLL) [17] were reported and a new method for analysis of the enzyme kinetics were established. In this study we performed imaging with AFM liquid cell of BSA monolayers adsorbed on mica solid supports, before and after flushing the liquid cell with the enzyme solution. We obtained real time images at different time intervals which represent a direct visual evidence for the Savinase action.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) was purchased from Sigma–Aldrich (Germany) in form of lyophilized powder. According to producer's specifications BSA has molecular mass about 69 kDa, contains 583 amino acid residues and total impurities of less than 0.01% fatty acid. BSA was dissolved in acetic acid, 0.1 M acetic acid to the concentration of 1 mg/mL. Tris(hydroxymethyl)aminomethane (TRIS), used in the buffer solution (TRIS/HCl) with pH 8, was supplied by Merck (Darmstadt, Germany). HCl was purchased from Theokom (Sofia, Bulgaria) and was used without further purification. In all experiments pure water from Milli-Q system (Millipore Corporation, Boston, USA) was used. Savinase was provided by Novozymes A/S (Bagsværd, Denmark) as a solution with concentration 1 g/L. This solution was injected in the subphase so to be diluted to the final concentrations of 2.5, 5, 17.5, and 35 nM. In monolayer preparation for the subphase TRIS buffer was also used.

2.2. Experimental methods

2.2.1. Surface pressure (constant area) and barostat (constant surface pressure) measurements

Two sets of measurements during the enzyme hydrolysis of the adsorbed BSA layers were performed. In the first one the surface pressure change ($\Delta\pi$) versus time was measured by Wilhelmy method. In the second set of experiments the decrease of the surface area (ΔA) versus time (t) at constant surface pressure (π) was measured. We used an automatic homemade surface trough equipped with Sartorius analytical balance model BP211D (Sartorius, Germany) and a platinum plate. All the setup was connected to a personal computer and provided with user software for real time data acquisition and feedback control.

Experimentally, at first using Hamilton microsyringe we spread 4 μL of BSA solution with concentration 1 mg/mL on a subphase of TRIS buffer in a Teflon rectangular trough (length 7.5 cm, width 4 cm and depth 1.8 cm). When we measured the change of the surface area (ΔA) versus time (t) at constant surface pressure (π), first we spread the BSA monolayer and left it until the equilibrium value of the surface pressure was reached. Then, the barostat setup was turned on in order to keep this value constant. As a next step with a Hamilton microsyringe the Savinase solution was injected into the subphase and the change of the surface area was recorded. In the other set of experiments the barostat was left off. When the enzyme solution was injected into subphase, the change of the surface pressure was followed. The subphase was continuously stirred with a magnetic rod at 250 min^{-1} to homogenize the Savinase solution. In both sets of experiments we observed a decrease of the surface area and a decrease of the surface pressure which were related to the kinetics of the interfacial reorganization of the BSA monolayer induced by the enzyme action.

2.2.2. AFM measurements

Multimode Nanoscope IV AFM (Veeco Metrology Group, now Bruker AXS) equipped with J scanner with maximum imaging range of 120 μm was used. For liquid imaging a fluid cell with sealing silicon O-ring was used. The estimated volume of the cell chamber was about 20 μL. The sample imaging was carried out in tapping mode using oxide-sharpened silicon nitride tips (NanoProbes, Santa Barbara, CA) with cantilever spring constant about 0.38 N m^{-1}. The tuning of the resonance frequency was around 9 kHz. The scan rate was 2 Hz (i.e. 4.2 min/image). As a solid supports for BSA adsorption were used mica discs Grade V-4 Muscovite with diameter of 12 mm (Structure Probe Inc./SPI Supplies, West Chester, PA). The mica dick was glued to the metal pad. After cleaving the mica, the disc was placed into the liquid cell and the cell was flushed with pure water. Subsequently the BSA solution (0.02 mg/mL) was injected by syringe into the liquid cell. After 20 min, the liquid cell was flushed again with TRIS buffer and left for another 20 min for thermal equilibration. Afterwards the AFM was switch to contact mode and XY scanning size was set at 1 μm × 1 μm. The set point was manually adjusted so that the force between tip and the sample to be maintained at maximum value. The scanning rate was set to 20 Hz, and when the scanning started the feedback control was turned off. The surface was extensively scanned for 5 min. The idea of this procedure was to scratch the sample and to form a defect in the BSA monolayer with a square shape 1 μm × 1 μm. After that the tip was disengaged and the AFM was switch to tapping mode, the cantilever was tuned at the resonant frequency of 9 kHz and the scanning size was set at 5 μm × 5 μm. If the square defect on the BSA sample was clearly formed than as a next experimental step, the Savinase solution with 10 nM concentration was flushed trough the liquid cell. As a control experiment, at the end of hydrolysis, the scanning area was increased as a check that no mechanical impact on the sample within the previously scanned area has been exerted by the tip. The obtained images have been flattened and corrected for baseline tilt and bow using the Nanoscope software. The WSXM 5.0 software package was also used for section analysis and image representation [18].

3. Results and discussions

At Fig. 1 is presented a typical curve of the surface pressure change during spreading of BSA and subsequently after injection of Savinase solution. After spreading of 4 μL of BSA solution on TRIS buffer the surface pressure gradually increases and reaches equilibrium value of 11.5 (±0.2) mN m^{-1}. This equilibrium value was previously reported by Krause and Schwenke [19], although from dynamic BSA isotherms of the surface pressure versus surface area, Boury et al. [20] reported 13 mN m^{-1} at the inflection point of the isotherm. This slightly small difference between the equilibrium values of the surface pressure could be explained with the different grades of BSA used.

At $t \sim 600$ s the solution of Savinase was injected into the subphase. Initially for about 100 s a small drop of the surface pressure (about 0.5 mN m^{-1}) is noticed (Fig. 1). This drop is further followed by profound decrease of the surface pressure as a result of hydrolytic action of Savinase. The enzyme cuts portions of the BSA molecules and subsequently, the chopped amino acid segments dissolve in the liquid subphase. As a result the surface pressure decreases.

The dependence of the surface pressure decrease $\Delta\pi(t)$ on the enzyme concentration (E_0) in the subphase is measured at con-

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