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Adsorption of *Candida rugosa* lipase at water-polymer interface: The case of poly(DL)lactide

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ABSTRACT

Insights into the interactions between biological macromolecules and polymeric surfaces are of great interest because of potential uses in developing biotechnologies. In this study we focused on the adsorption of a model lipolytic enzyme, *Candida rugosa* lipase (CRL), on poly-(D,L)-lactic acid (PDLLA) polymer with the aim to gain deeper insights into the interactions between the enzyme and the carrier. Such studies are of particular relevance in order to establish the optimal conditions for enzyme immobilization and its applications. We employed two different approaches; by analyzing the influence of adsorbed CRL molecules on the thermodynamic behavior of Langmuir films of PDLLA deposited at air–water interface, we gained interesting information on the molecular interactions between the protein and the polymer. Successively, by a systematic analysis of the adsorption of CRL on PDLLA nanoparticles, we showed that the adsorption of a model lipase, CRL, on PDLLA is described in terms of a Langmuir-type adsorption behavior. In this model, only monomolecular adsorption takes place (i.e. only a single layer of the protein adsorbs on the support) and the interactions between adsorbed molecules and surface are short ranged. Moreover, both the adsorption and desorption are activated processes, and the heat of adsorption (the difference between the activation energy for adsorption and desorption) is independent from the surface coverage of the adsorbing species. Finally, we obtained an estimate of the number of molecules of the protein adsorbed per surface unit on the particles, a parameter of a practical relevance for applications in biocatalysis, and a semi-quantitative estimate of the energies (heat of adsorption) involved in the adsorption process.

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1. Introduction

Polylactic acid (PLA) is one of the most commonly used polymers for biotechnological applications [1,2]. In fact, it possesses features like non-toxicity, biodegradability and biocompatibility that make it an ideal material for a wide number of biotechnological applications [3].

Moreover, the biodegradation of PLA leads to pharmacologically inactive substances, which are absorbed by the body or removed by metabolism [4]. PLA is derived from renewable sources, such as corn starch and sugar cane, and its use in the preparation of bioplastics, usefulness for producing loose-fill packaging, compost bags, food packaging, and disposable tableware, qualify it as a sustainable alternative to petrochemical-derived products [5,6]. PLA monolayer isotherms have been recently studied [7]. They show a feature that looks similar to the well-known monolayer main transition of fatty acids or phospholipids, with analogous temperature dependence, although PLA has a completely different molecular structure. The dependence of the main transition on the length of the tails of the molecules is mirrored by the dependence on the length of the polymer chain (or molecular weight). Materials of different molecular weight are miscible in monolayers and the main transition like feature is shifted in proportion to the concentration. This is similar to the behavior of mixtures of biologically relevant lipids and suggests the possible use of polymers as membrane materials for artificial applications because polymers should be able to provide much more durable membranes than natural or small molecule films can afford.

All cellular properties are strongly dependent from the specific functional features provided to the subcellular space by interfaces. Lipases are soluble enzymes comprising a category of the most frequently studied interfacial enzymes [8,9], mainly acting at the oil–water interface (surface of oil droplets) and not at the level of cellular membranes, on the contrary to some phospholipases. Having a catalytic action which is strictly dependent upon the presence of a water–lipid interface, lipases are an example of the importance of studying protein adsorption and protein–surface interactions [10–14]. However most of the published

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studies on lipase–monolayer interface interactions concern the interactions with lipid films that is with the substrate. Moreover, lipase immobilization for biocatalytic applications is particularly important and represents a very active research area [15–17]. When lipases are used as immobilized enzymes, their substrate may also be solubilized in organic solvent, thus increasing dramatically the number of their potential applications [18–20]. *Candida rugosa* lipase (CRL) is one of the most important industrial enzymes, thanks to its ability to produce chiral chemicals with high enantiomeric purity [21,22]. Having the GRAS status, its multiple applications of commercial interest cover a broad range of industrial fields, from food, to chemical synthesis, to skin care [23].

It is well established that a hydrophobic interface can improve lipase stability. Moreover, recent studies have demonstrated that the interaction of lipolytic enzymes with nanostructured materials can enhance the activity of the adsorbed protein [24]. The mechanisms likely to occur during the adsorption of lipase onto monomolecular films at the air–water interface have been studied to some extent [25–27]. Further investigations of protein interactions with synthetic interfaces may inform the design of protein-based biosensors and, since adsorbed proteins mediate the interaction of cells with a surface, complement our understanding of cell–surface interactions.

Adsorption of proteins at an interface can be investigated by using a number of methods. The Langmuir balance is a useful technique for model studies of molecular interactions at interfaces because surface pressure-area isotherms reflect the intermolecular forces operating in two-dimensional arrays of macromolecules, and also provide information about their organization and conformational changes [28–31]. When lipases are used as immobilized enzymes, their substrate is usually solubilized in organic solvents. Therefore, a double interest in studying lipase adsorption arises: one can study adsorption in relation with the mode of action of the enzyme on its natural substrate, but also in relation with enzyme immobilization for other purposes. In this context, the adsorption of an enzymatic protein, *candida rugosa* lipase (CRL), on poly-D,L-lactic acid (PDLLA)-based polymeric surfaces, was investigated. Qualitative information on the interactions between the protein and the polymeric surface as well as on their mutual organization was obtained. CRL adsorption isotherms on PDLLA nanoparticles were also studied and analyzed to gain quantitative thermodynamic information and an estimate of the heat of adsorption.

2. Experimental section

2.1. Materials

The polylactide employed in this study, a copolymer of poly-D and L-lactic acid, PDLLA, (MW 75–120 kDa), and the lipase from *Candida rugosa* (CRL), type VII (MW 57 kDa) were purchased from Sigma-Aldrich (St. Louis MO). Bradford Reagent and bovine serum albumin (BSA), chloroform, dimethylformamide and other solvents, analytical grade, were also purchased from Sigma. Na_2HPO_4 and KH_2PO_4 were purchased from Carlo Erba Analyticals (Milano, Italy). All the chemicals were used without further treatment. Water used in all experiments and cleaning procedures was purified using a Milli-Q system (Billerica, MA), to a specific resistance of 18 MΩ cm^{-1}. Phosphate buffered saline (PBS, pH 7.6, I 0.1) was used. Protein samples were centrifuged at 14000 rpm for 3 min at 4 °C. Following the centrifugation, a UV/Visible spectrophotometer Pharmacia Biotech Ultrospec 4000 (Stockholm, SE) was used to determine the protein concentration by the Bradford method using bovine serum albumin (BSA) as a standard.

2.2. Methods

2.2.1. Thermodynamic measurements on Langmuir films

Surface measurements were carried out using a thermostated KSV Langmuir Mini-trough system (KSV LTD, Finland), placed on an anti-vibration table and enclosed in a Plexiglas box to avoid impurities and dust deposition. In this system, compression is achieved with the symmetric movement of two opposing barriers. In all experiments the compression rate was 20 cm^2/min. A rectangular trough with a total area of 246.4 cm^2 was used. Trough and barriers were thoroughly cleaned before each measurement with appropriate solvents, and rinsed with ultrapure water. Prior to film deposition, the surface was cleaned repeatedly by slowly sweeping the barriers and vacuum aspirating the surface in between, until no change in surface pressure was detectable comparing the closed and open positions. Due to the rather small effect of the presence of PDLLA on the surface pressure, the cleaning process was a critical step of the measurement procedure.

PDLLA was dissolved in chloroform. A known amount of the solution was carefully spread with a micro-syringe onto the clean air–water interface. All the measurements were performed at 25 ± 0.2 °C. The solvent was allowed to evaporate for about 10 min before starting the compression. The desired subphase temperature was controlled by a water circulating bath (C25, Haake, Karlsruhe, Germany). Surface pressure measurements were carried out by the Wilhelmy method, using a roughened platinum plate, to an accuracy of 1 mN/m. Due to unavoidable experimental uncertainties, the maximum difference between every two repeated curves is contained in a band of ~±2.5 mN/m on the average.

Surface potential measurements were performed using the noncontact vibrating plate capacitor method, originally introduced by Kelvin and improved by Yamins and Zisman [32,33]. We used a computer-controlled device (SPOT1, KSV LTD, Finland), with a 17 mm diameter active electrode, placed at less than 3 mm above the air–water interface, and a stainless steel reference electrode immersed in the subphase [34].

Surface pressure and surface potential were measured simultaneously during film compression; at the beginning of each experiment, the surface potential of the aqueous phase was measured, and this value was assumed as a reference. Before starting the compression, the solvent was allowed to evaporate for about 10 min for a good stabilization of the initial potential value. The probe parameter setting was adjusted in order to reduce the stray capacitance effect, due to the small variation of the electrode distance from the interface in different measurements, to a negligible extent. The reproducibility of surface potential measurements was within 5 mV.

The interaction of CRL with PDLLA films was studied by comparing the thermodynamic behavior of PDLLA films spread on pure PBS subphase and on CRL-containing subphase. In a different series of experiments, changes in the surface parameters of PDLLA induced by the CRL were investigated by monitoring the effect of injecting different concentrations of the protein beneath the compressed films at different target pressures of compression (5, 10, and 15 mN/m). More in detail, a fixed volume of CRL buffered solution (at the concentration required to obtain the final desired one in the whole subphase volume) was carefully injected in the subphase by a syringe ending in a thin Teflon tube placed in the subphase well below the film, thus avoiding any perturbation of the film [28]. The increase in surface pressure upon CRL injection was monitored until it reached an equilibrium value. All experiments were repeated at least three times.

2.2.2. Adsorption on PDLLA-nanoparticles

PDLLA nanoparticles were prepared by using a recently patented methodology [35]. In brief, the commercially available polymer was dissolved in dimethylformamide. The obtained solution (5 mg/ml) was then transferred in a dialysis bag and dialyzed against water, which is a non-solvent for the polymer (solvent/non solvent ratio 1:20). After 5 days at 4 °C the precipitate was recovered, rinsed several times in water, centrifuged and freeze-dried. The size and morphology of the obtained nanoparticles (average diameter 220 ± 5 nm) were characterized by scanning electron microscopy and dynamic light scattering as described elsewhere [24]. Adsorption experiments of CRL onto PDLLA nanoparticles were performed in Pyrex tubes containing a known

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