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Physisorption of enzymatically active chymotrypsin on titania colloidal particles

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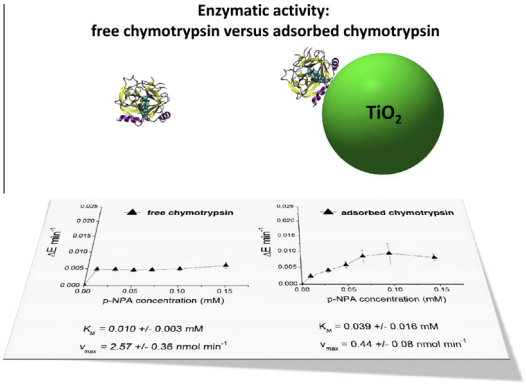
GRAPHICAL ABSTRACT



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ABSTRACT

In this study we use a straightforward experimental method to probe the presence and activity of the proteolytic enzyme α-chymotrypsin adsorbed on titania colloidal particles. We show that the adsorption of α-chymotrypsin on the particles is irreversible and pH-dependent. At pH 8 the amount of adsorbed chymotrypsin is threefold higher compared to the adsorption at pH 5. However, we observe that the adsorption is accompanied by a substantial loss of enzymatic activity, and only around 6–9 % of the initial enzyme activity is retained. A Michaelis–Menten kinetics analysis of both unbound and TiO_2-bound chymotrypsin shows that the K_M value is increased from ~10 μM for free chymotrypsin to ~40 μM for the particle bound enzyme. Such activity decrease could be related by the hindered accessibility of substrate to the active site of adsorbed chymotrypsin, or by adsorption-induced structural changes. Our simple experimental method does not require any complex technical equipment, can be applied to a broad range of hydrolytic enzymes and to various types of colloidal materials. Our approach allows an easy, fast and reliable determination of particle surface-bound enzyme activity and has high potential for development of future enzyme-based biotechnological and industrial processes.

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1. Introduction

Immobilization of enzymes onto water-insoluble carriers is of increasing importance for a wide range of applications including bioanalysis, biosensoring, biotechnology, medical therapy, diagnostic, pharmaceutics and proteomics [1–4]. Enzyme immobilization offers several advantages including more convenient handling of the enzyme, facile separation from the enzymatic products, efficient recovery and reuse of costly enzymes. In the past decades, different immobilization approaches have been designed to limit activity loss, decrease the response rate to selected substrates or avoid possible irreversible damage to the enzyme during the immobilization steps. One of the major challenges is still the need of an easy and straightforward method that makes it possible to determine the activity of adsorbed or surface-bound enzymes. The activity of surface-bound enzymes may be altered by several factors, including denaturation, orientation on the surface, and steric hindrance [3,5–13]. Conventional assays for direct measurement of the activity of free enzymes are often based on UV/Vis spectrometry. These assays are not reliable if enzymes are immobilized on particles, because the particles increase light scattering and consequently can lead to false results. This problem can be circumvented if a so-called end-point-mode assay is used, which consists of removing the particles from suspensions before measuring the product generated by the particle-bound enzymes [14–17].

Here we investigate the adsorption of the serine protease α-chymotrypsin on colloidal TiO_2 particles. TiO_2 colloidal particles are frequently used as a carrier because of their lack of toxicity, their stability and easy availability, as well as their relevance for several applications including bio-inorganic hybrids [18–20], catalysis [21], bioanalytical devices [22] and proteomics [23]. Chymotrypsin represents a good model for studying the modulation of activity after immobilization due to its well-defined structure, well characterized enzymatic properties [24–26], and its practical applications [16,27].

In this study chymotrypsin was immobilized by physisorption to TiO_2 particles to avoid interference by additional chemicals or coupling reagents with the activity assay [15,28]. A well-known enzymatic assay based on the hydrolysis of the artificial substrate p-NPA [29–31] was then optimized to determine chymotrypsin activity before and after adsorption of the enzyme on TiO_2 particles. Our data demonstrate that chymotrypsin adsorbs efficiently to TiO_2 colloidal particles in a pH-dependent and concentration-dependent manner, but only little enzymatic activity is retained after binding on the particle surface.

2. Experimental section

2.1. Materials

TiO_2 particles (DuPont™ Ti-Pure® R-101, 97 wt% rutile, Lot. No. 811969T.07) were obtained from DuPont de Nemours GmbH (Germany). Lyophilized α-chymotrypsin type II from bovine pancreas (molar weight 25,300 g mol^{-1}, purity 94.1 wt%, Lot. No. 60M7007V, 65.622 units mg^{-1} protein, whereby one unit will hydrolyze 1 micromole of N-benzoyl-L-tyrosine ethyl ester per minute at pH 7.8 at 25 °C), p-nitrophenol acetate (p-NPA; CAS No. 830-03-5, Lot. No. 0001422901), potassium dihydrogen phosphate (⩾99 wt%, CAS No. 7778-77-0) and 1,4-dioxane (>99.8 wt%, CAS No. 123-91-1, Lot. No. STBB3939) were purchased from Sigma–Aldrich (Germany) and used without any modifications. Pierce™ bicinchoninic acid protein assay kit (BCA assay) was obtained from Thermo Fisher Scientific GmbH (Germany). All other chemicals were purchased from Fluka (Switzerland) or Merck (Germany) at analytical grade. For all aqueous solutions double deionized water (ddH_2O) with a conductivity of 0.04 μS cm^{-1} was used as the solvent (Millipore Synergy®, Millipore Corporation, Germany).

2.2. Methods

2.2.1. Characterization of TiO_2 colloidal particles

Prior to the investigations, the TiO_2 colloidal particles were calcinated at 400 °C for 4 h with a heating and cooling rate of 3 °C min^{-1} (oven L3/11/S27, Nabertherm, Germany) to remove any possible organic contaminants. The particle size was determined by dynamic light scattering (DLS, Malvern Zetasizer Nano SP, Malvern Instruments Ltd, UK) using 0.003 vol% TiO_2 suspension at pH 8. Before each DLS measurement, the suspensions' conductivity and pH were adjusted to avoid or to minimize particle agglomeration. The pH was adjusted to pH 5 and pH 8 with 1 M KOH or 1 M HCl using a pH-meter (Five Easy™ FE20, Mettler-Toledo GmbH, Switzerland). The conductivity was set at 500 μS cm^{-1} using 3 M KCl, which corresponds to an ionic strength of 3 mM.

The particles' specific surface area (SSA_{BET}) was obtained by volumetric nitrogen adsorption measurements using a BELsorp-mini II device (Bel Japan Inc, Japan) and assuming a cross-sectional area of the nitrogen molecule of 0.162 nm^2 [15]. Adsorption isotherms were recorded at −196 °C. All samples were out-gassed at 100 °C under flowing argon for 24 h before measurement.

Zeta potential (ζ-potential) measurements were carried out using an electroacoustic spectrometer DT1200 (Dispersion Technology Inc., USA), as previously reported [32]. ζ-Potential measurements were carried out using 1 vol.% TiO_2 suspensions with an initial conductivity of 500 μS cm^{-1} and the pH was varied with the integrated titration unit using 1 M HCl and 1 M KOH. The quantity of hydroxyl groups present on the surface of TiO_2 particles was determined by potentiometric titrations (TitraLab® TIM 840 Titration Workstation, Hach Lange GmbH, Germany) according to Hidber [33,34]. The TiO_2 particle density was measured by a helium pycnometer (Pycnomatic ATC, Porotec GmbH, Germany).

Transmission electron microscopy (TEM) imaging was performed using a FEI Titan 80/300 kV (FEI, The Netherlands) equipped with a Cs-corrector for spherical aberration of the objective lens at 300 kV and a vacuum of 1.3×10^{-7} mbar. TiO_2 particles were deposited on graphene-coated copper grids (Graphene Supermarket, New York, USA). The particle morphology was analyzed by scanning electron microscopy (SEM; field-emission SEM SUPRA 40, Zeiss, Germany) operating at 2.00 kV mounted on carbon tape.

The hydrophilic/hydrophobic surface properties were investigated by volumetric water and n-heptane adsorption measurements using a BELsorp 18-3 device (Bel Japan Inc, Japan) according to [35,36]. Amounts of adsorbed water and n-heptane are reported as a ratio p/p_0 of 0.95, to most accurately simulate atmospheric conditions. Prior to the adsorption measurements, all samples were out-gassed at 100 °C under flowing argon for 24 h. The average and standard deviations of three single measurements are given.

2.2.2. Hydrophobic/hydrophilic properties of chymotrypsin

Chymotrypsin representation of secondary structure, spatial surface potential distribution and surface hydrophilic/hydrophobic regions were visualized using the software Visual Molecular Dynamics (VMD, Version 1.9.1) [37]. The atomic coordinates of the proteins were taken from the Brookhaven Protein Database (PDB-ID: 4CHA). After a 1 ns Molecular Dynamics simulation in explicit solvent, the projection of the hydrophobic and hydrophilic amino acids on the "solvent accessible surface area" were