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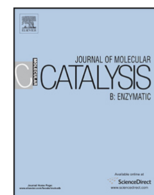

Turbidimetric analysis of the enzymatic hydrolysis of polyethylene terephthalate nanoparticles

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ABSTRACT

The heterogeneous enzymatic hydrolysis of polyethylene terephthalate (PET) by a polyester hydrolase (TfCut2) from *Thermobifida fusca* KW3 was determined by measuring the change of intensity of transmitted light due to the scattering effect of PET nanoparticles immobilized in an agarose gel. Nanoparticles with a mean diameter between 100 and 160 nm were prepared from PET samples of different crystallinity to provide a large surface area for the adsorption of the enzyme. The turbidity decrease of the PET nanoparticle suspensions was correlated to the surface erosion process resulting from the enzymatic degradation, and enabled a direct estimation of the kinetic parameters of the enzymatic hydrolysis of PET based on a model for heterogeneous biocatalysis. A comparison of the hydrolysis rate constants and the adsorption equilibrium constants of the enzymatic hydrolysis of PET nanoparticles prepared from recycled PET granulate, film and fibres showed that the biodegradability of PET was mainly influenced by the mobility of the polyester chains, which determined the affinity and accessibility of the ester bonds to the enzyme. Differential scanning calorimetric analysis of the partially hydrolyzed PET nanoparticles provided indirect evidence for an endo-type hydrolytic mechanism of TfCut2 in the heterogeneous degradation of aromatic polyesters.

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1. Introduction

Polyethylene terephthalate (PET) is a synthetic thermoplastic polymer. The aromatic polyester can be composed of both amorphous and crystalline domains. PET shows high resistance to many chemicals and to mechanical stress [1,2]. As a consequence, high energy-consuming processes are required for the recycling of post-consumer PET [2]. Recently, polyester-hydrolyzing enzymes active at temperatures above 50 °C derived from fungi [3,4] and from thermophilic bacteria have been reported [5–12].

In contrast to chemical hydrolysis processes of PET, the enzymatic hydrolysis of the insoluble polyester is a surface erosion process [13–15], since due to their size the enzyme molecules are unable to penetrate into the bulk polymer [15]. Thus, the rate of degradation can be drastically accelerated by enlarging the available polymer surface [16], e.g. by using nanometer-scale polyester particles [14].

The enzymatic hydrolysis of polyesters using nanoparticles has been previously studied by Wu and co-workers [17–19]. They investigated the enzymatic hydrolysis of poly(ε-caprolactone) (PCL) nanoparticles with a lipase from Pseudomonas cepacia by laser light scattering and found that the enzymatic degradation rate was accelerated by a factor of 10^3 when PCL nanoparticles were used instead of a film. Later studies of the enzymatic hydrolysis of nanoparticles prepared from several aliphatic and aromatic polyesters by a lipase using a pH titration method have been reported [14,15], where the influence of the chemical structure and thermal properties of the polyesters on their biodegradability was described. It has been shown that the enzymatic hydrolysis rate of these polyesters is primarily determined by the mobility of the polymer chains correlated with the degree of crystallinity of the material. The enzymatic hydrolysis of polyester nanoparticles can be considered as a surface erosion process and has been described in terms of a two-step kinetic model [20,21]. The same model has also been applied to study the enzymatic hydrolysis of PET films monitored by titration of the carboxyl groups formed during the reaction [3], and by fluorimetric detection of derivatized terephthalic acid (TPA) [22], one of the final products of the PET hydrolysis reaction. Furthermore, esters of TPA such as mono 2-hydroxyethyl terephthalate (MHET) and bis 2-hydroxyethyl terephthalate (BHET) have also been detected by HPLC as water-soluble degradation products [6,7,23].

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In this study, the enzymatic hydrolysis of PET nanoparticles by a polyester hydrolase, TfCut2, from *Thermobifida fusca* KW3 [24] was investigated. Since the enzymatic hydrolysis of polyesters is controlled by the chain mobility of the polymer influenced by its crystallinity [3,14,25–30], nanoparticles were prepared from PET samples with low crystallinity (PET film) and high crystallinity (recycled PET granulate and PET fibres). The PET nanoparticles were immobilized in an agarose gel for rapid enzymatic hydrolysis without requiring agitation of the reaction mixtures. The decrease of turbidity caused by the enzymatic hydrolysis of the nanoparticles was determined at 600 nm using a spectrophotometer. Initial reaction rates and the corresponding kinetic constants for the enzymatic hydrolysis reaction were directly calculated from the rates of turbidity decrease of the reaction mixture. A differential scanning calorimetric characterization of the PET samples and the corresponding nanoparticles was performed to compare their properties influencing the enzymatic degradability of the PET polymers.

2. Materials and methods

2.1. Preparation and characterization of PET nanoparticles

PET nanoparticles were prepared using recycled PET granulate (TEXPET A P800, Texplast GmbH, Wolfen, Germany) with a particle size of 4–8 mm and a crystallinity of 34–36% [31], PET fibres (Amtex Ltd., Barcelona, Spain) of about 30% crystallinity [32] and low-crystallinity (7%) PET film [3] (Goodfellow GmbH, Bad Nauheim, Germany, product number 029-198-54, 250 μm in thickness) using a precipitation and solvent evaporation technique as described elsewhere [24]. The PET nanoparticle size was determined at 21 °C by dynamic light scattering using a Zetasizer 3000 HS (Malvern, Herrenberg, Germany) at a wavelength of 633 nm. Results were expressed as the mean values of three determinations. To calculate the specific surface area of each type of nanoparticles based on their size, a density of PET of 1.38 g/cm^3 was used [33]. The particle concentration of the final suspension was determined by weighing of the pellet obtained from an aliquot of 3 mL of PET nanoparticle suspension by centrifugation and drying at 50 °C.

2.2. Differential scanning calorimetry (DSC)

PET samples (recycled granulate, film and fibres) and the corresponding nanoparticles before and after enzymatic hydrolysis were analyzed by DSC (Polymer DSC, Mettler Toledo, Giessen, Germany) equipped with an autosampler and an intercooler system. Dry samples of approximately 1 mg were subjected to a temperature program composed of a first heating, cooling, and a second heating cycle under a nitrogen atmosphere. The first heating cycle between −20 and +300 °C was run at a rate of 5 K/min. The subsequent cooling to 0 °C and second heating cycle to 300 °C was performed at a constant rate of −10 K/min and 10 K/min, respectively. The resulting thermograms were analyzed with StarE® software (Mettler Toledo, Giessen, Germany). From the first heating cycle, the temperature of the PET melting endotherm was obtained. If applicable, the glass transition temperature and the crystallization temperature were also calculated at the first heating cycle. The measurements were performed in duplicate.

2.3. Enzymatic hydrolysis of PET nanoparticles

TfCut2 solution in 0.5 M Tris–HCl (pH 8.5) was added to suspensions containing 0.5 mg PET nanoparticles in a total volume of 1 mL. The final enzyme concentration was 100 μg/mL. The enzymatic hydrolysis was performed at 60 °C with a constant agitation at 1000 rpm per min for 30 min. The residual nanoparticles were collected by centrifugation at 18,000 rpm for 10 min. The nanoparticle pellet was dried overnight at 50 °C and then analyzed by DSC.

2.4. Turbidimetric determination of enzymatic hydrolysis of PET nanoparticles

The recombinant polyester hydrolase TfCut2 from *T. fusca* KW3 was expressed and purified as described previously [24]. 2% (w/v) agarose (Carl Roth GmbH, Karlsruhe, Germany) was melted in 0.5 M Tris–HCl (pH 8.5) and cooled to 60 °C in a water bath. The reaction mixture contained in a spectrophotometer cuvette was composed of the PET nanoparticle suspension, TfCut2 dissolved in 0.5 M Tris–HCl (pH 8.5) and 1.5 mL of 2% agarose, and cooled immediately to 4 °C after thoroughly mixing with a plastic rod until the agarose solidified. The final TfCut2 concentration was between 0 and 80 μg/mL and the final concentration of PET nanoparticles was between 0 and 0.3 mg/mL. The enzymatic hydrolysis reaction was started by incubating the cuvette at 60 °C. The relative turbidity of the samples was calculated from the optical density determined at 600 nm (OD_{600}) every 5 min for a total reaction time of 90 min using a Cary 50 Bio spectrophotometer (Varian, Darmstadt, Germany) with

$$turbidity(\%)_t = \frac{|OD_{600} - OD_{600,blank}|_t}{|OD_{600} - OD_{600,blank}|_0} \times 100 \quad (1)$$

where zero refers to the starting time of the reaction, and t to the reaction time in the course of enzymatic hydrolysis. The blank sample contained only buffer and agarose gel.

2.5. Derivation of the kinetic model for the enzymatic hydrolysis of nanoparticles

The kinetic model describing the heterogeneous biocatalysis [14,21] was applied to analyze the enzymatic hydrolysis of PET nanoparticles. V_0, the initial rate of polyester hydrolysis, can thereby be defined as

$$V_0 = -\frac{d(n_{EB})}{dt} = k[ES] = k\rho_{EB}A_0\theta \quad (2)$$

where n_{EB} is the concentration of accessible ester bonds at the surface of the polyester, k is the hydrolysis rate constant, and $[ES]$ is the concentration of the enzyme-substrate complex. For the biocatalytic hydrolysis, $[ES]$ can be estimated to be proportional to A_0 [14], the initial surface area, ρ_{EB}, the molar density of ester bonds in the polymer that is a constant for a given polyester substrate, and θ, the fraction of the polyester surface area occupied by hydrolytically active enzyme protein.

Based on previous studies [14,21,34,35], the adsorption behavior of enzymes on insoluble substrates during hydrolysis can be described by a Langmuir-type model with

$$\theta = \frac{K_A[E]}{1 + K_A[E]} \quad (3)$$

where $[E]$ is the dissolved enzyme concentration, and K_A is the adsorption equilibrium constant, defined as the ratio of the rate constant of adsorption to that of desorption [21]. Incorporating the Langmuir isotherm θ into Eq. (2), the initial rate of polyester hydrolysis can be expressed as

$$V_0 = k\rho_{EB}A_0\frac{K_A[E]}{1 + K_A[E]} \quad (4)$$

The turbidity τ of a particle suspension describes the decrease of the intensity of the transmitted light resulting from scattering by the particles. For a simplified system consisting of non-absorbing

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