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Substrate specificities of cutinases on aliphatic–aromatic polyesters and on their model substrates

Research Paper

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The enzymatic hydrolysis of the biodegradable polyester ecoflex and of a variety of oligomeric and polymeric ecoflex model substrates was investigated. For this purpose, substrate specificities of two enzymes of typical compost inhabitants, namely a fungal cutinase from *Humicola insolens* (HiC) and a bacterial cutinase from *Thermobifida cellulosilytica* (Thc_Cut1) were compared. Model substrates were systematically designed with variations of the chain length of the alcohol and the acid as well as with varying content of the aromatic constituent terephthalic acid (Ta).

HPLC/MS identification and quantification of the hydrolysis products terephthalic acid (Ta), benzoic acid (Ba), adipic acid (Ada), mono(4-hydroxybutyl) terephthalate (BTa), mono-(2-hydroxyethyl) terephthalate (ETa), mono-(6-hydroxyhexyl) terephthalate (HTa) and bis(4-hydroxybutyl) terephthalate (BTaB) indicated that these enzymes indeed hydrolyze the tested esters. Shorter terminal chain length acids but longer chain length alcohols in oligomeric model substrates were generally hydrolyzed more efficiently. Thc_Cut1 hydrolyzed aromatic ester bonds more efficiently than HiC resulting in up to 3-fold higher concentrations of the monomeric hydrolysis product Ta. Nevertheless, HiC exhibited a higher overall hydrolytic activity on the tested polyesters, resulting in 2-fold higher concentration of released molecules. Thermogravimetry and differential scanning calorimetry (TG-DSC) of the polymeric model substrates revealed a general trend that a lower difference between melting temperature (T_m) and the temperature at which the enzymatic degradation takes place resulted in higher susceptibility to enzymatic hydrolysis.

Introduction

Q2 The legislative and environmental pressure to reduce polymer and packaging waste is increasing. Consequently, there is a strong demand to design and improve polyesters that are not only biodegradable but also meet the requirements of expected material properties. Aliphatic polyesters show, generally spoken, better biodegradability than aliphatic–aromatic copolyesters but sometimes lack the required thermal and mechanical properties [1]. Aromatic polyesters like polyethyleneterephthalate (PET) exhibit very good material properties but were believed to be inert to biological attack [2]. The polymer ecoflex is a polyester that shows promising material features [3] and various studies have already proven its biodegradability under composting conditions [4–6]. Nevertheless, considerably less is known about mechanistic aspects of the enzymatic hydrolysis involved in biodegradation by fungi and bacteria typically present in compost. Consequently, the

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substrate specificities of a fungal cutinase (HiC) and a bacterial cutinase (Thc_Cut1) from the compost inhabitants *Humicula insolens* [7] and *Thermobifida cellulosilytica* [8] were compared in this study.

In the late 70s, enzymatic hydrolysis of polyester by a lipase was reported for the first time [9]. Since then, different studies were conducted in order to identify enzymes hydrolyzing polyesters. Marten *et al*. investigated the parameters that are important for lipases to hydrolyze polyesters [10,11]. A kinetic model for the layer-by-layer degradation of aliphatic and aromatic polyester nanoparticles by lipases from *Candida cylindracea* and *Pseudomonas* sp. was described by Herzog *et al*. [12]. *Thermobifida fusca* was identified to be one potent microorganism that is responsible for biodegradation of aliphatic–aromatic copolyesters during composting [13]. A big breakthrough was the identification of polyester active enzymes from *Thermobifida* sp. A hydrolase from *Thermobifida fusca* was proven to be active on aliphatic–aromatic copolyesters [14] and cutinases from *Thermobifida cellulosilytica* [15], *Thermobifida alba* [16] and *Fusarium solani pisi* [17] showed activity on aromatic polyesters like PET leading to surface hydrolysis of the polyester [17]. Recently, also a cutinase-like enzyme from *Saccharomonospora viridis* [18,19] was reported to hydrolyze different polyesters including foils made out of PET and ecoflex. The fungal cutinase from *Humicula insolens* was already demonstrated to have a potential for hydrolysis of polyester [16] or deacetylation of poly(vinyl acetate) [20]. Moreover, HiC was also proven to be able to hydrolyze the polyester backbone of alkyd resins [21]. Other enzymes that were reported to be active on polyesters are, for example, a cutinase-like enzyme from *Pseudozyma antarctica* that degrades polybutylene succinate-*co*-adipate and polybutylene succinate [22]. Some mechanistic studies on the hydrolysis of PET exist about a nitrobenzylesterase from *Bacillus subtilis* [23] and cutinases from *Thermobifida cellulosilytica* [15], but so far, there is a lack of information about enzymatic hydrolysis of aliphatic–aromatic copolyesters. Nevertheless, cutinases are known as attractive biocatalysts for industrial applications including esterification, trans-esterification and hydrolysis [24] in, for example, textile industry and polymer chemistry.

Compost is an interesting source for cutinases that do not only hydrolyze the naturally occurring polyester cutin but are potentially active on synthetic polyesters. In this work, the substrate specificities of two different cutinases from a fungal and a bacterial compost inhabitant were studied in detail, employing systematically designed oligomeric and polymeric ecoflex model substrates. Detailed knowledge about substrate specificities of the enzymes will allow adaptation of the polymer structure towards efficient biodegradation and will also make an enzymatic recycling of polyester building blocks feasible.

Materials and methods

Expression and purification of the enzymes

Thc_Cut1 was expressed in *Escherichia coli* BL21-Gold(DE3) as previously reported [15]. To harvest the cells, 100 ml of the culture were centrifuged (7000 × *g*, 4°C, 20 min) (Sorvall RC-5B Refrigerated Superspeed Centrifuge, Du Pont Instruments, USA). The pellet was resuspended in 10 ml buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazol, pH 8.0) sonicated under ice cooling with two 3-min pulses (duty cycle 80%, output 7–8) on a BRANSON Ultrasonics cell disruptor (USA). Cell fragments were removed through centrifugation (7000 × *g*, 4°C, 30 min). The enzyme was purified by means of affinity chromatography as previously described [15]. The second cutinase HiC was received from Novozymes, purified through ultrafiltration (30 kDa and 10 kDa molecular weight cut off Vivaspin, Sartorius AG, Germany) and purity was confirmed by gel electrophoresis (supplementary material Figure A1).

Protein quantification, activity and temperature stability

Protein concentrations of all tested enzymes were determined with the help of the Bradford-based Bio-Rad Protein Assay (Bio-Rad Laboratories GmbH, Munich, Germany) and bovine serum albumin as standard. The protein assay was performed according to the manufacturers' instructions.

Esterase activity of Thc_Cut1 and HiC was analyzed based on UV/Vis spectroscopy, using the soluble substrate *p*-nitrophenyl butyrate (*p*NPB). 200 μl of 100 mM potassium phosphate buffer pH 7.0 containing the substrate were placed in a 96 well plate. The reaction was started by adding 20 μl of enzyme solution and the increase of the absorbance at 405 nm was recorded for 5 min on a plate reader (Tecan infinite M200, Tecan Austria GmbH). The experiments were conducted at 25°C. A blank reaction containing 20μl of buffer instead of enzyme solution was measured simultaneously. The hydrolysis of *p*NPB to *p*-nitrophenol leads to an absorbance increase at 405 nm indicating an esterase activity. The molar extinction coefficient for *p*-nitrophenolate 405 nm, 25°C in 100 mM potassium phosphate buffer pH 7.0 was measured to be 11.581 $mM^{-1}\,cm^{-1}$.

The activity of all tested enzymes was calculated in Units (U). One U is defined as the amount of enzyme that is needed to catalyze the conversion of 1 μmol of substrate per minute under the given conditions.

Temperature stability was determined by incubating the enzymes in 100 mM potassium phosphate buffer pH 7.0 at 50°C and 100 rpm. The initial specific enzyme activity was measured (time point 0) using *p*NPB as substrate and defined to be 100%. The remaining enzyme activities were then determined after 24 h, 48 h and 72 h.

Synthesis of oligomeric ecoflex model substrates

Synthesis of mono(4-hydroxybutyl) terephthalate (BTa) was accomplished in accordance with Padias *et al*. [25] with modifications. Detailed information is provided in Ref. [26].

The synthesis of BTaB and BTaBTaB was conducted as described by Hässlin *et al*. [27] with one exception: 10 eq instead of 3.3 eq of 1,4-butanediol were used. An alternative synthesis was described by Atfani *et al*. [28].

Syntheses of the BTaB derivates HaBTaBHa, DaBTabDa, TdaBTaBTda, HTaH and BaBTaBBa are described in detail by Perz *et al*. [26].

BaETaEBa was synthesized as previously described [29]. An overview of the chemical structures of all oligomeric model substrates is provided in Fig. 1.

Synthesis of ecoflex and polymeric ecoflex model substrates

The aliphatic–aromatic copolyester ecoflex (poly(butylene adipate-co-butylene terephthalate, short PBAT) contains adipic acid, 1,4-butanediol and terephthalic acid. The adipic acid:terephthalic

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