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Hydrolysis of Cutin by PET-Hydrolases

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Summary: Functionalisation of synthetic polymers by using enzymes has been recently demonstrated. The major advantage of enzymes over chemical processes lies in their surface specific and endo-wise mode of action. Surface hydrophilisation of PET with lipases and cutinases leads to a dramatic increase of the surfacial acid and hydroxyl group content while conventional chemical treatment does not cause any change. However, this PET-hydrolysing activity by enzymes from distinct classes has not yet been correlated to activity on natural polyesters. Here, we show that lipases, cutinases and a PHA-depolymerase are all capable of hydrolysing PET, while only lipases and cutinases also hydrolysed cutin to various degrees. Lipases showed a higher specificity for terminal fatty acids while the cutinases preferred hydroxy fatty acids during cutin hydrolysis.

Keywords: cutin; pet-hydrolase; polyester

Introduction

In the last few years the potential of enzymes for surface hydrophilization of polyalkyleneterephthalate based materials has been investigated.[1–5] For example, limited surface hydrolysis of PET fabrics can improve bonding of finishers or reduce amount of adhesives needed in coating with PVC.[6] In several of these studies it has been shown that conventional standard assays for lipases or esterases such as using p-nitrophenyl substrates do not correlate with this PET-hydrolase activity. Since many PET-hydrolases have been shown to be inducible by the plant polyester cutin,[7] the focus of this work was to compare activity of PET-hydrolases on cutin and polyethyleneterephthalates (PET) and to develop the required analytical procedures.

The plant cuticle is composed of chloroform- soluble waxes and insoluble cutin consisting of oxygenated C16 and C18 fatty acids cross-linked by ester bonds.[8] Cutin plays a major role in plant protection and its enzymatic degradation has proved to be one of the first steps in the infection process.[9] Cutin oligomers, resulting from cutin hydrolysis by small levels of constitutive cutinase activity, have been suggested to induce production of higher amounts of cutinases.[10] Fungal cutinases show both exo- and endo-esterase activity[11] and have first been purified and characterized from *Fusarium solani* pisi growing on cutin as a carbon source.[12] The hydrolysis catalyzed by cutinases results in release of chloroform- methanol- soluble monomers. The most common components of the C16 family of monomers are 16-hydroxyhexadecanoic acid and 9,10,16-dihydroxyhexadecanoic acid. Usually a mixture of medium chain length positional isomers of dihydroxy acids is also present. The major members of C18 cutin monomers are 18-hydroxy-C18-9-enoic acid, 18-hydroxy-C18-

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9,12-dienoic acid, 18-hydroxy-9,10-epoxy- C18 acid, 18-hydroxy-9,10-epoxy-C18 acid, 9,12,18-trihydroxy-C18 acid and 9,10,18-trihydroxy-C18-12-enoic acid.[11]

Material and Methods

Chemicals and Enzymes

Glacial acetic acid, methanol, diethyl ether and acetonitrile were purchased from Roth (Carl Roth GmbH, Karlsruhe, Germany) and VWR Prolabo, respectively. N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was from Fluka. All other chemicals were analytical grade, provided from Sigma- Aldrich.

The cutinase from *Fusarium solani* and the extracellular medium-chain-length PHA-depolymerase from *Pseudomonas fluorescens* GK13 were produced and purified as previously described.[13,14] Enzymes from *Thermobifida fusca* and *Thermobifida alba* were produced using the following procedure: 3mL of TSB medium (Caso-Boullion- Merk 5459, 30 g tryptic soy/L; pH 7.3) were inoculated and kept for 24 h at 50°C. Those 3 mL were added to 35 mL of TSB medium for another 24 h of incubation. The lipases from *Thermomyces lanuginosus* and from *Candida antarctica* and the cutinase from *Humicola insolens* were kindly supplied from Novozymes. In all comparative experiments, these enzymes were applied based on the same activity on para-nitrophenyl palmitate (PNPP) at the individual temperature optima.

Determination of Lipase Activity

Lipase activity was determined spectrophotometrically using PNPP as substrate.[15] The amount of released p-nitrophenol was determined by spectrophotometric quantification at 405 nm. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol PNPP per minute.

Enzymatic Hydrolysis of Cutin

Cutin from apples was prepared following a modified procedure of.[16] Peels were boiled for 24 h in oxalate buffer (4 g l^{-1} oxalic acid and 16 g l^{-1} ammonium oxalate). Collected cuticle was washed several times with distilled water, dried and minced. Thereafter, the cuticle was extracted using a mixture of chloroform and methanol (2:1), dried at 100 °C and again washed several times with distilled water. Cellulose and pectin were removed by treatment of the cuticle material with cellulase from *Aspergillus niger* and pectinase from *Rhizopus* sp. (both from Sigma Chemical Co., St. Louis). The incubation of the enzymes and cuticle was done in 50 mM acetate buffer, pH 4.0 at 22°C for 48 h.

The release of monomers from cutin was quantified using a modified method from,[17] incubating 10 mg cutin with 300 μL appropriately diluted enzyme solution to obtain a final activity of 100 U ml^{-1} at the individual incubation temperatures. The reaction mixture was incubated for 72 h at the temperature according to the optimum of each enzyme (enzymes from *F. solani, T. lanuguinosus* and *P. fluorescens* at 37 °C; enzymes from all *Thermobifida* species, *Candida antarctica* and *H. insolens* 50 °C). As a control, incubation of cutin in a 50 mM phosphate buffer, pH 7.0 was performed. After the incubation, 0.5 mL of glacial acetic acid was added into each reaction tube and after mixing, additionally 3 mL of chloroform was added. Samples were shaken vigorously and centrifuged. The organic layer was removed and taken to dryness under a stream of nitrogen. The residues were taken up in 1 mL mixture of $CHCl_3$: MeOH (85:15) and transferred into a glass auto sampler vial. The content of glass vials was taken to dryness under a stream of nitrogen and residues were dissolved in 1 mL of pyridine. The released monomers were silylated with N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA). After extraction, 100 μL of MSTFA was added into the injection glass vials containing 1 ml of pyridine and hydroxy fatty acids released during enzymatic degradation of cutin. The reaction mixture was heated to 60 °C for 1 h. After this time, the samples were cooled down to room temperature. Siliylated samples

were analysed with GC combined with MS (GC- MS). GC- MS determination was performed by using an Agilent (Waldbronn, Germany) 6890 gas chromatograph equipped with an HP7683 auto sampler and a split/ splitless injector operated in splitless mode. The injection volume was 1.0 μl. The capillary column used was an HP- 5MS, 30 m x 0.25mm id and 0.25um film thickness, using temperature programming from 60 °C - 150 °C at 25 °C min^{-1}, further from to 250 °C at 10 °C min^{-1} and held for 5 min and to 300 °C at 5 °C min^{-1} and held for 5 min. The gas chromatograph was coupled to an HP5973 mass selective detector with electron impact ionization, operated in single ion monitoring (SIM) mode, using the m/z values. Relative activities were calculated based on the peak areas and represent average values from triplicate measurements with a standard deviation below 5%.

Enzymatic Hydrolysis of PET

For PET hydrolysis monitoring, transparent RHS film, 75 μm (Hostaphan®, Mitsubishi Polyester Film GmbH, Wiesbaden,) was used. Films of size 1.0 / 0.5 cm, in order to clean and remove finishing agents, were washed with an aqueous sodium carbonate solution (2 g l^{-1}) at 37 °C for 0.5 h and twice washed with distilled water. The enzymatic reaction was started by addition of 100 U ml^{-1} of an enzyme solution into (1.5 mL) Eppendorf tubes containing the film sample. The reaction was carried out at the temperature suitable for the enzyme (like indicated above) for a time of six days. The reaction was terminated and the samples prepared like described by monitoring of hydrolysis of the PET model substrate.

For detection of hydrolysis products released during the reaction, HPLC-UVD was used. The equipment was from DIONEX P-580 PUMP (Dionex Cooperation, Sunnyvale, USA), with an ASI- 100 automated sample injector and a PDA- 100 photodiode array detector. For analysis of the terephthalate acid (TA), mono-(2-hydroxyethyl) terephthalate (MHET) and benzoic acid (BA) a reverse phase column RP- C18 (150 x 4.6 mm, 5 μm with pre-column, Supelco, Bellefonte, USA) was used. Separation was achieved with 20% acetonitrile, 20% 10mM H2SO4 and 60% water as eluent by the flux of 1 mL min^{-1} and a temperature adjusted to and 25 °C. The products were detected at $\lambda = 241$ nm for TA and MHET and $\lambda = 228$ nm for BA and concentrations calculated based on standard curves. Data represent average values from triplicate measurements with a standard deviation below 3%.

Results and Discussion

Enzymatic hydrolysis of PET has been described for a variety of enzymes from distinct classes while the number of reports on the industrial application is increasing.[1] Here, we investigated whether cutin hydrolysis would be a common feature of all PET hydrolysing enzymes. All enzymes tested in this study except the *C. antarctica* lipase were able to hydrolyse PET (Fig. 1). A higher release of MHET was measured for the *T. lanuginosus* lipase which was previously correlated to a higher degree of endo-type hydrolysis.[3] In contrast, the *T. fusca* and *F. solani* enzymes seemed to be more exo-acting as indicated by a higher amount of terephthalic acid (TA) released. Differences in substrate specificities among the two *Thermobifida* species may be explained by the presence of different amounts of lipases and/or cutinases in the crude enzyme preparations. Interestingly, the PHA-depolymerase from *P. fluorescens* which hydrolyses a variety of different PHAs,[18] also showed some activity on PET. This demonstrates that enzymes from various classes show PET-hydrolase activity. Previously, a correlation between PET-hydrolase activity on synthetic short chain PET model substrate and PET has been found[19] while this characteristic has not yet been related to activity on a natural polymeric substrate.

Apart from the known cutinases from *F. solani* and *H. insolens*, the induction of PET-hydrolase activity by cutin was also described for *P. simplicissimum* and

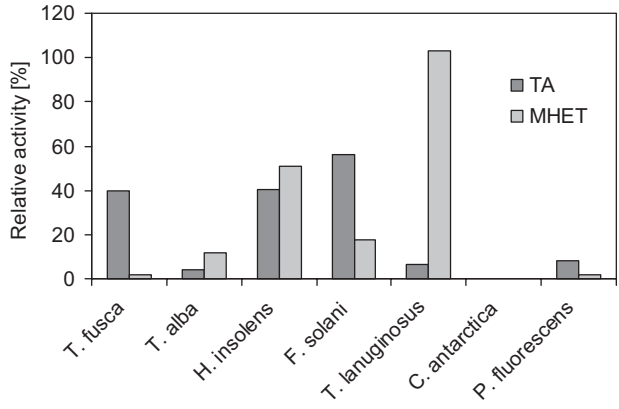

Figure 1.
Enzymatic hydrolysis of PET-films: Relative activity of different enzymes compared to the *T. lanuginosus* lipase set as 100% measured as release of terephthalic acid (TA) and mono-(2- hydroxyethyl) terephthalate (MHET).

T. fusca.[7,13,20] Thus, it was interesting to know, whether PET-hydrolases from other enzyme classes such as the lipase from *T. lanuginosus* [3] would also show cutinase activity. Consequently, a GC-MS based technique was successfully established and products released by the enzymes from cutin were analysed. Tetradecanoic, hexadecanoic and octadecanoic acids released were identified by means of the mass spectrometry library of Wiley while other hydroxyl acids were identified basing on interpretation of the mass spectrum. Among them, derivatives of 6,16-dihydrox-

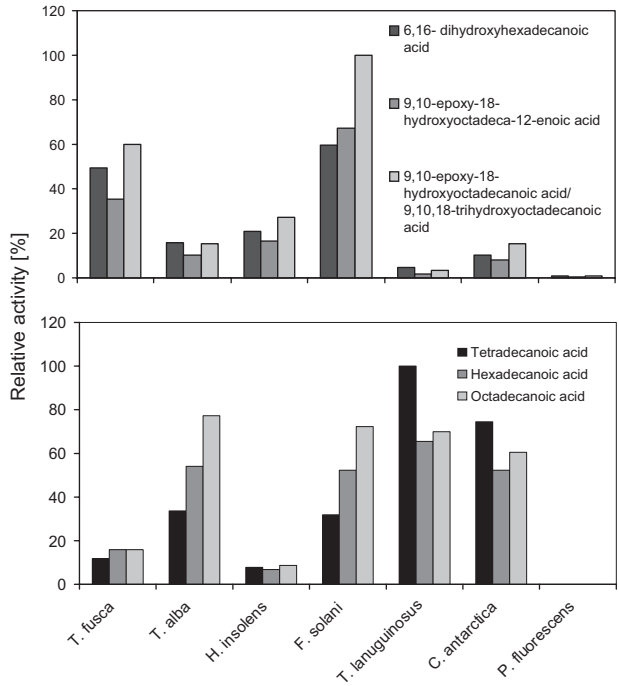

Figure 2.
Enzymatic hydrolysis of cutin: release of hydrolysis products (Maximum values set to 100%).

yhexadecanoic acid and 9,10- epoxy-18-hydroxyoctadeca-12-enoic acid, with their parent ions at m/z 489 and m/z 603 and prominent fragment ions at m/z 331 and 317 respectively, were detected. The third important monomer was characterized by a parent ion at m/z 605 and prominent fragment ion at m/z 317. Most likely this indicates the presence of 9,10- epoxy- 18-hydroxyoctadecanoic acid and/or 9,10,18-trihydroxyoctadecanoic acid.

All enzymes except the *P. fluorescens* PHA-depolymerase were able to release fatty acids from cutin with the lipases from *T. lanuginosus* and *C. antarctica* showing highest activity (Fig. 2). The cutinases from *F. solani* and *T. alba* released lower amounts while the activity was increasing with increasing chain length (highest activity on octadecanoic acid). In contrast to these "terminal" acids, a different pattern was seen for hydroxyl groups carrying acids building the backbone of cutin. Both lipases released clearly lower amounts of these acids when compared to the cutinases from *H. insolens* and *F. solani* and to the *Thermobifida* sp. enzymes. Classical lipases differ from cutinases in that they require interfacial activation while cutinases are active on both soluble and emulsified triglycerides.[21] The PHA-depolymerase from *P. fluorescens* showed only marginal activity on cutin in terms or release of hydroxyl fatty acids when compared to the *F. solani* cutinase.

Conclusion

Summarizing the results of this study it was shown that all PET-hydrolases from distinct enzyme classes were able to hydrolyze cutin. However, there is no clear correlation between activity on cutin and on PET. Lipases showed a higher specificity for terminal fatty acids while the cutinases preferred hydroxy fatty acids.

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[1] G. M. Guebitz, A. Cavaco-Paulo, *Trends Biotechnol.* **2008**, *26*, 32.
[2] A. Eberl, S. Heumann, R. Kotek, F. Kaufmann, S. Mitsche, A. Cavaco Paulo, G. M. Guebitz, *J.Biotechnol.* **2008**, *135*, 45.
[3] T. Brückner, A. Eberl, S. Heumann, M. Rabe, G. M. Guebitz, *J Polym Sci A: Polym Chem*, **2008**, *46*, 6435.
[4] M. Alisch-Mark, A. Herrmann, W. Zimmermann, *Biotechnology Letters* **2006**, *28*, 681.
[5] Y. B. Liu, G. F. Wu, L. H. Gu, *AATCC Review* **2008**, *8*, 44.
[6] E. Almansa, S. Heumann, A. Eberl, G. Fischer-Colbrie, L. Martinkova, J. Marek, A. Cavaco-Paulo, G. M. Guebitz, *Biocatal Biotrans*, **2008**, *26*, 365.
[7] R. J. Mueller, *Process Biochemistry* **2006**, *41*, 2124.
[8] W. F. Fett, C. Wijey, R. A. Moreau, S. F. Osman, *J. Appl. Microbiol.* **1998**, *86*, 561.
[9] C. M. L. Carvalho, M. R. Aires-Barros, J. M. S. Cabral, *Electr. J. Biotechnol.* **1998**, *1*, 160.
[10] T. S. Lin, P. E. Kolattukudy, *J. Bacteriol.* **1978**, *133*, 942.
[11] P. E. Kolattukudy, *Ann. l Rev. Plant Physiol. Plant Mol. Biol.* **1981**, *32*, 539.
[12] P. E. Kolattukudy, L. M. Rogers, D. X. Li, C. S. Hwang, M. A. Flaishman, *Proc. Natl. l Acad. Sci. USA* **1995**, *92*, 4080.
[13] R. Araujo, C. Silva, A. O'Neill, N. Micaelo, G. Guebitz, C. M. Soares, M. Casal, A. Cavaco-Paulo, *J. Biotechnol.* **2007**, *128*, 849.
[14] J. Ihssen, D. Magnani, L. Thoeny-Meyer, Q. Ren, *Biomacromolecules* **2009**, *10*, 1854.
[15] M. Alisch, A. Feuerhack, H. Muller, B. Mensak, J. Andreaus, W. Zimmermann, *Biocatal. Biotrans.* **2004**, *22*, 347.
[16] T. J. Walton, P. E. Kolattukudy, *Biochemistry* **1972**, *11*, 1885.
[17] W. F. Fett, H. C. Gerard, R. A. Moreau, S. F. Osman, L. E. Jones, *Appl. Env. Microbiol.* **1992**, *58*, 2123.
[18] K. E. Jaeger, A. Steinbuchel, D. Jendrossek, *Appl Env. Microbiol.* **1995**, *61*, 3113.
[19] S. Heumann, A. Eberl, H. Pobeheim, S. Liebminger, G. Fischer-Colbrie, E. Almansa, A. Cavaco-Paulo, G. M. Gubitz, *J. Biochem. Biophys. Meth.* **2006**, *69*, 89.
[20] S. Liebminger, A. Eberl, F. Sousa, S. Heumann, G. Fischer-Colbrie, A. Cavaco-Paulo, G. Guebitz, *Biocat. Biotrans.* **2007**, *25*, 171.
[21] C. Martinez, P. Degeus, M. Lauwereys, G. Matthyssens, C. Cambillau, *Nature* **1992**, *356*, 615.

