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Thiol-functionalization of acrylic ester monomers catalyzed by immobilized *Humicola insolens* cutinase

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ABSTRACT

Immobilized cutinase HiC from the ascomycete *Humicola insolens* was applied as a novel biocatalyst for the synthesis of functionalized acryclic esters by transesterification. As a model reaction, transesterification of methyl acrylate with 6-mercapto-1-hexanol at a high molar ratio in a solvent free system was chosen. Besides two minor Michael-addition by-products, 6-mercaptohexyl acrylic ester was identified as the main product with the thiol as the functional end group. Reaction conditions were optimized regarding the influence of water (0–1.72 M), temperature (22–50 °C), product inhibition and addition of the radical inhibitor butylated hydroxytoluol (BHT; 0.14–0.71 M) on conversion and by-product formation. Highest conversion of 6-mercapto-1-hexanol to 6-mercaptohexyl acrylic ester (95.4 ± 0.3%) was achieved after 6 h at 40 °C in the presence of 0.025% (w/w) water without formation of by-products in a solvent free system. Applying methyl methacrylate, transesterification with 6-mercapto-1-hexanol was significantly lower (43.6 ± 0.1%) compared to transesterification of methyl acrylate with 6-mercapto-1-hexanol.
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1. Introduction

Polymers with both, thiol and acrylate functional groups, based on standard acrylic monomers have a great potential for industrial application and, prepared using thiol-ene chemistry, are already in use as lacquers for improved scratch-resistance, adhesive formulations for high tack and instant bond strength or sizing of paper and textile for improved printability and reduced abrasiveness [1]. The synthesis of functionalized acrylic esters by chemical means is, however, hampered by undesired side-reactions like the Michael-addition of alcohols to acrylic esters and the thiol-Michael-addition of mercaptoalcohols to acrylic esters [2]. Enzymatic processes employing biocatalysts such as hydrolases could be most beneficial in acrylic ester functionalization since their high chemoselectivity allows for the introduction of functional groups into acrylic esters by transesterification.

The transesterification of acrylates with lipases has been described for the first time for sterically hindered diols with a lipase from *Chromobacterium viscosum* [3]. Moreover, CalB (*Pseudozyma antarctica* lipase B) has shown to be the superior catalyst out of 19 enzymes in the transesterification of methyl acrylate with 1-undecanol [4]. Patents by chemical companies like BASF emphasize the industrial interest in the field of enzymatic synthesis of acrylates [5,6]. Controlling the water activity as well as the choice of solvent are critical for high reaction rates of enzymatic acrylation [7,8], as it was shown for the acrylation of octanol in a solvent-free system with ethyl acrylate as donor of the acrylic group [8]. At water activities a_w of 0.06 and 0.45 over 95% conversion of octanol was achieved, but only 67% and 23% octyl acrylate, respectively, was obtained due to increased hydrolysis at high water activity. Besides optimizing critical reaction parameters, the enzymatic activity can be improved as well. Liu et al. applied rational protein design to construct CalB mutants showing lowered enantioselectivity and thus higher activity in acrylation of hydroxypropylcarbamate, a racemic mixture of enantiomers of primary and secondary alcohol, with methyl acrylate [9]. Direct routes to polymers with thiol and acrylate functional groups circumventing tedious chemical routes were already introduced using CalB [10,11].

Cutinases (EC 3.1.1.74) are hydrolytic, cutin-degrading enzymes. Cutin, found in higher plants, is a polymer of hydroxy and epoxy fatty acids with a chain length of primarily n-C_{16} and n-C_{18} [12]. These enzymes show a broad substrate spectrum including soluble synthetic esters and emulsified triglycerides, while preferring short-chain length substrates like *para*-nitrophenylbutyrate and tributyrin [13]. Based on studies on cutinase FsC from *Fusarium solani pisi*, it was shown that cutinases belong to the class of serine esterases and the α/β hydrolase fold superfamily [12]. With a

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molecular mass of around 22 kDa they are the smallest members of this group. Their catalytic triad consists of an active serine, an essential histidine and a carboxyl residue (usually aspartate) [12]. Besides hydrolysis, cutinases are able to catalyze esterification, transesterification and interesterification in organic media or at low water activities. Unlike most lipases, cutinases do not display interfacial activation and, for these reasons, are considered intermediates between esterases and lipases [14].

Biotechnological and industrial applications of cutinases have been comprehensively reviewed [12,15]. They offer potential applications in the dairy industry, industrial cleaning or household detergents, oleochemical industry, polymer chemistry, textile industry and laundry, food industry and in the synthesis of ingredients for personal-care products, agrochemicals and pharmaceuticals. Furthermore, cutinases could be applied, besides lipases, in the synthesis of functional polymers by transesterification of acrylates. Immobilization of cutinases allows for the recycling of these enzymes, which is especially interesting for industrial applications. Recently, immobilized cutinase from *Humicola insolens* (HiC) was successfully applied in polymer chemistry for polycondensation polymerizations of a series of diols and diacids and for lactone ring-opening polymerizations [16,17].

Here, we report on the functionalization of acrylic ester monomers with mercaptoalcohols yielding thiol-functionalized acrylic esters by using immobilized HiC. As a model reaction the transesterification of methyl acrylate with 6-mercapto-1-hexanol was chosen and reaction conditions were optimized regarding high conversion and less by-product formation.

2. Material and methods

2.1. Chemicals, strains and plasmids

All chemicals were of analytical grade or higher quality and purchased from Fluka (Buchs, Switzerland) or Sigma (Deisenhofen, Germany). Diaion HP20 was a gift from BASF AG (Ludwigshafen, Germany). *Escherichia coli* strain DH5α [F^- φ80/*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r^-_k m^-_k) *pho*A *sup*E44 λ^- *thi*-1 *gyr*A96 *rel*A1] was obtained from Clontech. The methylotrophic yeast *Pichia pastoris* X-33 (wild-type) and the expression vector pPICZαA were obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Cloning and expression of hic

The *hic* wild-type gene was cloned from pEF-Tu_HiC, kindly provided by Dr. Steffen Maurer (BASF). The gene was subcloned into pPICZαA using restriction enzymes *Eco*RI and *Kpn*I. The sequence was verified by DNA sequencing. *P. pastoris* X-33 cells were transformed with *Pme*I linearized plasmid pPICZαA-HiC according to the manufacturer's protocol (Invitrogen). Cells were spread on YPDS agar plates containing Zeocin™ (100 μg mL^{-1}) and incubated at 30 °C up to 5 d until colonies formed. Selected transformants were inoculated in 10 mL BMGY medium (buffered glycerol-complex medium for yeast, 2% peptone, 1% yeast extract, 10% potassium phosphate buffer pH 6.0, 1% glycerol) and cultivated at 30 °C and 190 rpm overnight. The preculture was used to inoculate 200 mL BMMY medium (buffered methanol-complex medium for yeast, 2% peptone, 1% yeast extract, 10% potassium phosphate buffer pH 6.0, 0.5% methanol) to an OD_{600} of 0.5. The culture was grown at 30 °C and 190 rpm in 1 L baffled flasks for 72 h. Methanol (100%) was added daily to a final concentration of 0.5% to maintain induction. Cells were harvested by centrifugation (1800 g, 4 °C, 15 min). The supernatant was concentrated at 4 °C with a PALL Minimate™ tangential flow filtration capsule (5 kDa nominal weight limit (NMWL)) according to the manufacturer's manual. A further concentration was achieved by ultrafiltration using an Amicon stirred cell model 8050 with a 5 kDa NMWL membrane according to the manufacturer's protocol. The protein concentration was determined using Bradford assay [18]. The purity of the enzyme was estimated by SDS-polyacrylamide gel electrophoresis using 16% polyacrylamide running gels. The enzyme volumetric yield reached 200 mg L^{-1} supernatant.

2.3. Cutinase activity assay

Cutinase activity was determined using *para*-nitrophenyl (*p*NP) esters of butyrate, octanoate and palmitate. Activity was measured by absorbance increase at 410 nm (ε = 13,200 M^{-1} cm^{-1}). The assay consisted of 800 μL Tris buffer (100 mM Tris pH 7.5 with 0.5% Triton X-100 and 0.1% gum arabic), 100 μL substrate (2.5 mM *p*NP ester in 100% DMSO) and 100 μL cutinase. One unit of enzymatic activity was defined as the amount of free, soluble cutinase that releases 1 μmol of *para*-nitrophenol in 1 min at room temperature. For stability tests, HiC (3.3 mg mL^{-1}) was incubated at temperatures ranging from 22 to 60 °C in a thermocycler for up to 48 h. Samples were taken at several time points and HiC was appropriately diluted prior to determination of activity versus *p*NPP. For kinetic data, activity of HiC towards *p*NP esters was measured at concentrations of 10 μM to 10 mM for *p*NPP and of 10 μM to 2 mM for *p*NPB and *p*NPO, respectively. Apparent kinetic constants K_m and k_{cat} were calculated using Origin 8.0 from the non-linear regression of the Michaelis–Menten plots. Standard deviations were calculated from at least three individual measurements and were below 6.2%.

Cutinase activity towards triglycerides tributyrin (C4:0), tricaprylin (C8:0) and triolein (C18:1) was determined using the pH stat titration method [19]. The substrates were emulsified for 15 min by dispersion (Ultraturrax T18 basic) in solution A (20 mM $CaCl_2$, 0.6 M NaCl, 1 mM sodium taurocholate and 10% (w/v) gum Arabic) to a final concentration of 30 mM [20]. A 20 mL aliquot of substrate emulsion was adjusted to pH 7.5 with 0.1 M NaOH. After addition of the enzyme solution, fatty acids liberated from triglycerides were automatically titrated with 0.01 M NaOH to maintain a constant pH of 7.5 at 30 °C (Titrando 842, Metrohm, Switzerland). One unit of cutinase activity was defined as the amount of enzyme releasing 1 μmol fatty acid per min at room temperature. All values were corrected for the autohydrolysis of the substrates at different assay conditions.

2.4. Immobilization

HiC was immobilized onto microporous resin Diaion HP20 (average particle size 440 μm, 30 nm pore radius) with an enzyme loading of 1% (w/w) or 4.5 μmol HiC. The resin was degreased using ethanol (100%) and dried under vacuum for 24 h at room temperature prior to use. Three mg of HiC were diluted in immobilization buffer (50 mM potassium phosphate buffer pH 7.8 containing 25% ethanol) to a final concentration of 0.25 mg mL^{-1}. Afterwards, 300 mg Diaion HP20 was added and the solution was incubated at 4 °C for 24 h under constant agitation. Subsequently, the immobilisate was recovered by filtration, washed twice with 15 mL potassium phosphate buffer (50 mM, pH 7.8) and dried in a desiccator over silica gel for 72 h at room temperature to achieve a low water activity a_w towards 0. The immobilisate was stored at 4 °C.

2.5. Transesterification and GC/MS analysis

In the standard reaction setup, methyl acrylate (300 μL, 10.3 M) as donor of the acrylic group and sole solvent, 6-mercapto-1-hexanol (20 μL, 275 mM) and 10 mg immobilisate (1% (w/w) enzyme loading; 4.5 μmol HiC) were applied. Reactions were carried out in screw-capped glass vials under constant shaking (700 rpm) at temperatures from 22 to 50 °C for up to 24 h. For optimization of reaction conditions ddH_2O (0.025–0.1% (w/w), 0.43–1.72 M), methanol (0.78–3.03% (w/v), 0.19–0.75 M), ethanol (0.78–3.03% (w/v), 0.13–0.52 M) and BHT (3–15% (w/w) acrylic ester, 0.14–0.71 M), respectively, were added. For stability tests, the immobilisate was incubated at 40 °C for 18–24 h in methyl acrylate, recovered and used in a standard reaction. At appropriate time points, 50 μL samples were taken, centrifuged for 1 min at 14,000 rpm, diluted in 1 mL diethyl ether dried over $MgSO_4$ (anhydrous) and analyzed by GC/MS measurements. GC/MS analysis was carried out on a Shimadzu GCMS-QP2010 (equipped with a FS-Supreme-5 column length 30 m, internal diameter 0.25 mm) using helium as carrier gas. The following GC program was used: 70 °C for 1 min, 15 °C min^{-1} to 280 °C, 30 °C min^{-1} to 310 °C. Standard curves for substrates were generated and conversion of mercaptoalcohols was calculated from the peak area ratios of mercaptoalcohols and products. Transesterification products were identified by their characteristic MS fragmentation patterns.

2.6. Product purification and identification by NMR and FT-IR

6-mercaptohexyl acrylic ester was purified using column chromatography with a mobile phase of hexane:diethyl ether (10:4). The structure was confirmed by ^{1}H and ^{13}C NMR measurements ($CDCl_3$ with TMS (1 vol.%)). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.2–1.6 (m, 9 H), 2.4 (dt, J = 7.5 Hz, J = 10.5 Hz, 2 H), 4.1 (t, J = 6.7 Hz, 2 H), 5.8 (dd, J = 1.3 Hz, J = 10.5 Hz, 1 H), 6.0 (dd, J = 10.5 Hz, J = 17.2 Hz, 1 H), 6.3 (dd, J = 1.3, J = 17.2, 1 H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 23 (C-9), 24 (C-6), 27 (C-7), 28 (C-5), 33, (C-8), 63 (C-4), 128 (C-1), 130 (C-2), 165 (C-3) ppm. FT-IR: 2936 (w), 2860 (w) 2562 (w, S-H), 2255 (w), 1961 (w), 1717 (m), 1637 (w), 1464 (w), 1409 (w), 1280 (w), 1197 (m), 1061 (w), 984 (w).

3. Results and discussion

3.1. Enzyme activity and stability

Prior to optimization of transesterification reaction conditions substrate spectrum, temperature optimum and thermal stability of HiC have been determined. Kinetic parameters of HiC, recombinantly expressed in *Pichia pastoris*, were investigated towards soluble *para*-nitrophenyl (*p*NP) esters and triglycerides. Recombinant HiC displayed a preference for shorter chain substrates (C_4

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