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Short communication

Biocatalytic functionalization of hydroxyalkyl acrylates and phenoxyethanol via phosphorylation

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ABSTRACT

The enzymatic phosphorylation of phenoxyethanol, 2-hydroxyethyl acrylate and 4-hydroxybutyl acrylate catalyzed by acid phosphatases PhoN-Sf and PiACP at the expense of inorganic di-, tri-, hexameta- or polyphosphate was applied to the preparative-scale synthesis of phosphorylated compounds. The reaction conditions were optimized with respect to enzyme immobilization, substrate concentration, pH and type of phosphate donor. The mild reaction conditions prevented undesired polymerization and hydrolysis of the acrylate ester moiety. Application of a continuous flow system allowed facile scale-up and mono-phosphates were obtained in up to 26% isolated yield with space-time yields of 0.89 kg L^{-1} h^{-1}.

1. Introduction

Phosphorylation by chemical means requires the use of activated phosphoric acid derivatives. Although novel mild catalytic methods employing metal- and organocatalysts have been disclosed recently (Murray et al., 2015), these methods usually lead to phosphate triesters requiring subsequent deprotection to yield the desired monophosphates or suffer from low regioselectivity. In contrast, enzymatic phosphorylation proceeds under physiological conditions, allowing for clean and selective reactions. Biocatalytic phosphorylation traditionally relies on kinases together with suitable ATP-recycling (Faber, 2011; Langer et al., 1976; Pollak et al., 1977). However, since these enzymes are predominantly involved in biological activation and messaging processes, their substrate spectrum is generally narrow (Crans and Whitesides, 1985a,b; Chenault et al., 1997; Drueckhammer and Wong, 1985). Together with the modest efficiency of ATP-recycling showing TTNs of several 100 at best (e.g. (Shih and Whitesides, 1977), this has set low limits for preparative-scale applications (Andexer and Richter, 2015). A modern variant of enzymatic phosphorylation makes use of phosphatases in the phosphate-transfer mode using cheap inorganic di- or triphosphate as phosphate-donor (Wever and van Herk, 2012). Since phosphatases are hydrolytic enzymes involved in biodegradation, their substrate profile is generally much broader compared to that of kinases. In order to avoid undesired phosphate ester (product) hydrolysis, kinetic reaction control as well as physical separation of product from enzyme by means of flow set-up were successfully applied (Babich, 2013; Babich et al., 2012a,b). Previously, this approach was first used for preparative purposes using calf intestine alkaline phosphatase (Pradines et al., 1988). Later, mutant acid phosphatases exhibiting reduced product hydrolysis activity were employed for the phosphorylation of nucleosides (Suzuki et al., 2007; Asano et al., 1999; Mihara et al., 2000). Non-specific acid phosphatases (NSAPs) from *Shigella flexneri* (PhoN-Sf) and from *Salmonella enterica* ser. *typhimurium* LT2 (PhoN-Se) accepted a broad range of alcohols including monoalcohols, diols and sugars (Murray et al., 2015; Tanaka et al., 2003; Tasnádi et al., 2016; van Herk et al., 2005) and were successfully implemented in cascade reactions (Babich et al., 2012b; Babich et al., 2011; Hartog et al., 2011; van Herk et al., 2006).

In order to employ this phosphate-transfer protocol to chemically sensitive *prim*-alcohol substrates, we studied the phosphorylation of 2-phenoxyethanol (ethylene glycol phenyl ether, **1a**) and hydroxyalkyl acrylates **2a** and **3a** using NSAPs PhoN-Sf (Uchiya

Abbreviations: PP_i, inorganic pyrophosphate; PPP_i, inorganic triphosphate; HMP, hexametaphosphate; polyP, polyphosphoric acid; STY, space-time yield; P-yield, phosphorylation yield; $cons_{ppi}$, consumption of PP_i.

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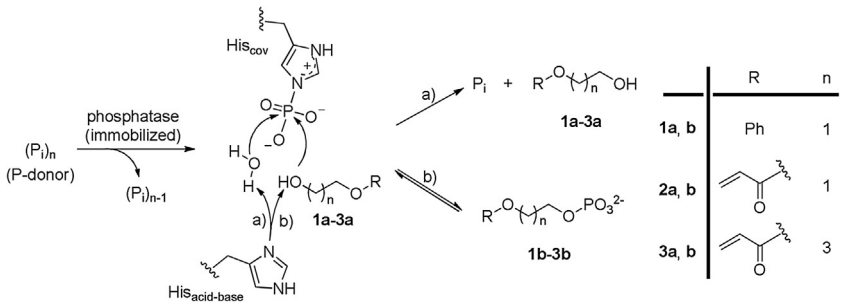

Scheme 1. Enzymatic phosphorylation of 2-phenoxyethanol (**1a**) and hydroxyalkyl acrylates (**2a**, **3a**). His$_{cov}$: catalytic histidine forming a covalent intermediate with phosphate (His207 in PhoN-Sf and His209 in PiACP). His$_{acid-base}$: histidine involved in activation of phosphate acceptor (His168 in PhoN-Sf and His170 in PiACP).

et al., 1996) and PiACP from *Prevotella intermedia* (Chen et al., 1999) (Scheme 1). PiACP was identified as a potent acid phosphatase having 56% identity to PhoN-Sf (Chen et al., 1999). Both enzymes rely on two catalytic histidine residues, which form a covalent enzyme-phosphate intermediate and act as a base, respectively (His$_{cov}$, His$_{acid-base}$, Scheme 1). Both enzymes were overexpressed in *E. coli*, subsequently purified to homogeneity using His-tag technique and immobilized on various supports. Various parameters, such as concentration of substrate, pH and type of phosphate donor (P-donor) were investigated using immobilized enzyme preparations. Upscaling was carried out by using the enzyme in a flow reactor allowing the isolation of the product phosphates (**1b**–**3b**) in gram amounts. Compound **1a** is commonly used in cosmetic products as a preservative (Lowe and Southern, 1994), while **2b** and **3b** are building blocks of polymeric dispersing agents (Gaedt et al., 2015).

2. Materials and methods

2.1. Materials

All chemicals were purchased from commercial suppliers and were used without further purification. Phenoxyethanol (**1a**), 4-nitrophenyl phosphate disodium salt hexahydrate (*p*-NPP), sodium pyrophosphate dibasic (PP_i), polyphosphoric acid (polyP, product number: 04101), hexametaphosphate (HMP) and Immobead 150 (particle size: 100–500 μm) were purchased from Sigma, Relizyme HA403/M (particle size: 200–500 μm, average pore size: 40–60 nm, functional group density: min. 600 μmol g^{-1} wet bead) was purchased from Resindion. Relizyme EP403/M (same technical specifications as HA403/M except functional group density: min. 30 μmol g^{-1} wet bead) was a kind gift from Resindion. Sodium tripolyphosphate (PPP_i) was from Alfa Aesar. Ni-NTA column for His-tag purification was from GE Healthcare Life Sciences. 2-Hydroxyethyl acrylate (**2a**) and 4-hydroxybutyl acrylate (**3a**) were synthesized at BASF, 50% aqueous glutaraldehyde solution is a BASF product. PhoN-Sf (A_{spec} = 66.9 U mg^{-1}) and PiACP (A_{spec} = 84.9 U mg^{-1}) were overexpressed as reported (Tasnádi et al., 2016).

2.2. Analytical tools

NMR spectra were measured on a Bruker Avance III 300 MHz NMR spectrometer. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS or H_3PO_4 as a reference. ^{31}P-NMR spectrum was taken using inverse gated decoupling. HPLC analysis was carried out on a Dionex Ultimate 3000 system equipped with Shodex RI-101 refractory index detector (HPLC-RI) (Table S1). Products were characterized on an Agilent 1260 Infinity system equipped with Agilent Q6120 quadrupole mass spectrometer using electrospray ionization (HPLC–MS, Zorbax 300-SCX cation exchanger column; eluent: 0.1% (v/v) formic acid; flow rate: 1 mL min^{-1}; 40 °C; injection volume: 10 mL) and via NMR (see SI).

2.3. Immobilization of PhoN-Sf and PiACP

Before immobilization, Relizyme HA403/M was activated via functionalization with glutaraldehyde. The beads (10, 25 or 300 mg wet beads) were mixed with 20 mM KP_i buffer pH 8 and 50% aqueous solution of glutaraldehyde (1% final concentration) for 1 h at room temperature (120 rpm), then washed twice with 20 mM KP_i buffer for 30 min at 120 rpm and room temperature and used immediately for immobilization.

To the activated beads (10, 25 or 300 mg wet beads) were added 1.25 M KP_i buffer pH 8 (0.5, 1 or 15 mL) and the purified enzyme (10, 25 or 300 U, resp.). The mixture was shaken at room temperature and 120 rpm overnight. Then the beads were washed twice with H_2O and once with 2 M glycine buffer pH 8.5 and shaken in this buffer overnight at room temperature and 120 rpm. The supernatant was removed; the beads were washed twice with H_2O and once with 100 mM KP_i buffer pH 7 and stored in this buffer at 4 °C until use.

2.4. Assay for phosphohydrolase activity (p-NPP assay)

Free and immobilized enzyme activities were assayed spectrophotometrically by measuring the dephosphorylation of 4-nitrophenyl phosphate (*p*-NPP) via release of *p*-nitrophenol. Soluble (100 μg mL^{-1}) or immobilized enzyme (10 μL settled wet beads) was added to maleate buffer pH 6.0 (100 mM final concentration) to a final volume of 480 μL followed by addition of 20 μL 250 mM *p*-NPP in H_2O (10 mM final concentration in buffer) and were mixed at 30 °C and 450 rpm. After 1 min incubation time, the reaction was quenched with 500 μL 1 M NaOH and the absorbance of 4-nitrophenol (*p*-NP) was recorded at 405 nm (ε = 18500 M^{-1} cm^{-1}). The activity tests were always performed in triplicates. One unit of phosphatase activity (U) corresponds to the amount of *p*-NP (micromoles) released per minute under assay conditions. Specific activity (A_{spec}) represents the phosphatase activity (U) of 1 mg protein or 1 g dry resin, respectively.

2.5. General conditions for enzymatic transphosphorylation

A standard reaction mixture contained substrate and P-donor in H_2O at a concentration indicated in captions of figures and 1% DMSO as internal standard at a given pH. The reactions were initiated by the addition of 1 mL reaction mixture to the immobilized enzyme preparation (10 or 25 mg) directly prepared in 1.5 mL screw-cap glass vials. The mixture was horizontally shaken at 30 °C and 450 rpm. Samples of 25 μL were taken at intervals and diluted to 500 μL with 5 mM H_2SO_4 followed by injection to HPLC-RI equipped with an Alltech IOA-2000 cation exchanger col-