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Investigations on the activity of poly(2-oxazoline) enzyme conjugates dissolved in organic solvents

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ABSTRACT

The use of enzymes in organic solvents offers a great opportunity for the highly selective synthesis of complex organic compounds. In this study we investigate the POXylation of several enzymes with different polyoxazolines ranging from the hydrophilic poly(2-methyl-oxazoline) (PMOx) to the hydrophobic poly(2-heptyl-oxazoline) (PHeptOx). As reported previously on the examples of model enzymes POXylation mediated by pyromellitic acid dianhydride results in highly modified, organosoluble protein conjugates. This procedure is here extended to a larger number of proteins and optimized for the different polyoxazolines. The resulting polymer-enzyme conjugates (PEC) became soluble in different organic solvents ranging from hydrophilic DMF to even toluene. These conjugates were characterized regarding their solubility and especially their activity in organic solvents and in some cases the PECs showed significantly (up to 153,000 fold) higher activities than the respective native enzymes.

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1. Introduction

Enzymes are very active and selective biocatalysts, which are increasingly applied for the regioselective and enantioselective synthesis of organic compounds. Because the modern biotechnology allows the large scale production of many proteins, enzymes have become serious competitors to organometal catalysts (Faber, 2011).

Due to their origin, enzymes are mostly used to catalyze chemical conversions in aqueous environments. However, the number of possible products can be greatly extended in organic media, because many enzyme-catalyzed reactions will not take place in aqueous media due to the low solubility of the substrates (Khmelnitsky and Rich, 1999). Organic solvents do not only increase the number of potential substrates, but allow also reactions that are not taking place in water-based systems, for example the reverse reaction of lipases or the acceleration of transesterification reactions (Zaks and Klibanov, 1985). Also selectivities are controllable by non-aqueous media. A major drawback is the usually lower activity of enzymes in organic solvents compared to their activity in aqueous systems (Klibanov, 1997).

In order to improve reactivity and stability of biocatalysts in organic media several approaches have been described. Enzymes were used in a monophasic organic solvent after lyophilization with or without the addition of lyoprotectants (Klibanov, 2001). Another possibility is the application in a mixture of water and a water miscible organic solvent (Dordick, 1989). Enzymatic reactions can also be carried out in two phase systems (Schulz et al., 2005) or in miniemulsions, which lead to higher reaction rates compared to classic emulsions (Gröger et al., 2006). Paradkar and Dordick (1994) dissolved enzymes in organic media by using the surfactant Aerosol OT achieving great activities for proteases. The most common way to improve enzyme activity in organic media is the immobilization on a solid support. A further way of working with enzymes in nonaqueous environments is the entrapment into polyurethanes or into silicates (May, 1997; Romaškevič et al., 2006) or alternatively in amphiphilic polymer conetworks (Bruns and Tiller, 2005, 2006; Bruns et al., 2008; Dech et al., 2012), or amphiphilic polymer beads (Savin et al., 2005; Schoenfeld et al., 2013). Alternatively, the chemical modification of proteins with polymers has been explored. The advantage of a chemical modification is that the enzymes become soluble in organic solvents leading to a higher catalytic activity due to the omission of the diffusion limitation of the substrate.

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There is also no steric hindrance of active sites by other nearby enzymes as it is the case in dispersed lyophilized enzyme particles and in solid supports (Klibanov, 1997). A further advantage of the conjugation with a polymer is the protection of the enzyme from the organic solvent and the retaining of essential water molecules (Takahashi et al., 1984). Thus, polymer enzyme conjugates (PEC) are more stable against denaturation (DeSantis and Jones, 1999).

The most common polymeric modifier for proteins is polyethylene glycol (PEG) (Roberts et al., 2002; Miyamoto et al., 1990). It is known to literature that several enzymes modified with PEG are soluble in organic solvents (Gaertner and Puigserver, 1988; Matsushima et al., 1984; Takahashi et al., 1984). However, PEG has the disadvantage that it is difficult to modify and cannot easily be altered to tailor the properties of the respective PEC. Poly-2-alkyl-2-oxazolines (POx) are a suitable alternative to PEG, because they allow the synthesis of well-defined polymeric structures and are easily accessible by microwave-assisted cationic ring-opening polymerization (Hoogenboom, 2009; Wiesbrock et al., 2004). The hydrophobicity of the polyoxazolines can be varied via nature of the monomers and amphiphilic block polymers are easily accessible. POx polymers can be tailored to be soluble in both organic solvents and depending of the substituent in water and show no toxicity (Mero et al., 2008). In addition, POx can be equipped with different functional groups by the initiation or the termination step (Kempe et al., 2011; Krumm et al., 2012; Waschinski et al., 2005, 2008).

Previously, we introduced a method to highly modify proteins with PMOx and PEtOx, respectively, in order to render the enzymes RNase A and lysozyme soluble in the organic solvents DMF and chloroform (Konieczny et al., 2012). In the present study, this concept was extended to a larger number of proteins by optimizing the method and the poly(2-alkyloxazoline) structure. The new POx-protein conjugates were explored regarding their enzymatic activity in the respective organic solvent.

2. Experimental

2.1. Materials

Chloroform was dried with activated alumina and subsequent distillation. The obtained chloroform contained less than 1 ppm of water (determined by Karl-Fischer titration) and was stored under argon over molecular sieve (4 Å). The monomers 2-methyl-2-oxazoline (MOx), 2-ethyl-2-oxazoline (EtOx), 2-butyl-2-oxazoline (BuOx) and 2-heptyl-2-oxazoline (HeptOx) were distilled twice from CaH_2 under reduced pressure and argon atmosphere and stored under argon at −20 °C over molecular sieve (4 Å). *N*,*N*-dimethylformamide (DMF) was dried, freed of amine by distillation and stored under argon over molecular sieve (4 Å). All other chemicals were purchased from Sigma–Aldrich, Applichem, Merck, ABCR, Acros, Armar Chemicals and Carl Roth and were of analytical grade or purer and used without further modification.

2.2. Measurements

^{1}H NMR spectra were recorded in DMSO-d_6 or Methanol-d_4 using a Bruker DRX-400 spectrometer with a 5 mm sample head operating at 400.13 MHz.

Size exclusion chromatography (SEC) of the polyoxazolines was performed on a Viscotek GPCMax system equipped with a refractive index (RI) detector in saline DMF (20 mM LiBr) at 60 °C with a flow rate of 0.7 mL/min. Two TSKgel GMHHR-M 7.8 mm × 300 mm columns and one precolumn were used. The calibration was performed using polystyrene standards.

2.3. Synthesis of 2-butyl-2-oxazoline

2-Butyl-2-oxazoline was synthesized as described in previous work (Krumm et al., 2013).

2.4. Synthesis of 2-heptyl-2-oxazoline

2-Heptyl-2-oxazoline was synthesized according to a modified protocol of Kempe et al. (2009). To this end, 70 mL (0.455 mol) of heptyl cyanide, 1.96 g of zinc acetate dihydrate (0.02 equiv.) and 33.3 mL of ethanolamine (1.2 equiv.) were heated under reflux in a microwave reactor to 140 °C for 25 h. After cooling to room temperature, the reaction mixture was dissolved in 500 mL cyclohexane and was subsequently washed 3 times with water (500 mL). In a final step the organic phase was washed with brine and dried over $MgSO_4$. After filtration the cyclohexane was evaporated and the crude product was distilled under reduced pressure. The product was obtained with a yield of 61–75% and characterized by ^{1}H NMR spectroscopy.

^{1}H NMR ($CDCl_3$): δ (ppm) = 4.09–4.00 (t, 2H, —O—CH_2—); 3.70–3.60 (t, 2H, —N—CH_2—); 2.14–2.05 (t, 2H, —CH_2—$(CH_2)_5$—CH_3); 1.52–1.42 (m, 2H, —CH_2—CH_2—$(CH_2)_4$—CH_3); 1.22–1.05 (m, 8H, —CH_2—CH_2—$(CH_2)_4$—CH_3); 0.78–0.66 (t, 3H, —$(CH_2)_6$—CH_3).

2.5. Synthesis of benzyl tosylate

The synthesis of benzyl tosylate was performed according to a modified procedure of Kazemi et al. (2007). The reaction was carried out in a mortar. To this end, 1.04 mL (10 mmol) of benzyl alcohol and 2.86 g (15 mmol) of tosyl chloride (TsCl) were added to 5 g of dry K_2CO_3 and grinded vigorously for 5 min. Then, 2.81 g of powdered KOH (50 mmol) were added and again vigorously grinded for 2 min. The mixture was suspended in diethyl ether and the solids were filtered off. After evaporating the solvent, crude product was further purified by twofold recrystallization from *n*-heptane. The product was obtained with a yield of 75% and characterized by ^{1}H NMR spectroscopy.

^{1}H NMR ($CDCl_3$): δ (ppm) = 7.85–7.74 (d, 2H, —S-*o*—C_6H_4—CH_3); 7.21–7.19 (m, 7H, —O—CH_2—C_6H_5, —S-*m*—C_6H_4—CH_3,); 5.03–5.07 (s, 2H, —CH_2—C_6H_5); 2.41–2.48 (s, 3H, C_6H_4—CH_3).

2.6. Synthesis of polyoxazolines (general procedure)

All reactions were carried out under argon atmosphere. The initiator benzyl tosylate and the respective monomer were dissolved in 20 mL of dry chloroform at room temperature. Then the reaction mixture was heated in a closed vessel in a CEM Discover synthesis microwave reactor.

After the polymerization, the reaction mixture was cooled to room temperature and 4.5 g (5 mL) of ethylenediamine (EDA) was added. Then, the solution was heated to 45 °C and kept for another 48 h. The poly-2-methyl-oxazoline (PMOx) and poly-2-ethyl-oxazoline (PEtOx) polymers were precipitated in diethylether, filtered off and reprecipitated in diethylether/chloroform (3 times). Then, the polymers were dialyzed against methanol for two days using ZelluTrans cellulose membranes with a molecular weight cut off (MWCO) of 1000 g mol^{-1}. In the cases of the PBuOx-*b*-PMOx diblock copolymer and the poly-2-heptyl-oxazoline (PHeptOx) the liquids were removed by evaporation and the polymers were directly dialyzed against methanol for three days using the same membranes. After dialysis all polymers were dried under reduced pressure obtaining slightly yellow solids characterized by ^{1}H NMR spectroscopy and size exclusion chromatography (SEC, Table 2). The basic ^{1}H NMR signals of the PMOx/PEtOx/PBuOx-*b*-PMOx/PHeptOx backbones are listed below. The POx were typically

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