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Organosoluble enzyme conjugates with poly(2-oxazoline)s via pyromellitic acid dianhydride

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ABSTRACT

The use of enzymes in organic solvents offers a great opportunity for the synthesis of complex organic compounds and is therefore in focus of current research. In this work we describe the synthesis of poly(2-methyl-1,3-oxazoline) (PMOx) and poly(2-ethyl-1,3-oxazoline) (PEtOx) enzyme conjugates with hen-egg white lysozyme, RNase A and α-chymotrypsin using a new coupling technique. The POXylation was carried out reacting pyromellitic acid dianhydride subsequently with ethylenediamine terminated POx and then with the NH_2-groups of the respective enzymes. Upon conjugation with the polymers, RNase A and lysozyme became fully soluble in DMF (1.4 mg/ml). These are the first examples of fully POXylated proteins, which become organosoluble. The synthesized enzyme conjugates were characterized by SDS-PAGE, isoelectric focusing, dynamic light scattering and size exclusion chromatography, which all indicated the full POXylation of the enzymes. The modified enzymes even partly retained their activity in water. With α-chymotrypsin as example we could demonstrate that the molecular weight of the attached polymer significantly influences the activity.

1. Introduction

Enzymes are highly active and selective biocatalysts, which can be used for the synthesis of complex organic compounds. Since modern biotechnology enabled large scale production of nearly any protein, enzymes have become serious competitors to organometal catalysts (Faber, 2011).

Due to their natural environment enzymes are mostly applied in water-based systems. By using of biocatalysts in organic solvents the number of possible substrates can be greatly extended, because many enzymatically catalyzed reactions cannot be carried out in aqueous solutions due to the low solubility of the substrates (Khmelnitsky and Rich, 1999). Media other than water afford enzymes to catalyze reactions that are not occurring in aqueous environments. For example it is possible to reverse the reaction of lipases in non aqueous solvents or to accelerate transesterification reactions (Zaks and Klibanov, 1985). A disadvantage is the usually lower activity of enzymes in nonaqueous solvents compared to the activity in aqueous buffers (Klibanov, 1997). There are several ways for the application of enzymes in organic solvents. In a monophasic organic solvent they are used after lyophilization with or without the addition of lyoprotectants (Klibanov, 2001). Enzymatic reactions can also be carried out in two phase systems (Schulz et al., 2005). The preparation of miniemulsions leads to higher reaction rates compared to the classical emulsions (Gröger et al., 2006). A way to dissolve enzymes into organic media is the use of the surfactant Aerosol OT (Paradkar and Dordick, 1994). Another possibility is the application of enzymes in a mixture of water and a water miscible organic solvent (Dordick, 1989). Mostly, enzymes are used immobilized on a solid support. Alternatively, biocatalysts can be activated in organic solvents by entrapping them into polyurethanes or into silicates (May, 1997; Romaškevič et al., 2006). A further approach is the entrapment in amphiphilic polymer conetworks (Bruns and Tiller, 2005, 2006; Bruns et al., 2008), or amphiphilic polymer beads (Savin et al., 2005). Also the chemical modification with polymers has been explored for this purpose. Such a modification has the advantage that the enzymes become soluble in the organic solvent which leads to a higher catalytic activity due to the omission of the diffusion limitation of the substrate. There is also no steric hindrance of active sites by other nearby enzymes as it is the case in dispersed lyophilized enzyme

Abbreviations: CT, α-chymotrypsin; DM, degree of modification; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; DP, degree of polymerization; DTT, dithiothreitol; EDA, ethylenediamine; EtOx, 2-ethyl-1,3-oxazoline; GPC, gel permeation chromatography; IEF, isoelectric focussing; MeOx, 2-methyl-1,3-oxazoline; MWCO, molecular weight cut-off; NMR, nuclear magnetic resonance; PADA, pyromellitic acid dianhydride; PDI, polydispersity index; PEC, polymer enzyme conjugate; PEG, polyethylene glycol; PETOx, poly-2-ethyl-1,3-oxazoline; pI, isoelectric point; PMeOx, poly-2-methyl-1,3-oxazoline; POx, polyoxazolines; RI, refractive index; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEC, size exclusion chromatography.

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particles (Klibanov, 1997). The modification also protects the enzyme from the organic solvent and inhibits the removal of the essential water (Takahashi et al., 1984). The resulting polymer enzyme conjugates (PECs) are more stable against denaturation, e.g., at high temperatures (DeSantis and Jones, 1999).

The most common polymeric modifier for proteins is polyethylene glycol (PEG) which is often used for different purposes (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 is difficult to modify and cannot easily be altered to tailor the properties of the respective PEC. Poly-2-alkyl-1,3-oxazolines (POx) are a suitable alternative to PEG, because they are easily accessible by microwave-assisted cationic ring-opening polymerization and allow the synthesis of well defined polymeric structures (Hoogenboom, 2009; Wiesbrock et al., 2004). This way synthesis of amphiphilic block polymers is readily possible and their hydrophobicity can be varied via the nature of the monomers. They are soluble in both organic solvents and depending of the substituent in water and show no toxicity (Mero et al., 2008). Furthermore POx can be equipped with different functional groups by the initiation or the termination step (Kempe et al., 2011; Waschinski et al., 2005, 2008) and can be grafted to other macromolecules (Bieser et al., 2011).

Some works have been published that state the successful modification with different polyoxazolines (Mero et al., 2008; Miyamoto et al., 1990; Tong et al., 2010; Viegas et al., 2011). Only one of them quantifies the solubility of the POXylated protein with 0.05 mg/ml in chloroform, which seems very low (Miyamoto et al., 1990).

In this study we report on the conjugation of an amine terminated polyoxazoline with enzymes using pyromellitic acid dianhydride as coupling agent. So far, pyromellitic acid dianhydride is only known for the hydrophilization of enzymes (Mozhaev et al., 1988). The aim of this enzyme modification was to synthesize fully organosoluble enzyme conjugates. In this work we used hen-egg white lysozyme, RNase A and α-chymotrypsin as model enzymes.

2. Experimental part

2.1. Materials

The chloroform was dried with conc. H_2SO_4 and activated alumina and subsequent distillation. This way, the chloroform contained less than 1 ppm of water (determined by Karl–Fischer titration). The solvent was stored under argon over molecular sieve (4 Å). The monomers 2-methyl-1,3-oxazoline (MOx) and 2-ethyl- 1,3-oxazoline (EtOx) were distilled from CaH_2 under reduced pressure and argon atmosphere. They were stored under argon at −20 °C over molecular sieve (4 Å). *N*,*N*-Dimethylformamide (DMF) was dried, freed of amine and stored under argon over molecular sieve (4 Å). All other chemicals were purchased from Sigma–Aldrich, Applichem, Merck, Serva and Carl Roth, were of analytical grade or purer and were used without further modification.

2.2. Measurements

Size exclusion chromatography (SEC) for the poly(2-oxazoline) 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. SEC for the enzymes and enzyme conjugates was performed on a LaChrom Elite HPLC system equipped with a diode-array detector in buffer (0.1 M KH_2PO_4 buffer, pH 7) at 30 °C with a flow rate of 1 ml/min. A Biobasic SEC-300 7.8 mm × 300 mm column and one precolumn were used. ^{1}H NMR spectra were recorded in DMSO-d^6 using a Bruker DRX-400 spectrometer with a 5 mm sample head operating at 400.13 MHz. The DLS measurements were performed on a Malvern Zetasizer nano S at 25 °C. All samples were measured in DMF and filtered by a 0.2 μm syringe filter. The equilibration time was 3 min.

2.3. Synthesis of poly(2-oxazoline)s (typical procedure)

All reactions were carried out under argon atmosphere. For poly(2-methyl-1,3-oxazoline) (PMOX), the initiator methyl-*p*-toluenesulfonate and the monomer was 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 cooling the reaction mixture to room temperature 5 ml (4.5 g, 75 mmol, 38–77 eq.) of ethylenediamine (EDA) was added to the reaction mixture. The termination was performed at 40 °C for 48 h. The raw polymers were purified by repeated reprecipitation in diethylether/chloroform (3 times) and further dialysis against distilled methanol for 2 days using ZelluTrans cellulose membranes with a molecular weight cut off (MWCO) of 1000 g mol^{-1}. After dialysis the polymers were dried under reduced pressure obtaining slightly yellow solids characterized by ^{1}H NMR spectroscopy and size exclusion chromatography (SEC, Table 4). The basic ^{1}H NMR signals of the PMOx/PEtOx are listed below. We synthesized PMOx with two different degrees of polymerization (DP) and one PEtOx. The respective reaction conditions are summarized in Table 1.

PMOx: ^{1}H NMR (DMSO-d^6): δ (ppm) = 3.65–3.10 (b, *n*·4H, N(CH_2)$_2$), 2.7–2.55 (b, 6H, CH_2—NH—CH_2—CH_2—NH_2), 2.10–1.85 (b, *n*·3H, NCO—CH_3).

PEtOx: ^{1}H NMR (DMSO-d^6): δ (ppm) = 3.75–3.10 (b, *n*·4H, N(CH_2)$_2$), 2.75–2.55 (b, 6H, CH_2—NH—CH_2—CH_2—NH_2), 2.45–2.05 (b, *n*·3H, $NCOCH_2CH_3$), 1.10–0.75 (b, *n*·2H, $NCOCH_2CH_3$).

2.4. Conjugation of lysozyme, α-chymotrypsin and RNase A with poly(2-oxazoline)s

Typically, the pyromellitic acid dianhydride (PADA) was dissolved in 3 ml of DMF and the respective amount of POx was added. The polymer amount was calculated with a ten-fold molar excess referring to the enzymes primary amino groups. The PADA amount was varied according to Table 3. The mixture was allowed to react for 12 h. The enzyme, which was lyophilized from a 0.5 mM sodium carbonate buffer with a pH of 9.65, was dispersed in 2 ml DMF and 3 ml POx/PADA mixture were added drop wise within 15 min.

For electrophoresis and isoelectric focusing the DMF was removed by dialysis against water using ZelluTrans cellulose membranes with an MWCO of 1000 g mol^{-1}. The samples were concentrated using Millipore (Merck) Amicon ultrafiltration units with an MWCO of 10,000 g mol^{-1}. In the case of modification in a mixture of DMF and buffer, 5 ml of the polymer/PADA mixture were added to the enzyme, which was dissolved in 2 ml of a carbonate buffer (0.5 mM pH 9.65). The analytical data of the used enzymes are summarized in Table 2.

2.5. Electrophoresis

The reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970).

The dissolved native lysozyme (5 μg in about 5 μl) and the dissolved conjugate (30 μg in max. 25 μl) were mixed with 2× loading buffer, heated to 96 °C for 10 min and treated with 2 μl 1 M dithiothreitol (DTT) per 10 μl of sample. The mixtures were