Improved Pharmacokinetics and Stability Properties of Catalase by Chemical Glycosidation with End-Group Activated Dextran

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ABSTRACT: Catalase was chemically modified with a monoactivated dextran derivative having a carboxylate group at its reducing end residue. The modified enzyme retained 77% of its initial specific activity and was 3-fold more resistant to tryptic degradation. The plasma half-life time was increased to 7.3-fold after glycosidation. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 4573–4576, 2006

Key words: catalase; dextran; drug delivery systems; enzymes; polysaccharides

INTRODUCTION

Several proteins are currently used as therapeutic drugs for numerous diseases, but their major application is limited by the rapid clearance of these biomolecules after *in vivo* administration. This clearance is mediated by different mechanisms such as the recognition by the immune system, proteolytic degradation, and glomerular filtration through the kidney.[1] That is why the development of new delivery systems for these drugs is required.

Modification of proteins with biocompatible polymers constitutes a successful approach for improving their pharmacokinetics and stability properties.[2] In special, poly(ethylene glycol) (PEG) has been widely used for site-directed modification of protein drugs, and there are many PEG-based protein conjugates in the market with increased circulatory half-life.[3] However, PEG is a non biodegradable macromolecule which is slowly cleared from the human body, and the occurrence of anaphylactic reactions and a severe bronchospasm have been reported for PEG-modified therapeutic enzymes.[4]

Chemical crosslinking of enzymes with polyactivated macromolecules have been extensively used as a method for stabilizing such kind of catalysts.[5–8] Through this approach the catalytic and functional activity of several enzymes have been improved.[5–8] However, the adducts prepared showed undefined chemical structure and in some case lower catalytic behavior.[9] For this reason, the development of alternative modification methods for preparing pharmacologically active polymer–protein conjugates with more defined composition receives considerable attention in pharmacological sciences.

In the present article, we describe the synthesis of an end-group monocarboxylated dextran derivative and its attachment to the surface of catalase (EC 1.11.1.6). Dextran is a biocompatible and biodegradable polymer widely employed in biomedical fields.[10] Among these applications, crosslinking of pharmacological active proteins is included.[11] As target enzyme we used catalase, a tetrameric antioxidant enzyme with potential application in the therapy of several diseases mediated by reactive oxygen species.[12]

MATERIALS AND METHODS

Materials

Bovine liver catalase (14.6 U/g) was from Roche Molecular Biochemistry (Mannheim, Germany). Dextran 5000 was from Serva Electrophoresis (Heidelberg, Germany). Bovine pancreatic trypsin and Fractogel EMD BioSEC (S) were from Merck (Darmstadt, Germany). All other chemicals were analytical grade.

Synthesis of end-group carboxylated dextran

Dextran (2 g), dissolved in 10 mL distilled water was treated with 1 g ε-aminocaproic acid and stirred for

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2 h. About 150 mg $NaCNBH_3$ was then added, and the reaction mixture was continuously stirred at room temperature overnight. The solution was further extensively dialyzed vs. distilled water using a Spectrapor 6 dialysis tubing (Serva, molecular weight cut-off 1000 Da), and finally lyophilized. The aminated dextran derivative was characterized by ^1H NMR spectrometry using a Varian 400 MHz apparatus.

Preparation of catalase–dextran conjugate

Thirty milligrams of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to reaction mixtures containing 200 mg of activated dextran and 8 mg catalase, dissolved in 4 mL of 50 m*M* potassium phosphate buffer, pH 6.0. The solution was stirred for 1 h at room temperature and further on for 16 h at 4°C, and applied to a gel filtration column Fractogel EMD BioSEC (S) (2.6 × 60 cm^2), equilibrated in 20 m*M* sodium phosphate buffer, pH 7.0, containing 100 m*M* NaCl. The fractions containing the polymer–enzyme conjugate were pooled and kept at 4°C.

Assays

Catalase activity was determined spectrophotometrically at 240 nm and 37°C by the rate of decomposition of H_2O_2 in 20 m*M* sodium phosphate buffer, pH 7.0.[13] Protein concentration was estimated as described by Lowry et al.[14] using bovine serum albumin as standard. Total carbohydrates were determined by the phenol-sulfuric acid method[15] using glucose as standard.

Kinetics of thermal inactivation

Catalase preparations were incubated at 55°C in 20 m*M* sodium phosphate buffer, pH 7.0 (1 mg protein/mL). Aliquots were removed at scheduled times, chilled quickly, and assayed for enzymatic activity.

Kinetics of proteolytic degradation with trypsin

Catalase preparations were incubated at 37°C in 50 m*M* Tris-HCl buffer, pH 8.0 (1 mg protein/mL) in the presence of 20 μg trypsin (0.72 U). Aliquots were removed at scheduled times and assayed for enzymatic activity.

Pharmacokinetics studies

Native and dextran-modified catalase preparations were administered intravenously (50,000 U/kg) in the tail vein of female Wistar rats (250–300 g, six animals per group). Blood samples (500 μL) were collected at scheduled times with heparinized capillary syringes from the ocular plexus, centrifuged, and assayed for catalase activity. Endogenous catalase activity was previously evaluated in each animal before enzyme inoculation. The plasma level of all catalase forms as a function of time was analyzed according to a non compartmental pharmacokinetics models by using a linear trapezoidal calculation method.[11]

RESULTS AND DISCUSSION

Chemical modification of catalase with water-soluble polymers has been reported as an effective method for improving the pharmacokinetics behavior of this enzyme, as well as for increasing resistance toward proteolytic degradation.[16,17] In the present work, dextran was functionalized at its reducing end-group D-glucose residue with a reactive carboxylate group by treatment with a molar excess of ε-aminocaproic acid in the presence of sodium cyanoborohydride. The activated dextran was further attached to the free amino groups located at the protein surface of catalase by using a water-soluble carbodiimide as coupling agent. It could be expected that this modification should mainly affect the *N*-terminal amino groups of the enzyme, attending to the higher reactivity of these groups at the value of pH used in the conjugation reaction, compared with those in Lys residues. Similar results have been reported for other proteins chemically modified with aminated polymers under similar reaction conditions.[18] The conjugate prepared, retained 77% of the initial catalase activity and contained an average of 4 mol of dextran per mol of tetrameric protein. Treatment of the modified enzyme with 6*M* urea solution and further analysis by gel filtration chromatography on Fractogel EMD BioSEC (S) (2.6 × 60 cm^2) revealed that an average of 1 mol of dextran was attached to each mol of monomeric protein subunit.

The site-specific glycosidation of catalase with dextran yields several positive changes on the functional stability and the pharmacokinetics properties of the enzyme. Figure 1 shows the kinetics of thermal inactivation of native and dextran-modified catalase at 55°C and pH 7.0. During 1 h of incubation, both enzyme preparations lost activity progressively with time, but the half-life of the transformed enzyme was slightly increased (from 60 min to 1.5 h) in comparison with the native counterpart. This improved thermostability could be associated with the formation of new stabilizing hydrogen bonds between the hydroxyl groups from the polymer and the hydrophilic residues of catalase at the protein surface of the enzyme. In addition, the attached polysaccharide residues could thermostabilize this conjugate by

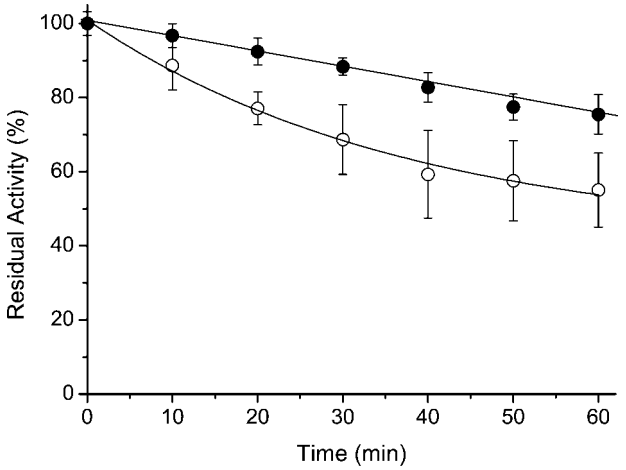

Figure 1 Kinetics of thermal inactivation of native (○) and dextran-modified catalase (●) at 55°C.

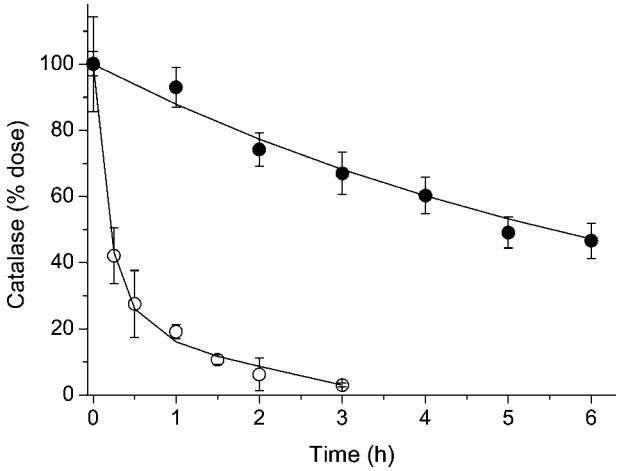

Figure 3 Pharmacokinetics profiles for native (○) and dextran-modified catalase (●) preparations after i.v. administration in rats (50,000 U/kg).

masking the hydrophobic clusters located at the surface of catalase, preventing both their unfavourable interaction with the surrounding water molecules and the occurrence of protein aggregation phenomenon at high temperatures.[19]

Figure 2 depicts the time-course of proteolytic inactivation of native and modified catalase preparations by incubation with bovine pancreatic trypsin at pH 8.0 and 37°C. As can be seen, both enzyme preparations showed the same inactivation pattern after treatment with the endoprotease, but the modification with the polymer prevents the proteolytic inactivation of the enzyme. In this sense, the half-life times for native and dextran-modified catalase were 40 min and 2.0 h after incubation with trypsin, respectively. This stabilizing effect could be explained by the steric hindrance provoked by the attached carbohydrate residues, which could markedly decrease the proteolytic attack of trypsin over the potential cleavage zones on the catalase surface.

Although modification of the redox enzyme with monoactivated dextran derivative yield improved stability against proteolytic attack and thermal treatment, this stabilizing effect could be higher by crosslinking the enzyme with a polyactivated dextran derivative.[20] Through this approach, the intra and intersubunit crosslinking could provide a more compact and stable protein structure. However, the approach here described for preparing a dextran–catalase conjugate is better from a pharmacological point of view.

Figure 3 reports the pharmacokinetics behavior of native and dextran-modified enzyme after intravenous administration in rats. All catalase preparations showed a loss of activity in plasma with time, and the data were fitted to a noncompartimental pharmacokinetics model. The results of these analyses are reported in Table I. The initial catalase concentration

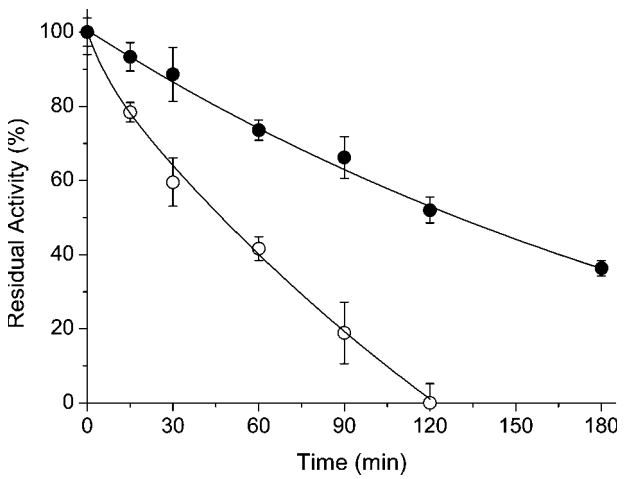

Figure 2 Kinetics of tryptic degradation of native (○) and dextran-modified catalase (●) at pH 8.0.

TABLE I
Pharmacokinetics Parameters of Native and Dextran-Modified Catalase Preparations After Intravenous Injection in Rats (50,000 U/kg)[a]

Parameter	Catalase	Catalase-dextran
Initial concentration in the plasma (U/mL)	3.3	23.4
$t_{1/2}$ (h)	0.7	5.1
AUC (U h/mL)	1.9	197
CL_{total} (mL/min)	110	1.1

$t_{1/2}$: half-life time; AUC: area under the plasma concentration curve; CL_{total}: total body clearance.

[a] Experimental details are given in the text. Pharmacokinetics parameters were calculated considering the initial concentration of the enzyme preparations in the plasma as 100%. The data represented are the means from six animals for sample, with standard error less than 10%.

in the plasma after i.v. administration was higher for the modified enzyme form. In addition, the native oxidoreductase showed a rapid clearance from the plasma with a half-life time of about 40 min. On the other hand, the dextran-modified enzyme preparation was retained in the blood circulation, and its half-life time was 7.3-fold higher than the corresponding native enzyme. The AUC was also increased (104-fold higher) and the total body clearance was noticeably reduced (100-fold) for the glycosidated enzyme.

The improved pharmacokinetics behavior showed by the dextran–catalase conjugate could be explained by the increased hydrodynamic radius of the adduct prepared by linking the polymeric chains of dextran to the enzyme, then reducing its glomerular filtration in the kidney.[21] In addition, the increased stability against proteases showed by the conjugate synthesized could also contribute to its higher half-life time and lower clearance, in comparison with the native counterpart. In fact, proteolysis is one of the main pathways of protein removal from the blood.[2]

From a practical point of view, this improvement in the pharmacokinetics behavior of catalase after site-specific glycosidation with aminated dextran, constitute a very interesting result that allows to evaluate these kind of enzyme–polymer adducts for biomedical applications.

CONCLUSIONS

In the present article, we described the synthesis of an end-group aminated polysaccharide derivative, and its further attachment to the protein surface of catalase. Through this site-specific glycosidation strategy we prepared a polymer–enzyme adduct with improved pharmacokinetics and stability properties. According to the results here reported, we suggest this modification approach as a successful method for preparing enzyme–dextran derivatives with prolonged life spar in blood circulation.

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