



## Transglutaminase-catalyzed site-specific glycosidation of catalase with aminated dextran

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### Abstract

An enzymatic approach, based on a transglutaminase-catalyzed coupling reaction, was investigated to modify bovine liver catalase with an end-group aminated dextran derivative. We demonstrated that catalase activity increased after enzymatic glycosidation and that the conjugate was 3.8-fold more stable to thermal inactivation at 55 °C and 2-fold more resistant to proteolytic degradation by trypsin. Moreover, the transglutaminase-mediated modification also improved the pharmacokinetics behavior of catalase, increasing 2.5-fold its plasma half-life time and reducing 3-fold the total clearance after its i.v. administration in rats.

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### 1. Introduction

During last decades, several proteins obtained from natural sources or through recombinant techniques have been evaluated as potential therapeutic drugs in different diseases. However, rapid clearance after in vivo administration, which is mediated by different mechanisms such as the recognition by the

immune system, proteolytic degradation, removal from the bloodstream by the liver and glomerular filtration through the kidney, currently limits the clinical application of the majority of the protein drugs (Caliceti and Veronese, 2003).

Chemical modification with biocompatible polymers constitutes a well-probed method for improving the pharmacokinetics properties of these bioactive polypeptides (Veronese and Morpurgo, 1999). In this respect, circulatory half-life of proteins has been increased by conjugation with natural and synthetic polymers such as poly(ethylene glycol)

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(PEG) (Veronese and Morpurgo, 1999; Kartre, 1993; Veronese, 2001; Fuertges and Abuchowski, 1990), dextran (Fujita et al., 1992), polyvinyl pyrrolidone (Caliceti et al., 1995), poly(styrene-co-maleic anhydride) (SMA) (Maeda, 2001), poly(divinyl ether-co-maleic anhydride) (DIVEMA) (Caliceti et al., 1996; Hirano et al., 1997), and *N*-(2-hydroxypropyl)methacrylamide (Ulbrich et al., 2000). Among these, PEG has been the polymer most extensively used due to the possibility to synthesize end-group activated PEG derivatives, able to give rise to polymer–protein conjugates with defined composition through site-specific chemical reactions (Caliceti and Veronese, 2003; Veronese and Morpurgo, 1999; Kartre, 1993; Veronese, 2001; Fuertges and Abuchowski, 1990; Roberts et al., 2002). However, PEG is a non biodegradable macromolecule slowly cleared from the human body and in addition the occurrence of anaphylactic reactions and severe bronchospasm have been reported after treatment with PEG-modified enzymes (Ho et al., 1986). On the other hand, chemical treatments often reduce the therapeutic effectiveness of the transformed proteins (Veronese, 2001), and the toxicity of the reagents commonly employed to synthesize polymer–protein conjugates forces to carry out arduous purification schemes to obtain adducts for biomedical applications. Therefore, the synthesis of new end-group functionalized derivatives of biodegradable macromolecules, as well as the development of alternative coupling procedures for preparing polymer–protein prodrugs, are receiving an increased attention in pharmacological sciences.

Recently we reported an enzymatic approach for synthesizing several trypsin–cyclodextrin complexes with improved catalytic and stability properties by using the enzyme transglutaminase (TGase, EC 2.3.2.13) (Villalonga et al., 2003a,b). TGase produces either intra- or intermolecular isopeptide bonds between the  $\gamma$ -carboxamide group of endoprotein glutamine residues acting as acyl donor and the  $\epsilon$ -amino group of endoprotein lysine residues which acts as acyl acceptor (Aeschlimann and Paulsson, 1994). Reactive lysines may be also substituted by several low molecular weight compounds containing primary amino groups, giving rise to a variety of protein-( $\gamma$ -glutamyl) derivatives (Folk and Chung, 1985). Thus, TGase has been employed to modify the biological activities of some peptides (Esposito et al., 1999;

Mancuso et al., 2001) and proteins (Porta et al., 1990; Mancuso et al., 1996; Tufano et al., 1996) by covalently linking polyamines to their reactive endo-glutamine residues.

In the present paper we describe a novel procedure for preparing a dextran–catalase conjugate by using TGase. In this sense, the synthesis of an end-group activated dextran derivative and its site-specific enzymatic attachment to the reactive glutamine residues of catalase (EC 1.11.1.6) is reported. Dextran is a natural and biodegradable polysaccharide widely employed in biomedical fields (Mehvar, 2000), including the cross-linking of bioactive proteins (Fujita et al., 1992; Marshall and Humphreys, 1979). On the other hand, catalase is an antioxidant enzyme with potential application in the therapy of several diseases mediated by reactive oxygen species (Shaked and Wolfe, 1988).

## 2. Materials and methods

### 2.1. Materials

Bovine liver catalase was purchased from Roche Molecular Biochemistry (Mannheim, Germany). Dextran 5000 was obtained from Serva (Heidelberg, Germany). TGase (8 U/mg based on the hydroxamate activity assay (Folk and Chung, 1985) from *Streptovorticillium* sp. was obtained from Ajinomoto Co. (Japan). Bovine pancreatic trypsin and Fractogel EMD BioSEC (S) were obtained from Merck (Darmstadt, Germany). All other chemicals were analytical grade.

### 2.2. TGase acyl donor substrate activity of catalase

The occurrence of reactive acyl donor glutamine residues in the catalase protein structure was assayed by a fluorometric method (Folk and Chung, 1985) based on the incorporation of monodansylcadaverin (MDC) as acyl acceptor. The composition of the reaction mixtures is described in the legend of Fig. 1. The separation of MDC from the reaction products and the visualization of the latter were achieved by SDS/PAGE (Laemmli, 1970) and ultraviolet illumination on a Fluor-S Multimager apparatus (BioRad, Richmond, USA).

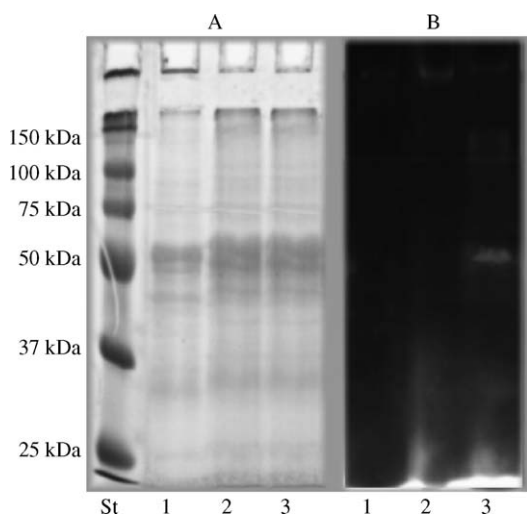


Fig. 1. TGase-dependent incorporation of MDC into catalase. Catalase (400  $\mu$ g) was incubated for 2 h at 37 °C in 70  $\mu$ l of 100 mM sodium phosphate buffer, pH 7.0, with TGase (40  $\mu$ g) in the presence (lanes 2 and 3) or absence (lanes 1) of 20 mM MDC control (lane 2) was boiled at 100 °C for 10 min after TGase addition. The mixtures were incubated at room temperature for 1 h. At the end of incubation the reactions were stopped by addition of 900  $\mu$ l of a solution of 9 M urea, 2% (m/v) SDS and 40 mM dithiothreitol in 50 mM Tris–HCl buffer, pH 7.1, and aliquots (20  $\mu$ l) of the samples analyzed by 12% SDS/PAGE. The gel was visualized by Coomassie staining (A) and ultraviolet illumination (B). St = Molecular weight standards:  $\beta$ -galactosidase from *E. coli* (150 kDa), phosphorylase B from rabbit muscle (100 kDa), bovine serum albumin (75 kDa), hen egg white ovalbumin (50 kDa), bovine carbonic anhydrase (37 kDa) and soybean trypsin inhibitor (25 kDa).

### 2.3. Synthesis of end-group aminated dextran

Dextran (2 g), dissolved in 10 ml distilled water, was treated with 1 ml hexylenediamine and stirred during 2 h. About 150 mg NaCNBH<sub>3</sub> was then added, and the reaction mixture was continuously stirred at room temperature overnight. The solution was then extensively dialyzed versus 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 <sup>1</sup>H NMR spectrometry using a Bruker AVANTE 500 MHz apparatus.

### 2.4. TGase-catalyzed synthesis of catalase–dextran conjugate

TGase (40  $\mu$ g) was added to 4 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 200 mg of dextran

and 8 mg catalase. The reaction mixture was stirred at 37 °C for 2 h and then applied to a gel filtration column Fractogel EMD BioSEC (S) (2.6 cm  $\times$  60 cm) equilibrated in 20 mM sodium phosphate buffer, pH 7.0, containing 100 mM NaCl. The fractions containing the polymer–enzyme conjugate were pooled and kept at 4 °C. The molecular weight of this conjugate was determined by analytical gel permeation chromatography (GPC) on TSK G3000 column (7.5 cm  $\times$  60 cm), calibrated with protein standards from the MW-GF-1000 kit (Sigma, USA).

### 2.5. Assays

Catalase activity was determined spectrophotometrically at 240 nm and 37 °C by the rate of decomposition of H<sub>2</sub>O<sub>2</sub> in 20 mM sodium phosphate buffer, pH 7.0 (Chance and Maehley, 1955). Protein concentration was estimated as described by Lowry et al. (1951) using bovine serum albumin as standard. Total carbohydrates were determined by the phenol-sulfuric acid method (Dubois et al., 1956) using glucose as standard.

### 2.6. Kinetics of thermal inactivation of proteolysis by trypsin

Native and dextran-modified catalase preparations were incubated at 55 °C in 20 mM sodium phosphate buffer, pH 7.0 (1 mg protein/ml). Aliquots were removed at scheduled times, chilled quickly, and assayed for enzymatic activity.

Different catalase preparations were incubated at 37 °C in 50 mM Tris–HCl buffer, pH 8.0 (1 mg protein/ml) in the presence of 20  $\mu$ g trypsin (0.72 U). Aliquots were removed at scheduled times and assayed for enzymatic activity.

### 2.7. Pharmacokinetic 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 each group). Blood samples (500  $\mu$ l) 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 enzyme

forms as a function of time was analyzed by a two-compartment model, fitting the experimental data to the equation  $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$  (Fujita et al., 1990), and assuming as 100% the initial enzymatic activity determined in the blood plasma after intravenous administration. The pharmacokinetic parameters  $A$ ,  $B$ ,  $\alpha$  and  $\beta$  were calculated by using a non-linear regression procedure based on the Marquardt-Levenberg method of iterative convergence included into the Microcal Origin 7.0 software (Microcal Software, Inc., MA, USA). The other parameters were calculated as follow: half-life in  $\alpha$  phase,  $t_{1/2}(\alpha) = (\ln 2)/\alpha$ ; half-life in  $\beta$  phase,  $t_{1/2}(\beta) = (\ln 2)/\beta$ ; area under the plasma concentration curve,  $AUC = A/\alpha + B/\beta$ ; total body clearance,  $CL_{total} = \text{dose}/AUC$ .

### 2.8. Statistical methods

Microcal Origin 7.0 software (Microcal Software, Inc., MA, USA) was used for all statistical analyses. The data were analyzed by ANOVA, and means were compared using Student's  $t$ -test. Differences were considered to be significant at  $p < 0.05$ .

## 3. Results

With the aim of evaluating the ability of bovine liver catalase to act as acyl donor substrate of microbial TGase, the incorporation of an aminated fluorescent marker (MDC) into its polypeptide chain was first investigated. Lane 3 of the Panel B of Fig. 1 shows the presence of a fluorescent band with the same electrophoretic mobility of catalase, indicating that the enzyme is able to act as a TGase amino-acceptor substrate.

The strategy employed in this work for end-group functionalization of dextran involves its treatment with a molar excess of hexylenediamine in the presence of sodium cyanoborohydride, in order to reduce only the new imine bonds formed at the reducing end of the polymer. This procedure has been previously used for preparing end-group activated pullulan derivatives (Bruneel and Schacht, 1995). The aminated dextran gave satisfactory  $^1\text{H}$  NMR spectra.

The activated dextran was further attached to the glutamine residues located at the protein surface of bovine liver catalase, under very mild conditions,

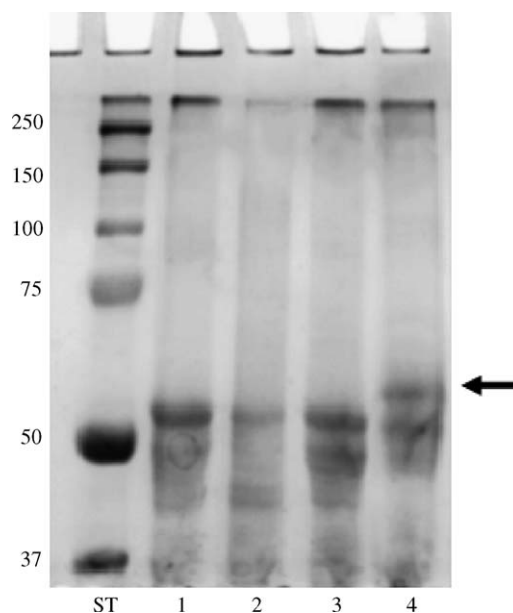


Fig. 2. TGase-mediated modification of catalase with end-group activated dextran analyzed by 12% SDS/PAGE. Lanes: (1) catalase, (2) catalase + TGase, (3) catalase + aminated dextran + TGase thermally inactivated, (4) catalase + aminated dextran + TGase, (St) molecular weight standards (as in Fig. 1). The reaction conditions are reported in Section 2.

through the formation of amide bonds using the enzyme TGase as coupling agent. The synthesis of the aminated dextran–catalase conjugate was demonstrated by SDS-PAGE, as shown by the occurrence in the lane 4 of Fig. 2, indicated by the arrow, of a protein band with a higher molecular mass than of unmodified catalase. In addition, no intermolecular cross-links between catalase molecules were detected after catalase treatment with TGase in the absence of the polymer (Fig. 2, lane 2). The aminated dextran–catalase adduct, was eluted as a single peak, containing carbohydrates and catalytic activity, from a gel filtration on Fractogel EMD BioSec (S) (data not shown).

Table 1 reports some structural and catalytic properties of native and TGase-modified catalase. The carbohydrate content of the conjugated catalase, determined by the colorimetric quantification of the attached D-glucose units, represented about 8.0% by weight of the dextran-modified enzyme form. According to the average molecular weight of the polysaccharide, this result represented an average of 4 mol of dextran per mol of protein, corresponding to 1 mol of polymer attached to

Table 1  
Structural and catalytic properties of native and modified catalase

Enzyme	Polymer content (mol/mol protein)	Molecular weight (kDa)	Specific activity (U/g)
Catalase	–	238	14.6
Aminated dextran–catalase	4	260	15.5

each protein subunit. This result is in agreement with the molecular weight determined for this catalase neo-glycoconjugate by GPC. Moreover, the specific activity of catalase was found slightly increased after its enzymatic glycosidation with aminated dextran.

The site-specific glycosidation of catalase with the end-group aminated dextran derivative yields also to several positive changes in the functional stability and pharmacokinetics properties of the enzyme. Fig. 3 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 native and modified enzyme lost activity progressively with time according to a first-order kinetics, whereas the half-life of the transformed enzyme was increased from 50 min to 3.2 h in comparison with the native counterpart.

Fig. 4 depicts the time-course of proteolytic inactivation of native and modified catalase by trypsin at pH 8.0 and 30 °C. Although both enzyme preparations showed a similar inactivation pattern after treatment with the endoprotease, the aminated dextran–catalase adduct seems to be more resistant to proteolytic inactivation. In this respect,  $t_{1/2}$  of the glycosidated catalase was calculated to be two-fold higher than that of native enzyme.

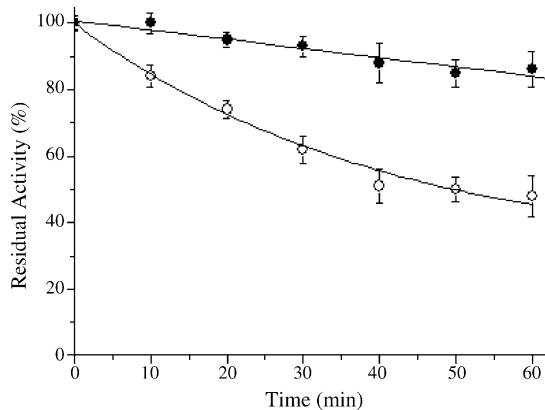


Fig. 3. Kinetics of thermal inactivation of native (○) and modified catalase (●) at 55 °C. Experimental details are given in the text.

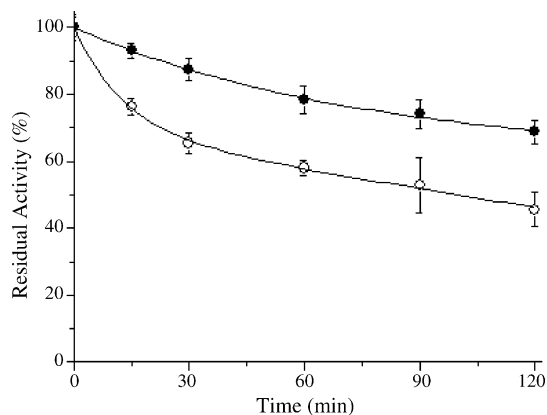


Fig. 4. Kinetics of tryptic degradation of native (○) and modified catalase (●) at pH 8.0. Experimental details are given in the text.

Fig. 5 reports the pharmacokinetics behavior of native and dextran-conjugated catalase after intravenous administration in rats. A biphasic elimination pattern from plasma was observed for both catalase preparations. Thus, the pharmacokinetics parameters were calculated assuming a bicompartamental pharmacokinetics model (Table 2). Although the

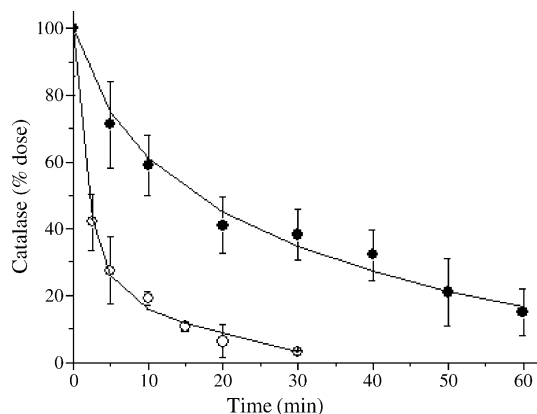


Fig. 5. Pharmacokinetics profiles of native (○) and modified catalase (●) after their i.v. administration in rats (50 000 U/kg). Experimental details are given in the text.

Table 2  
Pharmacokinetics parameters of native and modified catalase after intravenous injection in rats (50 000 U/kg)<sup>a</sup>

Parameter	Catalase	Aminated dextran–catalase
Initial concentration in the plasma (U/ml)	3.3	1.9
A	0.97	1.4
B	2.4	0.51
$\alpha$	0.60	0.24
$\beta$	5.4	1.9
$t_{1/2}(\alpha)$ (h)	1.2	3.0
$t_{1/2}(\beta)$ (h)	0.13	0.4
AUC (U h/ml)	2.1	6.1
CL <sub>total</sub> (ml/min)	110	36

<sup>a</sup> Experimental details are given in the text. The data are the means from six independent experiments, with standard error less than 10%.

initial catalase concentration in the plasma after i.v. administration was higher for the native preparation, the unmodified enzyme showed a rapid clearance from the plasma with a half-life time of about 1.2 h for the  $\alpha$  phase. By contrast, the dextran-conjugate catalase disappeared more slowly from the circulation and their  $t_{1/2}(\alpha)$  (3 h) and AUC (6.1 U h/ml) were significantly higher than those of the native enzyme. Finally, the total body clearance of the aminated dextran–catalase adduct was also reduced showing a value about three times lower than that of the native enzyme.

#### 4. Discussion

Previously the beneficial effect has been reported of chemical modification of catalase with some biocompatible polymers on its pharmacokinetics behavior and stability properties against proteolytic degradation (Marshall and Humphreys, 1979; Abuchowski et al., 1977; Maksimenko and Tischenko, 1997). The obtained improved characteristics favored the potential therapeutic action of polymer-modified catalase preparations toward many injuries mediated by hydrogen peroxide. Among these, the beneficial effects of the PEG–catalase complex in a rat model of lung injury due to asbestosis have been clearly demonstrated (Mossman et al., 1990). Treatment with PEG–catalase conjugates also reduced both proteinuria in rats with nephrotic syndrome (Beaman et al., 1987) and edema in the optic disc of guinea pigs with acute experimental allergic optic neuritis (Guy et al., 1994). Further poly-

mers, such as chondroitin sulfate (Maksimenko and Tischenko, 1997) and polyaldehyde dextran (Marshall and Humphreys, 1979), have been used as chemical modifiers for this enzyme.

In the present work we evaluated the possibility to employ an enzymatic approach for synthesizing a catalase preparation through the site-directed incorporation of an activated dextran derivative into the polypeptide structure of this enzyme via a TGase-catalyzed reaction. In this regard, the ability of catalase to act as acyl donor substrate of TGase was demonstrated by testing the enzymatic-mediated reactivity of its glutamine residues towards MDC. Thus, an end-group aminated dextran derivative was attached to catalase by using its characteristics to act as acyl acceptor TGase substrate. Therefore, an aminated dextran–catalase conjugate, with high purity and well-defined polymer content and molecular weight, was obtained.

In contrast to other polymer–catalase conjugates previously synthesized by chemical methods (Marshall and Humphreys, 1979; Maksimenko and Tischenko, 1997), the adduct prepared by TGase-mediated reaction showed an increased catalase activity in comparison with the native enzyme. A similar increase in the enzymatic activity has been previously described for cyclodextrin–trypsin conjugates obtained by a TGase-catalyzed reaction (Villalonga et al., 2003a,b).

The enzymatic crosslink of catalase with aminated dextran increased its resistance against thermal inactivation. The improved thermostability of the enzyme could be associated with the formation of new stabilizing hydrogen bonds between the hydroxyl groups of the polymer and the hydrophilic residues occurring on the protein surface of the enzyme. In addition, the TGase-catalysed attachment of dextran residues could also thermostabilize the conjugate by masking the hydrophobic clusters located at the surface of catalase, thus preventing both their unfavourable interaction with the surrounding water molecules and the occurrence of protein aggregation phenomena at high temperatures (Klibanov, 1983).

Furthermore, catalase was found to become more resistant to tryptic degradation after the described TGase-mediated modification. This stabilizing effect could be explained as a consequence of the steric hindrance determined by the attached carbohydrate residues, which could markedly protect the protein from the trypsin attack (Villalonga et al., 2003a,b).

The pharmacokinetics performance of catalase was also improved after TGase-catalyzed glycosidation with aminated dextran. The increased hydrodynamic radius of the adduct could be responsible for a reduced glomerular filtration of the enzyme in the kidney and, thus, for the observed increase of its  $t_{1/2}$  and AUC (Lote, 2000). Moreover, the increased stability of the aminated dextran–catalase conjugate against proteases could also contribute to its higher half-life time and lower clearance in comparison with the native counterpart (Veronese and Morpurgo, 1999). However, it should be noted that the observed prolongation of the serum half-life period was lower than that detected with the catalase adduct prepared by chemical conjugation with PEG residues of similar molecular weight (Abuchowski et al., 1977). This result could be a consequence of the low amount of dextran molecules covalently bound to each mol of protein, due to the limited amount of reactive glutamine residues occurring into the catalase polypeptide chain. By contrast, dextran biodegradability and lower toxicity, the higher catalytic activity showed by the prepared conjugate, as well as the benefits derived from the use of an enzyme catalyzed coupling reaction, constitute important advantages of the catalase glycoconjugate reported in present paper.

## 5. Conclusions

In the present paper, we described for the first time the use of TGase to covalently link end-group aminated polysaccharide moieties to enzymes. We report the enzymatic attachment of aminated dextran to catalase, and the consequent improvement of the pharmacokinetics and stability properties of this enzyme after the described glycosidation. The results here reported suggest that the TGase-catalyzed incorporation of end-group aminated dextran to reactive endo-protein glutamine residues might be an useful tool for preparing polysaccharide-protein prodrugs with potential pharmacological applications.

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