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Design of the Complex between Manganese Porphyrins and Catalase−Poly(ethylene glycol) Conjugates for a New Antioxidant

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Abstract: A new design of antioxidant, the complex between manganese (Mn) porphyrins and catalase−poly(ethylene glycol) (PEG) conjugates, is reported. Gel filtration chromatography and a Langmuir-type adsorption isotherm proved that the catalase−PEG conjugate formed the complex with the Mn-porphyrin. The resulting complex exhibited significant superoxide dismutase (SOD) and catalase activity. These results suggest that the Mn-porphyrin/catalase−PEG complex with dual enzymatic activity, i.e., SOD and catalase, is promising for a new class of antioxidants.

Keywords: Water-soluble manganese porphyrin; superoxide dismutase activity; catalase−poly(ethylene glycol) conjugate; complex formation; passive targeting

Here we have reported a new design of the complex between manganese (Mn) porphyrins and catalase−poly(ethylene glycol) (PEG) conjugates for a new antioxidant. Cells are protected against ROS by antioxidant enzymes such as Mn and Cu−Zn superoxide dismutase (Mn-SOD and Cu,Zn-SOD), catalase, and glutathione peroxidase.[1] In these antioxidant enzymes, SOD catalyzing the reduction from $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) is the key enzyme for the protection of oxidative stress. Fridovich and co-workers have reported that manganese porphyrins (Mn-porphyrins) are promising compounds as SOD mimics with chemical versatility.[2] Subsequently, we have synthesized a macromolecular Mn-porphyrin for enhancing half-life in blood circulation.[3] Moreover, we have modified a Mn-porphyrin with carbohydrate ligands for active targeting[4] or the signal peptide for mitochondrion targeting.[5] However, the resulting compounds are unable to eliminate the still reactive product of SOD, i.e., H_2O_2. Since H_2O_2 is considered a mediator of apoptotic cell death,[6] the elimination of the H_2O_2 is important for complete protection of oxidative stress. If a macromolecular antioxidant had dual antioxidative activity of SOD and catalase, which catalyzes the reduction from H_2O_2 to nontoxic water (H_2O), the radical approach[7] to therapy would be promising. In this study, we have designed the complex between Mn-porphyrin and catalase−PEG for a new macromolecular antioxidant with dual functions of SOD and catalase (Figure 1). This paper describes the design and characterization of the Mn-porphyrin/catalase−PEG complex in the context of its complex formation and dual catalytic activity; detailed studies on the mechanism of the complex formation and the continuous reduction from $O_2^{\bullet-}$ to H_2O are outside the scope of the present study.

To form the Mn-porphyrin/catalase−PEG complex, we first modified catalase with PEG.[8] The modification of PEG blocks renal clearance and increases the circulating enzyme half-life.[9] The biocompatible polymer PEG also reduces the antigenicity of the native protein and inhibits the hydrolysis

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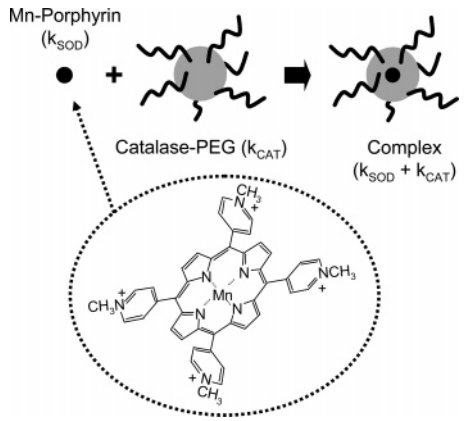

Figure 1. Complex formation between Mn-porphyrin and catalase−PEG.

of protease-sensitive proteins such as catalase.[10] Furthermore, the long-term stability of enzymes in aqueous solution is frequently increased by PEG conjugation.[11] The amino groups of catalase were therefore reacted with methoxy-PEG-succinimidyl propionate. Figure 2 shows the profile of gel filtration chromatography (GFC) of the isolated catalase−PEG conjugate. The GFC profile indicated that unreacted PEG macromolecules were removed by the purification process. The conjugation of catalase with PEG-chains was proved by the earlier shift of the elution volume (Figure 2) and the mass spectra of PEG fragment ions (m/z = 4600−6400) (results not shown). The spectrophotometric amino group determination with trinitrobenzenesulfonic acid[12] estimated that the modification degree of approximately 100 amino groups[13] on catalase was 10 mol %. Namely, approximately ten PEG-chains were bound to catalase.

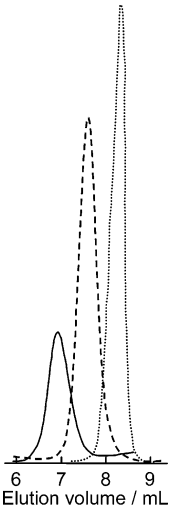

Figure 2. Gel filtration chromatograms of the isolated catalase−PEG conjugate (solid line), the unreacted catalase (dashed line), and the unreacted PEG (dotted line): column, Shodex OHpak SB-804 HQ; eluent, 0.1 M $NaNO_3$; flow rate, 1.0 mL/min; detection, refractive index.

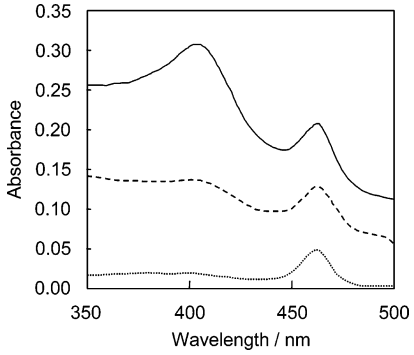

Figure 3. UV−visible spectra of the eluate from the column of GFC: early-stage eluate (solid line), middle-stage eluate (dashed line), and late-stage eluate (dotted line). The mixture of Mn-porphyrin and catalase−PEG was loaded into the column.

Then, the resulting catalase−PEG conjugate was incubated with the Mn-porphyrin 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphinatomanganese in phosphate-buffered saline (PBS, pH 7.4).[14] The mixture of Mn-porphyrin/catalase−PEG was loaded into a GFC column to examine the complex formation between Mn-porphyrin and catalase−PEG. Figure 3 shows the UV−visible spectra of the eluate from the GFC column using PBS as a mobile phase. The absorption spectrum of early-stage eluate had two peaks around 405

(8) A typical procedure is as follows: Catalase (60 mg; EC 1.11.1.6; from bovine liver; Sigma Chemical Co.) was dissolved in 8 mL of sodium borate buffer (50 mM, pH 9.1). Subsequently, methoxy-PEG-succinimidyl propionate (molecular weight = 5000; 2 mg/mL; Nectar Products) was added in the catalase solution. After 1 day incubation at 4 °C, the reaction mixture was loaded into a GFC column (TOYOPEAL HW-55F, Tosoh Co.) to remove unreacted PEG derivatives. During GFC, a phosphate buffer (50 mM, pH 7.4) as used as a mobile phase.

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(14) A typical procedure is as follows: The catalase−PEG conjugate (1 μM) and the Mn-porphyrin (0.6 mM) were incubated in PBS (pH 7.4) for 1 day at 4 °C. The resulting mixture was loaded into a GFC column (TOYOPEAL HW-55F, Tosoh Co.) to remove unbound Mn-porphyrin. During GFC, PBS (pH 7.4) as used as a mobile phase.

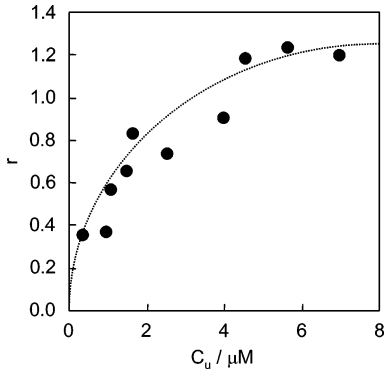

Figure 4. Analysis of the interaction between Mn-porphyrin and catalase−PEG: the *y*-axis is the number of Mn-porphyrins bound to one catalase−PEG molecule (*r*); the *x*-axis is the unbound Mn-porphyrin concentration (C_u).

and 462 nm; on the other hand, that of late-stage had one peak around 462 nm. The UV−visible spectrum of catalase exhibited an absorption with a peak position at 405 nm,[15] and the Mn-porphyrin had a Soret band at 462 nm.[2] These results suggest that the complex of Mn-porphyrin/catalase−PEG and unbound Mn-porphyrin existed in the early-stage and late-stage eluate, respectively.

To examine further the complex formation between Mn-porphyrin and catalase−PEG, we studied the Langmuir model for the sorption isotherm.[16] Figure 4 shows the plot of the number of Mn-porphyrins bound to one catalase−PEG molecule (r) against the unbound Mn-porphyrin concentration (C_u).[17] The r value reached a plateau, which was approximately 1.2. The result of the Langmuir-type adsorption isotherm of the Mn-porphyrin/catalase−PEG complex suggests that one Mn-porphyrin molecule was, on average, bound to one catalase−PEG macromolecule. The formation of the Mn-porphyrin/catalase−PEG complex was presumably mediated by the electrostatic interaction between cationic pyridyl groups of the Mn-porphyrin and acidic amino acid residues of the catalase−PEG as well as the hydrophobic interaction between a hydrophobic backbone of the porphyrin and hydrophobic amino acid residues of the catalase. The complex formation was stable in PBS, so that the construct is available as a drug used under physiological ionic strength without weakening electrostatic interaction.

Finally, we examined whether the resulting Mn-porphyrin/catalase−PEG complex worked as an antioxidant. To examine the enzyme activity of the complex, we first detected and quantified the SOD activity by the stopped-flow kinetic analysis, as is reported in our previous papers.[18] The k_{SOD} value for the Mn-porphyrin alone was 9.5×10^6 M^{-1} s^{-1}. On the other hand, that for the Mn-porphyrin/catalase−PEG complex was 9.2×10^6 M^{-1} s^{-1}. Under these experimental conditions, native catalase alone showed no SOD activity. Furthermore, we examined the catalase activity of the resulting complex. The dismutation of H_2O_2 is a first-order reaction, followed by the decrease in absorbance at 240 nm. The difference in absorbance (ΔA_{240}) per unit time is a measure of the catalase activity.[19] The k_{CAT} values for the resulting complex were 1.5×10^7 M^{-1} s^{-1}, which was not significantly different from that for native catalase. The results of the measurement of the enzyme activities are summarized in Table 1. It can be said that the Mn-porphyrin/catalase−PEG complex showed both significant SOD (k_{SOD}) and catalase (k_{CAT}) activity despite the complex formation between Mn-porphyrin and catalase−PEG.

Table 1. SOD and Catalase Activity of the Catalase−PEG/Mn-Porphyrin Complex

compound	k_{SOD} [10^6 M^{-1} s^{-1}]	k_{CAT} [10^7 M^{-1} s^{-1}]
Mn-porphyrin	9.5	
catalase		1.5
catalase−PEG/Mn-porphyrin[a]	9.2	1.5

[a] The decay of $O_2^{\bullet-}$ was spectrophotometrically monitored at 245 nm. The absorbance data (0.284−0.051; 0−20 ms) were processed to obtain an observed rate constant ($k_{SOD,obs}$) which was calculated from the slope of the plot of ln(absorbance at 245 nm) versus time. The k_{SOD} was determined from the slope of a plot of $k_{SOD,obs}$ versus the concentration (0−4 μM) of the Mn-porphyrin. Furthermore, the decay of H_2O_2 was spectrophotometrically monitored at 240 nm. The absorbance data (0.537−0.116; 0−30 s) were processed to obtain an observed rate constant ($k_{CAT,obs}$) which was calculated from the slope of the plot of ln(absorbance at 240 nm) versus time. The k_{CAT} was determined from the slope of $k_{CAT,obs}$ versus the concentration (0−7.5 nM) of the catalase−PEG.

In conclusion, we have designed the complex between Mn-porphyrin and catalase−PEG for a new class of antioxidant. The dual enzymatic activity of the resulting Mn-porphyrin/catalase−PEG complex suggests that $O_2^{\bullet-}$ could be continuously reduced to H_2O in the blood stream, which is the topic of our future investigation. The new antioxidant designed in this study is expected to be the first drug/carrier complex with dual enzymatic activity in the molecular pharmaceutics field.

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