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Preparation and Characterization of Cu,Zn-Superoxide Dismutase Covalently Modified by Polyunsaturated Fatty Acids

Z. J. Zhan, Z. G. Zhou, and W. G. Shan*

College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, P. R. China; fax: 86-571-8832-0913; E-mail: tianranyaowu@zjut.edu.cn

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Abstract—To improve its stability and lipophilicity, Cu,Zn-superoxide dismutase (SOD) was chemically modified with linoleic and α-linolenic acids using two different methods. Higher retained enzymatic activity has been observed compared with SOD modified by macromolecular substances. Enhanced heat stability, acid and alkali resistance, and anti-pepsin/trypsin ability of the modified SOD were observed compared with those of the natural enzyme, the apparent oil–water partition coefficient being especially increased. The results characterize SOD modified with polyunsaturated fatty acids as a promising pharmacological tool.

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Key words: Cu,Zn-superoxide dismutase, polyunsaturated fatty acids, chemical modification, stability, partition coefficient

It has long been recognized that high levels of excessive reactive oxygen species are associated with the development of cancer [1], various cardiovascular diseases [2], and several neurodegenerative diseases [3]. Superoxide dismutases (SOD) are essential enzymes eliminating superoxide radical and thus protecting cells from damage induced by free radicals; they have been proposed as ideal pharmaceuticals for preventing and treating these diseases [4-7]. Unfortunately, the practical application of SOD has been limited by its short plasma half life of less than 6 min *in vivo* [8], insufficient stability, and membrane impermeability. Consequently, numerous water-soluble macromolecular substances have been successfully employed to modify SOD and improve its pharmacological properties, such as polyethylene glycol [9], chondroitin sulfate [10], low molecular weight heparin [11], and mannan [12]. Nevertheless, there has been little research reported about lipophilic and micromolecular substances used to modify SOD.

Linoleic acid (LA) and α-linolenic acid (α-LA) are *n*-6 and *n*-3 series polyunsaturated fatty acid (PUFA), respectively. Clinical and epidemiological studies suggest that both *n*-6 and *n*-3 PUFA play major roles in aspects of regulating cardiovascular system [13, 14] and membrane lipids [15] and having anti-inflammatory [16, 17], anti-tumor [18, 19], and anti-diabetic actions [20]. The PUFAs possess high antioxidant activity, and thus they could reduce cellular damage and prevent oxidation-related pathologic events [21].

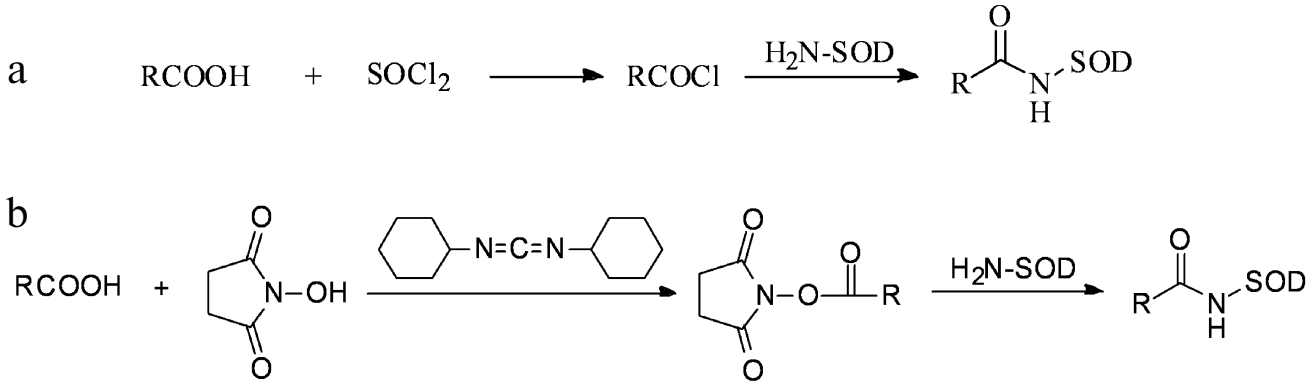
In this paper we report the chemical modification of SOD with LA and α-LA through two different synthetic procedures that improve heat stability, acid and alkali resistance, anti-protease stability, and increase apparent oil–water partition coefficient. We expect that the pharmacological properties of PUFA-SOD will be superior to that of native SOD, providing synergistic protection against the pathological effects of reactive oxygen species.

MATERIALS AND METHODS

Chemicals. Cu,Zn-SOD (Tieling Junkun Biological Engineering Ltd., China), LA (Shanghai Laser Fine Chemicals Factory, China), α-LA (Henan Yuanhua Biotechnology Co., Ltd, China), pyrogallol (Guizhou Zunyi Chemical Co., Ltd, China), 2,4,6-trinitrobenzenesulfonic acid (Sigma, USA), trypsin (Sinopharm Chemical Reagent Co., Ltd, China), pepsin (Shanghai Bio Life Science & Technology Co., Ltd, China), and BSA (Sino-American Biotechnology Co., China) were

Abbreviations: DCC, dicyclohexylcarbodiimide; LA, linoleic acid; PUFA, polyunsaturated fatty acid; SOD, superoxide dismutases.

* To whom correspondence should be addressed.



Synthesis of PUFA-SOD_{ACC} (a) and PUFA-SOD_{NHE} (b) conjugates

used in this study. All other chemicals and reagents were of analytical grade.

Synthesis of PUFA-SOD conjugates. PUFA-SOD_{ACC} (see Scheme (a)): 40 mg of bovine erythrocyte SOD were dissolved in 10 ml of 0.2 M sodium carbonate buffer (pH 9.0) and then 150 mg fatty acid previously activated with thionyl chloride [22] was added. At the same time, 0.2 M NaOH was added to keep the pH of the solution at 9.0. The mixture was agitated at 25°C for 1 h. After workup, it was supplemented with 30 ml of cooled acetone (–20°C) and centrifuged for 10 min at 8000 rpm. The precipitate was dissolved in distilled water, and the solution was centrifuged again, dialyzed against distilled water for 24 h, and freeze-dried.

PUFA-SOD_{NHE} (see Scheme (b)): 20 mg of SOD was dissolved in 10 ml of 0.1 M sodium phosphate buffer (pH 8.0). To this solution, 20 mg of the fatty acid activated with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide (DCC) [23] dissolved in 1 ml of dimethyl sulfoxide was added, and the reaction was carried out under stirring at 25°C for 24 h. After reaction, the mixture was purified by gel filtration on a Sephadex G-100 column equilibrated with 0.05 M sodium phosphate buffer (pH 7.5) and then purified as described for the PUFA-SOD_{ACC} conjugate (see procedure of acetone precipitation above).

Assays. SOD activity was assayed according to the improved pyrogallol autooxidation method [24]. One unit of SOD activity was defined as the amount of enzyme required to inhibit the autooxidation of pyrogallol by 50%. The percentage of protein in PUFA-SOD conjugates (protein content) was estimated as described by Lowry et al. [25], using BSA as standard. The modification degree of amino groups was determined by measuring the number of free amino groups in SOD before and after modification with PUFA by 2,4,6-trinitrobenzenesulfonic acid [26].

Heat treatment of PUFA-SOD conjugates and native SOD. The PUFA-SOD conjugates or native SOD were dissolved in a 0.2 M sodium phosphate buffer (pH 7.0) and then incubated at 75°C for 120 min. Aliquots were removed at scheduled times and then assayed for SOD activity.

Acid and alkali treatments of PUFA-SOD conjugates and native SOD. The PUFA-SOD conjugates or native SOD were dissolved in a 0.2 M Na_2HPO_4/0.1 M citric acid buffer (pH 5.2) or in 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 10.8) and then incubated at 25°C for 6 h. Aliquots were removed at 2-h intervals and then assayed for SOD activity.

Anti-pepsin and anti-trypsin abilities of PUFA-SOD conjugates and native SOD. The PUFA-SOD or native SOD were dissolved in 2 ml 0.1 M glycine-HCl buffer (pH 1.34) containing 6 mg pepsin or in 0.2 M Na_2HPO_4/NaH_2PO_4 solution buffer (pH 7.84) with 2 mg trypsin and then incubated at 25°C for 60 min. Aliquots were removed at 20-min intervals and then assayed for SOD activity.

Apparent oil–water partition coefficient determination. The apparent oil–water partition coefficient (*P*) of PUFA-SOD or native SOD was determined by the shake-flask method [27]. *P* is defined as the ratio of SOD concentrations in *n*-octanol and water phases. Each *P* value is an average of five replicate measurements.

RESULTS AND DISCUSSION

Synthesis of the PUFA-SOD conjugates. In the present research, thionyl chloride and N-hydroxysuccinimide in the presence of DCC were used to activate polyunsaturated fatty acids for coupling them to free amino groups at the protein surface of SOD (Scheme). Considering the first strategy (Scheme (a)), highly reactive chloride derivatives of fatty acids are instable and inconvenient to store. When it was used for coupling with SOD, hydrochloric acid was released quickly and caused the enzymatic activity of SOD to decrease. Conversely, the modification of

Table 1. Enzymatic activity, modification degree of NH_2 groups, and protein content of PUFA-SOD

Sample	Specific activity, U/mg	Modified NH_2 groups, %	Protein content, %*
Native SOD	5170	–	–
LA-SOD_{ACC}	4596	77	73
α-LA-SOD_{ACC}	4467	72	64
LA-SOD_{NHE}	5399	72	76
α-LA-SOD_{NHE}	5074	70	75

* Percentage of protein in PUFA-SOD conjugates.

Table 2. Effects of acidic (pH 5.2) and alkaline (pH 10.8) treatments at 25°C on the enzymatic activity of native and PUFA-modified SOD

Sample	Residual enzymatic activity, % (acidic/alkaline)		
	2 h	4 h	6 h
Native SOD	92/78	72/64	53/58
LA-SOD_{NHE}	94/91	89/90	78/73
α-LA-SOD_{NHE}	93/90	85/84	79/78

SOD with N-hydroxysuccinimide as activating reagent (Scheme (b)) was effective and less toxic, which is important in the application of SOD in medicine and food industry.

Enzymatic activity, modification degree of NH_2 groups, and protein content of PUFA-SOD. The enzymatic activity, modification rate of NH_2, and protein content of the PUFA-SOD are listed in Table 1. LA-SOD_{ACC} and α-LA-SOD_{ACC} retained 89 and 86% of the initial enzymatic activity, respectively. Interestingly, LA-SOD_{NHE} and α-LA-SOD_{NHE} retained 104 and 98% of its initial enzymatic activity, respectively. In this study, higher enzymatic activity was retained compared with SOD modified with macromolecular substances such as SOD-CMCH (57% residual activity) [28], LMW-SOD (64% residual activity) [29], and SOD-mannan (52% residual activity) [12]. The reason for higher retained enzymatic activity might be that PUFAs display synergistic anti-oxidation activity.

Stabilities of PUFA-SOD conjugates to heat, acid, alkali, and protease treatment. As the figure shows, native SOD was rapidly inactivated under heat (75°C) treatment, losing 51% of its activity after 2-h incubation. However, the LA-SOD and α-LA-SOD retained 77 and 76% activity after 2-h incubation under the same conditions, demonstrating that SOD modified with PUFA is more stable than native SOD towards heat treatment.

The stabilities under acidic or alkali condition of native SOD and PUFA-SOD are reported in Table 2. The data show that PUFA-SOD is more stable than native SOD towards acid and alkaline treatments.

As Table 3 shows, native SOD was rapidly inactivated on treatment with pepsin or trypsin, losing 78 and 45% of its enzymatic activity after 60 min, respectively. In contrast, PUFA-SOD retained more than 50 and 70% activity after 60 min under the same conditions, demonstrating an improvement in anti-pepsin/trypsin stabilities of SOD modified with PUFA.

The marked stability of PUFA-SOD to heat, acid, alkali, and protease treatment observed in the present investigation suggest that SOD modified with PUFA might be more stable *in vivo*, which would prolong the half-life and improve bioavailability. Possible reasons are that PUFA might decrease the conformational freedom of SOD. Furthermore, PUFA form a shielding layer on the enzyme surface, and hence hinder the contact of protease with SOD.

Apparent oil–water partition coefficient (*P*) of SOD and PUFA-SOD conjugates. The average values of log*P* ($n = 5$ and experimental error) are listed in Table 4, which shows that LA-SOD and α-LA-SOD have increased log*P*. The log*P* is an important parameter related to the

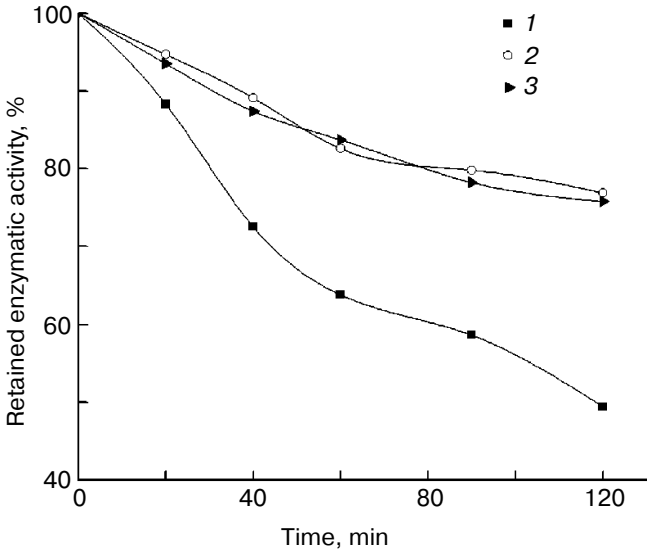
Table 3. Effects of pepsin (pH 1.34) and trypsin (pH 7.84) treatments at 25°C on enzymatic activity of native and PUFA-modified SOD

Sample	Residual enzymatic activity, % (pepsin/trypsin)		
	20 min	40 min	60 min
Native SOD	52/81	40/70	22/55
LA-SOD_{NHE}	80/86	69/78	54/74
α-LA-SOD_{NHE}	79/88	62/78	51/73

Table 4. Apparent oil–water partition coefficient

Sample	log*P* ± SD
Native SOD	−1.09 ± 0.04
LA-SOD_{NHE}	−0.91 ± 0.03
α-LA-SOD_{NHE}	−0.93 ± 0.04

Note: Mean *P* value and standard deviation (SD) were calculated from the results of five experiments.



Effect of heat (75°C) on enzymatic activity of native SOD (*1*), LA-SOD (*2*), and α-LA-SOD (*3*)

transfer of a drug through membrane. Improved lipophilicity can bring better membrane permeability and enhance bioavailability *in vivo*.

In conclusion, LA and α-LA are suitable substances for modifying native SOD. Enhanced heat stability, acid and alkali resistance, and anti-pepsin/trypsin stability of the modified SOD were observed compared with the natural SOD, and the apparent oil–water partition coefficient was especially increased, which should improve the pharmacological properties and practical application.

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