Liposome-Recruited Activity of Oxidized and Fragmented Superoxide Dismutase

Le Quoc Tuan, Hiroshi Umakoshi, Toshinori Shimanouchi, and Ryoichi Kuboi*

Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan

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The peptide fragment of H_2O_2-treated Cu,Zn-superoxide dismutase (SOD) was found to be reactivated with liposomes prepared by 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). The fragmentation of SOD was observed by 2 mM H_2O_2 treatment as well as by SOD inactivation and the loss of an α-helix in the neighborhood of its activity center. The H_2O_2-treated SOD, which lost its activity at different incubation times, was dramatically reactivated only by adding POPC liposomes, resulting in 1.3−2.8 times higher enzymatic activity. The ultrafiltration analysis of H_2O_2-treated SOD co-incubated with liposomes shows that some specific peptide fragments of the oxidized SOD can interact with POPC liposomes. A comparison of the fractions detected in reverse-phase chromatography shows that specific SOD fragments are able to contribute to the reactivation of oxidized and fragmented SOD in the presence of POPC liposomes. The liposomes can recruit the potentially active fragment of SOD among the lethally damaged SOD fragments to elucidate the antioxidative function.

Introduction

Superoxide dismutase, SOD, which is an intracellular enzyme vital to every cell in the body, is a metalloenzyme containing copper and zinc, and sometimes manganese or iron, at the active site. SOD plays an important role in the protection of all aerobic biological systems against oxygen toxicity and free radicals derived from oxygen, catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide.[1]

The overexpression of Cu,Zn-SOD in vivo has been reported to be related to negative responses of cells such as cell death,[2] the enhancement of lipid peroxidation,[3] the reduced transport of biogenic amines,[4] and the decomposition of brain neurons.[5] In in vitro experiments, it has previously been reported that the enzyme is inhibited by one of the reaction products, hydrogen peroxide, in the absence of catalase.[6] Some researchers have attempted to relate the H_2O_2-induced loss of enzymatic activity of SOD[7,8] to the structural changes in the enzyme active site (Cu^{2+} binding),[9] including increased proteolytic susceptibility,[10−12] fragmentation,[13] and oxidation of the active site.[14] Cu,Zn-SOD has also a free radical-generating function that utilizes its own product, H_2O_2, as a substrate.[15,16] If hydroxyl radicals are generated by the Cu,Zn-SOD and H_2O_2 system, they can react with superoxide dismutase itself and other molecules in the vicinity of their generation sites. As a result, the fragmentation of SOD is elucidated at high concentrations of hydrogen peroxide.[17,19,20] The fragments produced associate with the H_2O_2-induced active site (∼5 and ∼10 kDa) and the nonactive site (smearing between 3 and 16 kDa).[9]

In a biological system, SOD seems to be moderately controlled on the biomembrane. For example, extracellular SOD is known to be anchored to biomembranes by binding with heparin in the membranes, although the amount of extracellular SOD expressed is significantly lower than the amount of intracellular SOD expressed.[18] The extracellular SOD may thus have a strong resistance to oxidation and may maintain suitable oxidative conditions outside the cell. The role of biological membranes during SOD activity modulation should be clarified, especially under oxidative stress conditions. Using an in vitro approach, Nagami et al. recently reported that Cu,Zn-SOD retains 80% of its initial activity in the presence of liposomes after incubation with hydrogen peroxide for 24 h.[17] Under relatively mild stress conditions (<2 mM hydrogen peroxide), the liposomes were shown not only to prevent the oxidation of SOD but also to maintain its activity.[17] It is important and necessary to clarify the mechanism of the conformational change of Cu,Zn-SOD as well as the enzyme activity with the support of the liposome membrane under strong oxidative stress.

In this study, the peptide fragment of oxidized SOD, without enzymatic activity, was shown to be reactivated by adding liposomes, where specific fragments of SOD can be recruited on the liposome surface.

* Corresponding author. E-mail: kuboi@cheng.es.osaka-u.ac.jp. Tel & Fax: +81-(0)6-6850-6285.

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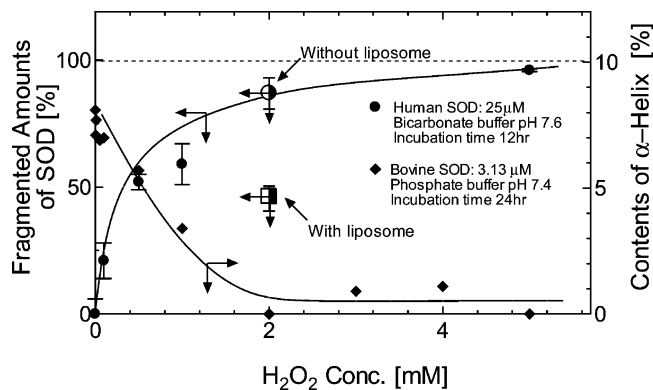
Figure 1. Dependence of H_2O_2 concentrations of the fragmentation of SOD determined from SDS-PAGE analysis. Data of fragmented amounts of SOD (●)[19] and α-helix contents (♦)[17] were replotted from previous studies. The fragmentation of SOD was conducted by the incubation of 2 μM bovine SOD in the presence of 2 mM POPC (▪) or in the absence of POPC liposomes (◐), with phosphate buffer at pH 7.4 and an incubation time of 24 h. The reaction was stopped by adding catalase to a final concentration of 100 μg/mL, and an aliquot was analyzed by SDS-PAGE. The stained-gel bands for SDS-PAGE analysis were evaluated by Scion Image Software, relying on the density of images (Materials and Methods section). Data represent the mean $\pm$ SD ($n = 3-5$).

Results and Discussion

The effects of the addition of liposomes on the enzymatic activity of SOD were first investigated in the presence of hydrogen peroxide. Figure 1 shows the responses of SOD as a function of H_2O_2 concentration: fragmentation of SOD[18] and the α-helix content of SOD.[17] The α-helix neighboring the active center of SOD was shown to be destroyed with increased H_2O_2 concentration and was completely lost at more than 2 mM (Figure 1). Simultaneously, the fragmentation of SOD was observed from the data of SDS-PAGE analysis according to a previous report,[18] resulting in an estimated 75% of the SOD being fragmented at more than 2 mM. A similar result (81% fragmentation) was also obtained by the SDS-PAGE analysis of the H_2O_2-treated SOD (Figure 1, half black−white circle). In contrast, the fragmentation of SOD reached only 46% in the presence of the POPC liposomes, despite the higher concentrations of H_2O_2 (2 mM) (Figure 1, half black−white square). The above results show that the POPC liposomes can positively act on SOD under the oxidative stress condition to protect the enzyme from being inactivated,[17] inducing conformational change and fragmentation.

The activity of SOD fragments in the presence of POPC liposomes was further studied by analyzing the time course of SOD activity during its oxidation and after liposome addition. The liposome-assisted activity of SOD has previously been modeled as the SOD−liposome interaction with the partially denatured SOD, which loses the α-helix content in the neighborhood of the active site of SOD (as observed in the circular dichroism spectra analysis).[17] However, the fragmentation of SOD was herewith observed in addition to the inactivation and loss of secondary structure, especially at H_2O_2 concentrations greater than 2 mM (Figure 1). It is hypothetically considered that some kinetic intermediates of SOD are formed during the oxidation of SOD with H_2O_2 and that these fragments are able to display their activity through their interactions with the liposome membrane. The oxidation of SOD and its interaction with liposomes were separately analyzed by employing a sequential treatment. After 2 μM SOD was incubated with 2 mM H_2O_2 (preoxidation), 2 mM POPC liposomes was added to the reaction bath at 2, 6, and 12 h (liposome addition). The results in Figure 2a show the time course of the relative SOD activity
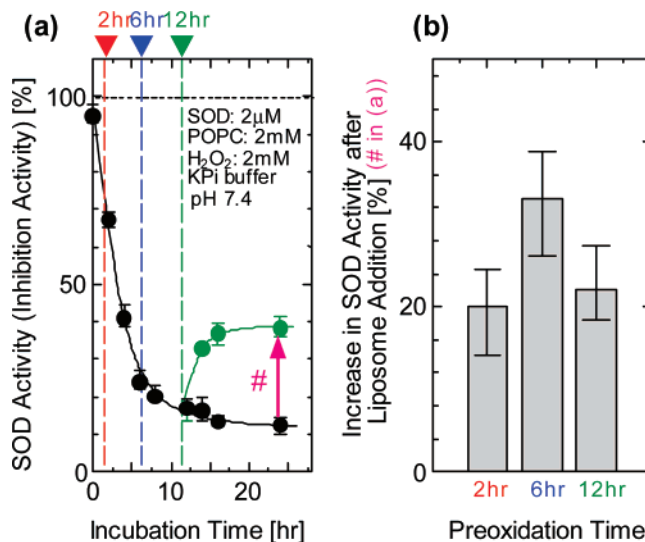

Figure 2. Response of SOD activity after the sequential treatment of preoxidation and liposome addition. (a) Time course of SOD activity. A sample of 2.0 μM SOD was treated with 2 mM H_2O_2 in phosphate buffer (pH 7.4) for 24 h at 37 °C for the appropriate time (2, 6, or 12 h) as a preoxidation treatment, and 2 mM POPC liposomes was added to the solution. The filled black circle is the control (without POPC); the filled green circle is the recovery of SOD activity after liposome addition. (b) Summary of the increase in SOD activity after liposome addition, followed by preoxidation. All of the data represent the mean $\pm$ SD ($n = 3-5$).

during preoxidation and after liposome addition. In the absence of liposomes, the SOD activity was drastically reduced and reached a saturated value ($<20\%$) in approximately 7 h. When the liposomes were added 12 h after the preoxidation, the decrease in SOD activity (15%) was reversed and reached 40% (2.66-fold more). Similarly, the SOD activity in different preoxidation periods was also increased by the addition of liposomes from 70 to 90% (1.28-fold) and from 22 to 60% (2.72-fold) for liposome addition at 2 and 6 h, respectively, after preoxidation (Figure 2b).

It has been reported that the α-helix content of SOD is lost by H_2O_2 in both the absence and presence of liposomes.[17] Our data show that SOD fragmentation can also occur in the presence of H_2O_2, as previously reported,[18] resulting in the inactivation of enzymatic activity of SOD.[21,22] During oxidation, there are some possibly temporal intermediates such as (i) SOD with a partially destroyed conformation (nonfragmentation) and (ii) specific fragments. These two possible states can be related to the liposome-assisted activity that was varied according to the preoxidation time in the above sequential treatment (Figure 2b). At lower levels of oxidative stress (i.e., a short treatment time (2 h) at 2 mM H_2O_2 or treatment at lower concentrations), the POPC liposomes can interact with the partially destroyed SOD and similarly protect it from further oxidation, as described previously.[17] This function of the POPC liposomes is supported by the suppression of fragmentation in the presence of liposomes (Figure 1). At higher oxidation levels (12 h of preoxidation), the specific fragments containing the active site of SOD are able to be recruited on the liposomes, and its conformation can be repaired for the continuous expression of SOD-like function. It is thought that both effects are elucidated after 6 h of preoxidation, resulting in a maximal value in increased SOD activity after liposome addition (Figure 2b). In the following investigations, the possibly

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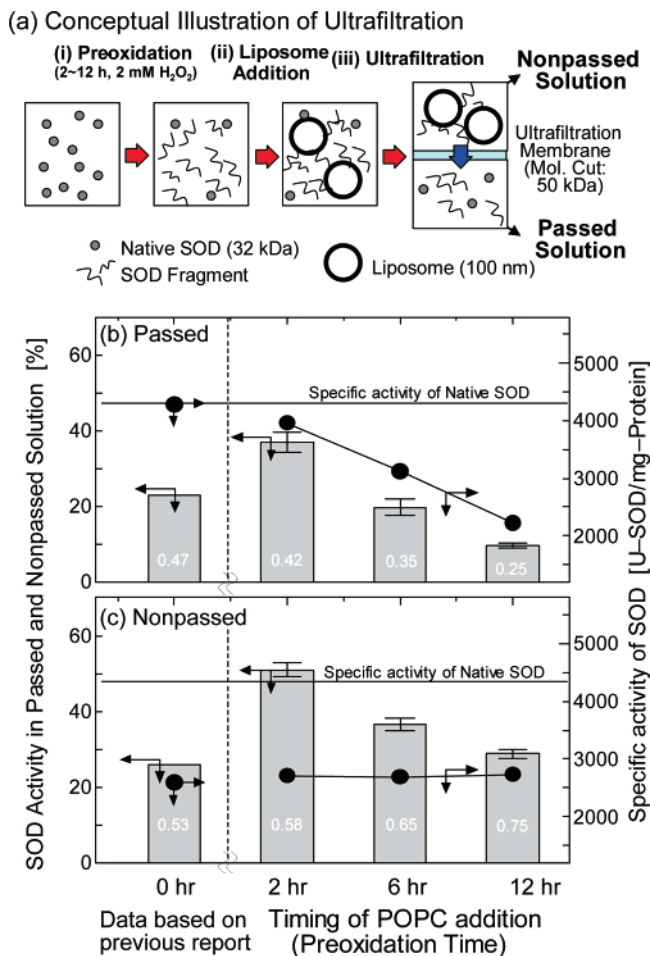**(a) Conceptual Illustration of Ultrafiltration**

Figure 3. Oxidized SOD activity and specific activity with POPC liposomes before/after the ultrafiltration operation. (a) Conceptual scheme of preoxidation, liposome addition, and ultrafiltration treatments. A sample of 2.0 μM SOD was incubated with 2 mM H_2O_2 for the appropriate time (2, 6, or 12 h) in phosphate buffer (pH 7.4). After 2 mM POPC liposomes was added to the solution, the final aliquots after 24 h of incubation were subjected to ultrafiltration to separate the passed solution, including molecules smaller than 50 kDa and the nonpassed solution including larger molecules or liposomes. In the preliminary experiments, native SOD (32 kDa) and its oxidized molecules were confirmed to be included in passed solution. (b, c) SOD activity and specific activity of SOD in the passed and nonpassed solutions, respectively. The samples treated by ultrafiltration were then used for the RP-HPLC analysis (Figure 4).

specific fragment of SOD at the higher levels of oxidative stress was further determined.

The binding of the peptide fragment of SOD on the liposomes was confirmed through ultrafiltration analysis. After the addition of liposomes, followed by preoxidation, the sample was treated by ultrafiltration (50 kDa molecular mass cutoff filter), which enables us to separate the liposomes and the fragments bound on it from the nonbound fragments (Figure 3a). The SOD activity and protein concentrations were measured in both "passed" and "nonpassed" solution after the ultrafiltration experiment. The lower activity of SOD (25−45% of total SOD activity) was observed in the passed solution; furthermore, both the activity and specific activity of SOD decreased with increases in the preoxidation time (Figure 3b). The majority of the SOD activity was detected in the nonpassed solution, whereas the SOD activity was reduced by increasing the preoxidation time (Figure 3c). However, the specific activity of the SOD in nonpassed solution was not changed, regardless of the increase in the preoxidation

time, in contrast to that in passed solution. Furthermore, the relative ratio of SOD activity in nonpassed to that in passed solution increased with increases in the preoxidation time (Figure 3b,c). The above phenomenon clearly shows that the liposomes can gather specific fragments of oxidized SOD and reactivate the specific fragment on the liposome membrane. Nagami et al. have previously reported that the liposome-assisted activity of H_2O_2-treated SOD is maintained by the interaction between the POPC liposomes and oxidized SOD.[17] It has also been demonstrated in a series of previous work that liposomes can perform a molecular chaperone-like function to assist in the refolding of partially denatured proteins.[23−25] However, the SOD was found to be irreversibly fragmented with H_2O_2 in just a few hours (Figure 2a). Another possible explanation based on the molecular chaperone-like function of the liposomes and the obtained results is the assemblage and conformational rearrangement of the intermediates of SOD on the liposome surface under a stress condition. When the POPC liposomes are added to the SOD treated with H_2O_2, the liposome membrane can recruit the fragments of oxidized SOD, including the specific residues of the active sites and nonactive parts, and modulate its conformation, leading to the display of new SOD-like activity (Figure 2).

Normal and oxidized forms of SOD after ultrafiltration were further analyzed by reverse-phase HPLC. Figure 4A shows the chromatograms of (a) normal SOD, (b) oxidized SOD solution, and (c) passed SOD solution after ultrafiltration of a preoxidized, liposome-treated sample. The results indicate that some oxidized species such as P2, P3, and P4 were formed after the oxidation of SOD. It has been reported that a 10 kDa fragment including the active center and other fragments (5 kDa) of SOD can be formed by H_2O_2 addition as a result of the fragmentation of SOD.[9] The active site contains four His residues with Cu^{2+} and Zn^{2+}, catalyzing the dismutation of the toxic superoxide radical into molecular oxygen and hydrogen peroxide without a significant energy cost.[26] A UV spectral analysis of the fragmented SOD separated by HPLC indicated that the fragmented SOD contains histidine residues or other residues with aromatic rings (data not shown). The detected peaks in oxidized SOD correspond to the oxidized fragments reported previously. Among the above fragments, the P4 fragment is thought to be relatively hydrophobic, judging from its long retention in an octadecyl column. As shown in Figure 4A(c), a part of P3 and the whole P4 fraction were not detected in the passed solution after ultrafiltration, showing that these fragments interact with the liposome membrane and continue to function as SOD together with liposomes because they contained active-site fragments. For further investigation, the P4 fragment of SOD, recovered from the RP-HPLC fraction, was co-incubated with the POPC liposomes together with Cu and Zn ions at 10 μM for the supplementation of metals to modify the active site. Similarly, the SOD activity was found to be increased to 32% when the P4 fragment (inactive) was incubated with POPC and metal ions. Otherwise, the enzymatic activity of this fragment has increased to nearly 4% in control experiments with the addition of Cu^{2+} and Zn^{2+} or POPC only (Figure 4B). The specific conformation of the P4 fragment with the POPC liposomes was also observed through circular dichroism analysis (data not shown). Binding of the P4 fragment with liposomes

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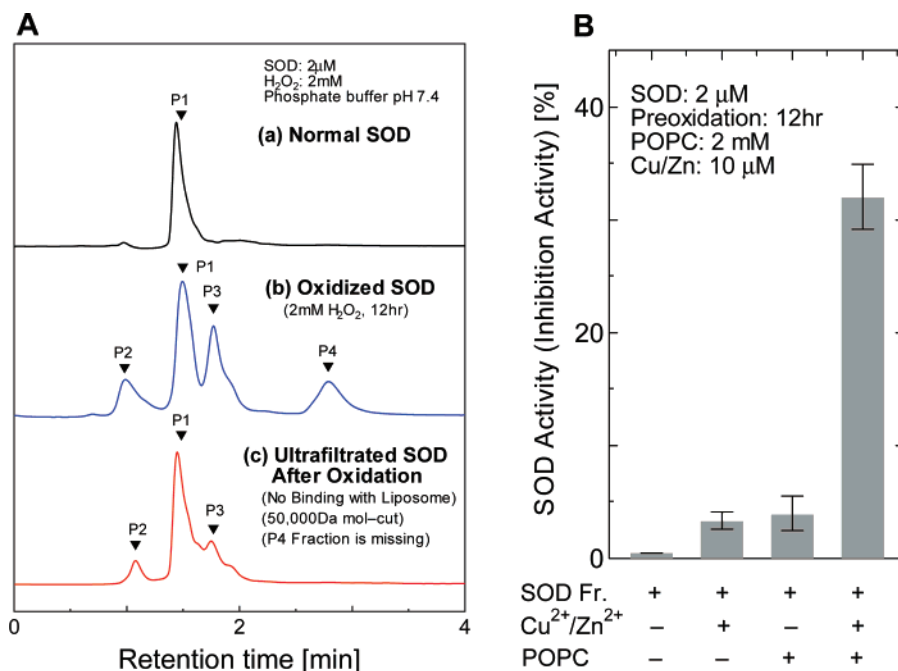
Figure 4. (A) C18-RP-HPLC analysis of SOD and oxidized SOD. The above samples were compared with that of oxidized SOD after ultrafiltration. P1, native SOD; P2, P3, and P4, derived from fragmentation. A part of P3 and all of P4 were retained by the liposomes after filtration. Acetonitrile/water (7/3 v/v) was applied to run RP-HPLC at 25 °C with a flow rate of 1 mL/min and a wavelength of 220 nm for absorption. (B) SOD activity of a specific fragment (SOD Fr) recovered by RP-HPLC with and without POPC liposomes and metal ions. All of the data represent the mean $\pm$ SD ($n = 3-5$).

and its resulting conformational change can elucidate the reactivation of the inactive SOD fragment.

From previous studies[9,17,19] and our present results, we can conclude that the specific fragment (P4), which has a hydrophobic site and a catalytic site of approximately 10 kDa, still displays the original SOD-like function after recruitment on the liposome membrane surface. The above results also imply the possible significant role of the membrane, as a fail-safe function, in the biological system during the response under lethal stress conditions.

Materials and Methods

Materials. Bovine erythrocyte Cu,Zn-SOD (EC 1.15.1.1), purchased from Sigma-Aldrich with a specific activity of 4470 U/mg (product no. S2515-30KU, lot no. 125K740), was used without purification. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and was used for liposome preparation. All other reagents of analytical grade were purchased from Wako Pure Chemicals (Osaka, Japan).

Analysis of Cu,Zn-SOD Fragmentation by H_2O_2. Cu,Zn-SOD (2 μM) was incubated with H_2O_2 (2 mM) in phosphate buffer (pH 7.4) at 37 °C for various periods. The enzymatic activity and protein-concentration-fragmented SOD were determined after the incubation of SOD with H_2O_2. The SDS-PAGE technique was used to analyze SOD fragmentation.

For the SOD activity, a highly water-soluble tretrazolium salt, WST-1 [2-(4-lopophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], produces a water-soluble formazan dye upon reduction with a superoxide anion, where the rate of reduction with O_2^- is linearly related to the xanthine oxidase (XO) activity and is inhibited by SOD. The absorption spectrum of WST-1 formazan was measured at 450 nm, and the SOD activity as an inhibition activity can be quantified through the decrease in color development.[27]

A BCA protein assay kit was used to determine protein concentrations. The protein was precipitated in a cold acetone solution to separate it from contaminants so that a more accurate estimation of protein content in the sample could be obtained. The mixture was then centrifuged at 15 000 rpm for 20 min. Pellets solubilized in 50 μL of H_2O were added to 1000 μL of BCA reagent solution and incubated for 30 min at 37 °C, after which the absorbance at 562 nm was measured. A standard curve was set up to analyze the protein concentrations.[28]

For SDS-PAGE analysis, aliquots of the sample were solubilized with the denaturing buffer (0.25 mM Tris-HCl, 8% SDS, 40% glycerol, 20% L-mercaptoethanol, and 0.01% bromophenol blue) and were boiled at 100 °C for 10 min before electrophoresis. An aliquot of each sample was subjected to SDS-PAGE as described by Laemmli[29] using a slab gel (PhastGel High Density, <0.2% acrylamide). The gels were stained with 0.2% Coomassie brilliant blue R-250. The amount of protein was quantitatively characterized by the staining intensity using the method described in the ref 30 and Scion Image software obtained at http://www.scioncorp.com/.

Ultrafiltration of H_2O_2-Treated SOD. Ultrafiltration using a Millipore Ultrafree-MC filter with a molecular mass cutoff of 10 or 50 kDa was applied for fragmentation analysis. SOD, after being treated with H_2O_2, was applied to the ultrafiltration tube and centrifuged at 15 000 rpm for 30 min at room temperature. The upper portion (nonpassed solution) had fragments larger than 50 kDa in molecular mass, which also bound liposomes (vesicles 100 nm in diameter), and the lower portion (passed solution) had fragments smaller than 10 or 50 kDa in molecular mass (called free fragments or free SOD). Both were then analyzed by reverse-phase HPLC and their enzymatic activity was determined.

As a control experiment, it was confirmed that the activity of native SOD (32 kDa) was detected in the passed solution across the ultrafiltration membrane and that neither measurable protein

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concentrations nor the activity was detected in the nonpassed solution. As a negative control experiment, the oxidized SOD solution was also treated by ultrafiltration, resulting in both the SOD activity and measurable protein concentrations being detected only in the passed solution. These results of the control experiments indicated that the oxidized SOD did not form large aggregates at more than 50 kDa. The mass balance of protein concentration was checked to confirm that neither SOD nor fragmented SOD was adsorbed on the ultrafiltration membrane.

For reverse-phase HPLC, a Shimadzu HPLC system equipped with an FCV-10AL pump and a DGU-20A$_3$ degasser and both an SPD-10A UV−vis detector and an LC-10AD liquid chromatograph were used. Elution profiles were monitored at 220 nm on the UV detector. The mobile phase of acetonitrile/water (7/3 v/v) with a flow rate of 1 mL/min was applied at 25 °C. An STR ODS-M column (0.46 cm × 15 cm), in which the particle surface was octadecylated, was used throughout this study.

Statistical Analysis. Results are expressed as the mean ± standard deviation (SD). All experiments were performed at least in triplicate.

The data distribution was analyzed, and statistical differences were evaluated using the Student t test. A P value of <0.05% was considered to be significant.

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