Separation of Superoxide Dismutase by Size-Exclusion Chromatography Column Packed with Regenerated Cellulose Gels

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ABSTRACT: A preparative size-exclusion chromatography (SEC) column (500 × 10 mm) packed with regenerated cellulose gel particles was used for the separation of superoxide dismutase (SOD) from pig blood. The fraction F-5, with an M_w of 12.4×10^4, composed of more aspartic acid (*Asp*), glutamine (*Glu*), and tyrosine (*Tyr*) but less cysteine (*Cys*), has the highest specific activity (204 units per mg protein), which was higher than that of the commercial product (175 units per mg protein). The change of fluorescence intensities at zero concentration of the fractions was identical to that of the activities. The results from amino acid sequence analysis and size-exclusion chromatography combined with static laser light scattering (SEC–LLS) analysis indicated that the separation mechanism of SOD is based on both size exclusion and ion affinity. They also showed that the activity of SOD is determined mainly by its composition. At a flow rate of 0.62 mL min^{-1}, 42 mg of a high specific activity product can be obtained from crude SOD in 1 day.
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Key words: chromatography; enzymes; separation of polymers; fluorescene; gels

INTRODUCTION

Reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) are generated in *vivo* through the incomplete reaction of oxygen during aerobic metabolism or from exposure to environmental agents such as radiation, redox cycling agents, or simulated host phagocytes.[1–3] These oxygen species can cause widespread damage to biological macromolecules, leading to lipid peroxide, protein oxidation, enzyme inactivation, and DNA strand breaks.[4,5] Superoxide dismutases (SODs), which are metalloproteins, containing iron or manganese or copper plus zinc as the prosthetic groups, play an essential role in allowing organisms to survive in the presence of O_2. These enzymes catalyze the dismutation of highly reactive superoxide anion radicals to molecular oxygen and hydrogen peroxide in all oxygen-metabolizing organisms and in some anaerobes.[6] Three distinct types of SODs have been reported, such as copper–zinc SODs (CuZn,SODs),[7] manganese SOD (MnSOD),[6,8] and iron enzyme (Fe-SOD).[9] The Cu plus Zn enzyme (CuZn,SOD) is an efficient substance for protecting humans from various diseases such as arteriosclerosis, cataracts, and retinal damage.[4] Scientific research and applications of CuZn,SOD have attracted much attention.[10–15] Some ways to isolate and purify CuZn,SOD have been reported, such as *O*-diethylaminoethyl (DEAE)–cellulose separation,[16,17] weaken anion-exchange HPLC,[18] and hollow-fiber ultrafiltration.[19] Among them, the column packed with DEAE–cellulose has been conventionally used to prepare SOD with high activity.[6,19] The properties of SOD can be characterized by biological[16,20] and chemical methods.[21] But both the separation and analysis methods above-mentioned are too expensive in industrial applications. In our laboratory, a preparative size-exclusion chromatography (SEC) column packed with regenerated cellulose gel particles was used for the fractionation of a dextran in water[22] and β-(1→3)-D-glucan from poria cocos in dimethyl sulfoxide,[23] and the fraction range of the stationary phase covered M_w from 3×10^3 to 1.1×10^6 (ref. 24).

To explore an easy and cheap route to separate biopolymers with high bioactivity, in the present study, we attempted to isolate CuZn,SOD from erythrocytes of pig blood using a preparative SEC column packed with regenerated cellulose gel particles. The enzymatic bioactivities were determined by the spontaneous autoxidation of the pyrogallic acid method[25] and fluorescence spectroscopy. The amino acid se-

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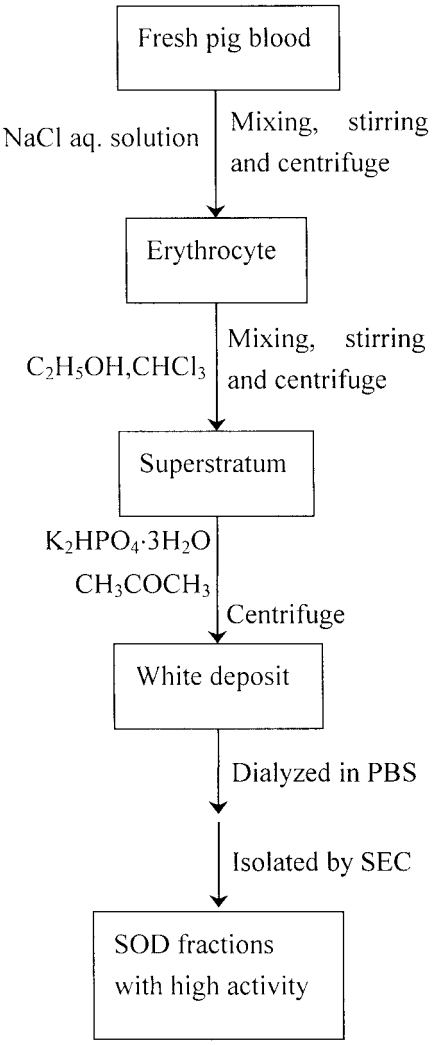
quence analysis and analytical SEC combined with laser light scattering (LLS) were used to characterize the fractions.

EXPERIMENTAL

Preparation of SEC column

An eight weight percent cellulose cuoxam solution (I), an 8 wt % silk fibroin cuoxam solution (II), and a 20 wt % PEG2000 aqueous solution (III) were prepared according to a previous method.[22,24] The mixture of I and II (1:1 v/v) was spun into a 10 wt % aqueous NaOH solution and then regenerated in a 5 wt % H_2SO_4 aqueous solution to obtain clear regenerated cellulose fibers. The fibers were cut into small particles with a length of 0.5–1.5 mm and then washed with distilled water. The cellulose gel particles prepared from solutions I, the mixture of I and II, and the mixture of I and III were coded as RCG-1, RCF-1, and RCG-2, respectively. The mixtures of RCG-1, RCG-2, and RCF-1 (2:2:1, v/v) were packed in a glass column (600 × 10 mm) to form a 500-mm gel bed. The distilled water was the elution phase, and the flow rate was adjusted to 1.5 mL min^{-1} during the runs to stabilize the column for 1 week.



Scheme 1 Procedure of SOD.

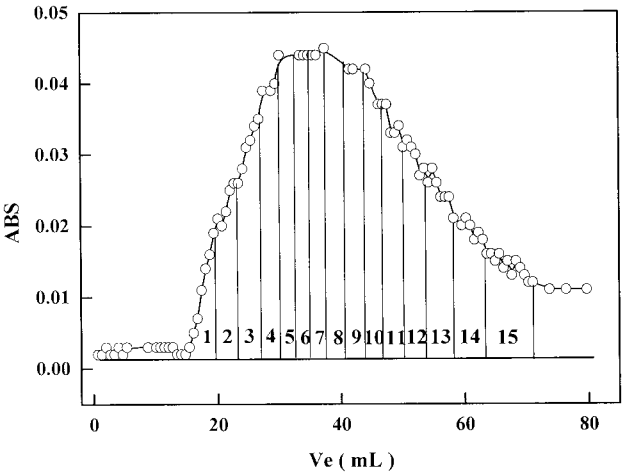


Figure 1 Elution pattern of SOD (0.5 wt %) in K_2HPO_4—KH_2PO_4 aqueous solution by preparative SEC column.

Isolation and purification of SOD from pig blood

Two liters of fresh pig blood, obtained from a local abattoir, was mixed with an equal volume of a 0.9 wt % NaCl aqueous solution immediately, and the mixture was centrifuged at 3000 rpm at 5°C for 20 min. The serum protein was removed from the erythrocyte by three times repeating of the process mentioned above. The underlayer erythrocyte was added with an equal volume of distilled water and stirred energetically in an ice bath for 40 min. Precooled 625 mL (0.25 times of volume) alcohol and 375 mL (0.15 times of volume) chloroform were added to the mixture gradually when being stirred, and, subsequently, the mixture was centrifuged at 3000 rpm at 5°C for 20 min. The superstratum solution was mixed with $K_2HPO_4 \cdot 3H_2O$ (43 g/100 mL), and then the superstratum layer of the mixture was centrifuged at 3500 rpm at 5°C for 25 min. Precooled acetone, 911 mL (0.75 times of volume), was added to the obtained 1214 mL solution and stirred and then centrifuged at 3000 rpm at 5°C for 20 min to get a white deposit. The white deposit was dissolved in 800 mL distilled water and dialyzed extensively against 2.5 m*M* phosphate buffer (PBS) (pH 7.6) for 3 days. The 669 mL crude SOD solution was rotary-evaporated under diminished pressure at 37°C and, finally, freeze-dried. The obtained light green crude enzyme was stored at −30°C until analysis. The overall process is outlined in Scheme 1.

Separation of SOD

The crude enzyme was dissolved in 2.5 m*M* PBS with a 0.5 wt % concentration. A 3 mL solution was injected

TABLE I
Properties of RCG-1, RCF-1, and RCG-2

Fiber	Mean particle size (μm)	Mean pore size (nm)	Pore volume (mL/g)	Porosity (%)
RCG-1	320 × 1390	108	6.17	90.1
RCF-1	400 × 1170	787	8.12	92.1
RCG-2	330 × 1180	545	7.27	92.6

into the SEC column mentioned above, which had been carefully equilibrated with 2.5 m*M* PBS and eluted with 2.5–200 m*M* PBS gradients at a flow rate of 0.62 mL/min at 25°C. The effluent was monitored at 280 nm using an UV detector (UV-1601, Shimadzu, Japan). The elution profile from the preparative column is shown in Figure 1. It was separated into 15 fractions according to an equally covered area. The fractions were coded as F-*n* (*n* is the fraction number such as 1, 2, . . . , 15).

Protein and SOD activity assay

To clarify the purity of the SOD, the protein was determined spectrophotometically according to the Bradford method using bovine serum albumin (BSA) as the standard.[25] The activity assay for crude and SOD fractions was based on the inhibitory effect of SOD on the spontaneous autoxidation of pyrogallic acid in 50 m*M* Tris–HCl buffer. One unit of the SOD is defined as that amount which requires 50% inhibition of the rate of pyrogallic acid autoxidation in the Tris–HCl buffer. Specific activity is defined as the units of activity/milligram of protein.

Fluorescence measurements were performed on a Shimadzu RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) with the sample compartment maintained at 25°C. A 150-W xenon source was used. The intrinsic fluorescence intensities of the crude and purified SOD fractions with different concentrations were routinely measured in 50 m*M* Tris–HCl, pH 7.6. The slit-width was fixed at 5 nm for excitation and emission. Samples were excited at 280 nm, and the emission was monitored between 300 and 400 nm. Each recorded spectrum was an average of three separate scans and was corrected for the background fluorescence of the relevant control.

Molecular weight determination

SEC–LLS was used for the determination of the weight-average molecular weight (M_w) of the crude and SOD fractions. The SEC–LLS measurements were carried out on a DAWN®DSP multiangle laser photometer (λ = 633nm; DAWN®DSP, Wyatt Technology Co., USA) combined with a pump p100 (Thermo Separation Products, San Jose, CA) equipped with a TSK-GEL G3000 PWXL column (7.88 × 300 mm), a TSK-GEL G5000 PWXL column (7.88 × 300 mm), and a differential refractive index detector (DAWN®DSP) at 25°C. The eluent was 2.5 m*M* PBS with a flow rate of 1.0 mL/min. The refractive index increment (*dn/dc*) was measured with an Optilab refractometer (DAWN®DSP) at 633 nm and 25°C. The value *dn/dc* of SOD in 2.5 m*M* PBS was determined to be 0.103 mL g^{-1}. Astra software was utilized for data acquisition and analysis.

Amino acid sequence analysis

The SOD fractions were subjected to acid hydrolysis, and then amino acids were analyzed by an amino acid autoanalysis instrument (Hitachi 835-50, Japan). The amino acids shown in Table IV were divided into four groups: First, the group with a positive charge (+) was composed of lysine (Lys), arginine (Arg), and histidine (HIS); second, the group with a negative charge (−) was composed of aspartic acid (Asp) and glutamic acid (Glu); threonine (Thr), Serine (Ser), glycine (Gly), glutamine (Glu), and cysteine (Cys) composed the third polarity group (↑) with no charge; and the no polarity and no charge composed the fourth group (→), such as alanine (Ala), valine (Val), Leucine (Leu), isoleucine (Ile), proline (Pro), phenylalanine (Phe), and methionine (Met). The quantity of the four groups were calculated as follows:

TABLE II
Activities and Yield of SOD Isolated from Pig Blood

Purification step	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Total activity × 10^{-3} (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Extract	1214	5.14	6240	177	28.3	100	1
After PBS dialysis	670	2.74	1236	144	116.5	81.4	4.1

TABLE III
Weight-average Molecular Weight (M_w), Polydispersity Index (d), Specific Activity, Total Activity, and Activity Percentage of the Fractions and the Crude SOD and the Commercial Product

Sample	$M_w \times 10^{-4}$	d	Specific activity (units/mg)	Total activity (units $\times 10^3$)	Activity percentage (%)
F-1	20.1	2.30	102.0	75.0	4.3
F-2			82.8	69.6	4.0
F-3	17.1	2.25	150.7	189.9	10.9
F-4	14.7	2.69	201.7	305.6	17.5
F-5	12.4	2.43	203.9	333.4	19.1
F-6			169.9	214.1	12.3
F-7	10.8	2.16	132.6	151.1	8.6
F-8			79.5	76.3	4.4
F-9	8.0	1.49	71.8	58.2	3.3
F-10			67	46.2	2.6
F-11	7.7	1.88	67.9	38.7	2.2
F-12			67.9	36.7	2.1
F-13	11.7	1.45	25.3	14.4	0.8
F-14			34.6	18.2	1.0
F-15	6.8	1.37	34.1	16.4	0.9
Crude SOD	10.8	2.33	116.5	1747.5	100
Commercial SOD	17.1	1.53	175.3	—	—

$$Q = \sum_i \frac{p}{M} \quad (1)$$

where Q is the quantity of the group; p, the percentage of the amino acid; and M, the molar mass of amino acid.

RESULTS AND DISCUSSION

It was reported[22,24] that the apparent pore sizes of RCF-1 and RCG-2 were obviously larger than those of RCG-1 because of the silk fibroin and PEG as the pore former, and the properties of the gels are summarized in Table I. The mean pore sizes of the gel particles are 135 nm for RCG-1, 370 nm for RCG-2, and 525 nm for RCF-1. The size-exclusion curve for the preparative SEC column (500 × 10 mm) packed with those gels is represented as follows[24]:

$$\log M = 6.287 - 0.0492Ve \quad (2)$$

The activities and yield of the crude SOD isolated by the column are listed in Table II. The specific activity of the crude SOD reached 116.5 units/mg, which was 4.1 times that of protein before PBS dialysis, and 81.4% of the total SOD activity was recovered. The elution profile (Fig. 1) shows one wide peak, indicating SOD alone from pig blood. The specific activity and activity percentage of the fractions and the crude SOD and the commercial product are summarized in Table III. The dependence of the specific activity and the total activity of the fractions on the fraction number is shown in Figure 2. The specific activities of F-4 and F-5 reached

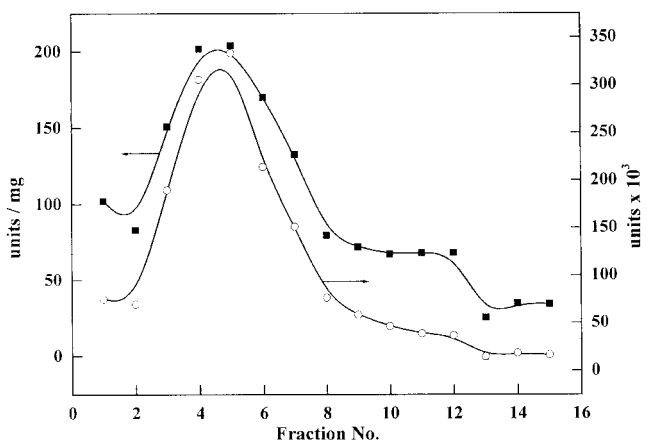


Figure 2 (■) Specific activity and (cir;) total activity of the fractions.

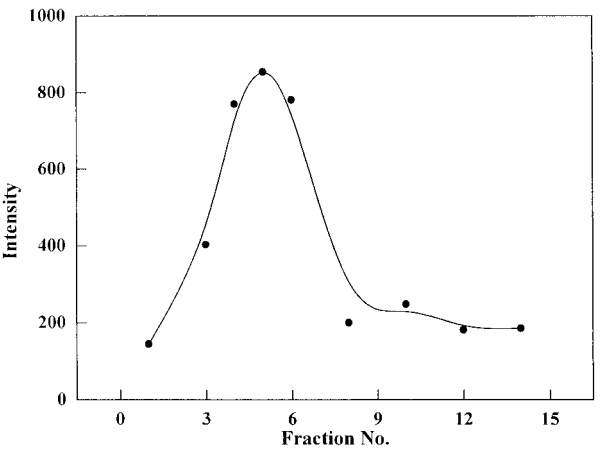


Figure 3 Various intercepts for the fractions.

the higher value (more than 200 units/mg), and activity of the two fractions occupied 36.6% of the whole. It was worth noting that the value was higher than 175 units/mg of the commercial product (Sigma Chemical Co., USA, EC 1.15.1.1) that was determined in the same way. The specific activities of F-3, F-4, F-5, F-6, and F-7 were higher than that of the crude SOD, and their total activity was 68.3% that of the injected protein.

The fluorescence intensities of the fractions with different concentrations at 280 nm were measured. After the linear regression analysis of the fluorescence intensities versus concentrations, the various intercepts for the fractions were obtained (Fig. 3). The tendency is just similar to the activity curves of the protein, which further confirms that the highest specific activity was achieved for fractions 4 or 5 regardless of the concentration of SOD.

The results from SEC–LLS and the activity assay are listed in Table III. The M_w values of the fractions decreased with the progress of the fractionation, suggesting that the separation mechanism follows the primary principle of size exclusion. The M_w's and polydispersity index (d) of the crude SOD and the commercial SOD were 10.8×10^4, 2.33, and 17.1×10^4, 1.53, respectively. The M_w of F-4 and F-5 were 14.7×10^4 and 12.4×10^4, and they are larger than that of the crude SOD and are close to the commercial product. This indicated that this column was an effective media to extract SOD with high specific activity.

The results of the amino acid sequence analysis are shown in Table IV. It can be seen that the fractions contain different amino acids with different percentages. There was no tryptophan (*Trp*) in any of the fractions. The fractions F-3, F-4, and F-5 with higher specific activities were composed of more aspartic acid (*Asp*), glutamine (*Glu*), and tyrosine (*Tyr*) but less cysteine (*Cys*). It is well known that the fluorescence spectrum of protein is presented by those of tryptophan, glutamine, and alanine.[26] These fractions with a higher glutamine percentage thus have higher fluorescence intensities and intercept values. This result is consistent with the fluorescence measurement described above.

TABLE IV
Amino Acid Composition of the SOD Fractions

	Fraction					
Amino acid	F-1	F-3	F-4	F-5	F-9	F-15
Asp (%)	6.8	10.6	10.3	11.1	8.9	8.7
Thr (%)	4.0	5.5	4.9	5.5	5.3	5.7
Ser (%)	4.1	4.8	4.6	4.7	3.6	3.9
Glu (%)	9.2	15.1	13.7	14.3	11.3	10.6
Gly (%)	5.4	6.7	6.2	6.6	6.5	7.0
Ala (%)	4.4	4.6	4.0	5.3	4.4	5.0
Cys (%)	4.5	0	0.4	0.6	1.8	2.0
Val (%)	6.0	7.1	6.8	6.9	6.7	8.0
Met (%)	2.3	0.3	1.1	0.7	0.3	1.6
ILE (%)	3.5	3.9	3.7	4.4	3.7	4.8
Leu (%)	6.4	7.4	6.3	7.8	5.6	5.9
Tyr (%)	0	2.1	2.7	2.0	15.1	1.8
Phe (%)	4.3	4.6	4.9	4.7	4.0	4.2
Lys (%)	4.1	6.7	6.9	6.6	5.6	5.6
NH_3 (%)	33.1	6.6	10.3	5.8	7.1	16.3
HIS (%)	1.0	4.3	4.3	3.9	3.5	3.0
Arg (%)	1.9	4.1	3.6	4.3	3.2	2.9
Pro (%)	0	5.7	5.1	4.8	3.3	3.1

TABLE V
***Q* Values of the SOD Fractions**

Group	F-1	F-3	F-4	F-5	F-9	F-15
$Q(+)$	0.0928	0.0976	0.0948	0.0947	0.0790	0.0739
$Q(-)$	0.1132	0.1814	0.1698	0.1800	0.1433	0.1366
$Q(\uparrow)$	0.1815	0.1920	0.1894	0.1948	0.2589	0.2048
$Q(\rightarrow)$	0.2163	0.2759	0.2593	0.2829	0.2301	0.2662

The different quantities of the four groups of amino acids for the fractions are listed in Table V. The amino acid residues of the SOD contained 72–79% hydrophilic groups and 21–28% hydrophobic groups. The intermolecular interaction between functional groups (such as COO^-, NH_4^+, and —NHCO—) in the fractions of SOD and —OH groups of cellulose might lead to selective separation in the preparative SEC column. F-3, F-4, and F-5, with higher specific activities, possessed more charge groups in the SOD molecule, especially the negative electric charge. The specific activities of F-4 and F-5 were higher than those of both crude and commercial SOD. This implies that the composition and molecular weight of SOD plays an important role in their activities.

CONCLUSIONS

A preparative SEC column packed with regenerated cellulose gel particles was satisfactorily used to separate the pig superoxide dismutase. The fractions F-4 and F-5 extracted from the crude SOD occupied 36.6% of the total activity, and their activities were higher than that of the commercial product. The specific activities of SOD were influenced mainly by its composition and molecular weight. At a flow-rate of 0.62 mL min^{-1}, 200 mg crude SOD can be separated and 42 mg of a high specific activity product can be obtained in 1 day. Therefore, the cellulose gel particles have a promising potential in the separation of protein (enzyme) or other biopolymers to obtain the fraction having higher bioactivities or functional efficiency.

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