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PURIFICATION AND CHARACTERIZATION OF MANGANESE SUPEROXIDE DISMUTASE FROM *GANODERMA MICROSPORUM*

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SUMMARY. Manganese superoxide dismutase (Mn-SOD) in the mycelium of *Ganoderma microsporum* was purified to homogeneity by heat treatment at 70 °C, ammonium sulfate fractionation, DEAE-52 anion-exchange chromatography, and Sephacryl SH-200 chromatography. The molecular mass of its native form was estimated to be 98 kD by size-exclusion chromatography. This enzyme is tetrameric composed of four subunits of equal size of 25 kD. The pI of this purified Mn-SOD was located at pH 6.34 and 5.06 by isoelectric focusing. Comparisons of 17 amino acids from the N-terminus of Mn-SOD subunit with the derived amino acid sequences from the reported Mn-SOD cDNA clones of other sources indicated a high degree of homology among the *Ganoderma* genus but the Mn-SOD from *G. microsporum* showed a high variation when compared with other organisms.

Key words: *Ganoderma microsporum*, Manganese superoxide dismutase (Mn-SOD)

INTRODUCTION

Superoxide dismutases (EC 1.15.1.1; SOD) catalyze the dismutation of superoxide radical to molecular oxygen and hydrogen peroxide [1,2]. These enzymes are critical in functioning as a defense mechanism against oxygen radicals [3,4,5]. SODs are a group of metalloproteins containing three distinct types of SODs with either Mn, Fe or Cu plus Zn as a prosthetic metal [6,7]. Furthermore, each type of SOD has multiple forms. CuZn-SODs are sensitive to CN^- and H_2O_2, whereas Fe-SODs are sensitive to H_2O_2 but insensitive to CN^-. On the other hand, Mn-SODs are insensitive to both chemicals. This selective inhibition by CN^- and/or H_2O_2, makes it possible to distinguish the three types of SOD in crude homogenates [8]. Because SODs enzymes are capable of removing superoxide anion to preclude formation of the peroxyl radical, they

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prevent lipids, proteins and nucleic acids from peroxidation and autoxidation [9]. Mn-SOD is considered to be present primarily in procarytoes and mitochondria [10], and it has been purified from pea [11], maize [12], spinach [13], Scots pine [14] and yeast [10]. However, little work has been done with fungi.

Ganoderma species are considered as medicinal fungi, and over 250 *Ganoderma* species have been described worldwide with most of them from the tropics. Medicinal benefits of *Ganoderma* species have been reviewed by Jong and Birmingham [15]. In the Orient, such as China, Japan and Korea, *Ganoderma* species are used in folk medicine to cure various diseases, and several strains are commercially cultivated for the preparation of health tablets or drinks. The fruiting bodies and culture mycelia are prescribed to treat chronic hepatopathy, hypertension and other diseases, several *Ganoderma* species are used as a tonic and sedative drug widely in Taiwan. Therefore, *Ganoderma* species have received a wide interest for studies ranging from identification [16], phylogenetic relationship [17,18]; to the content of natural products and their biological activities [19].

Because of medicinal usage, protocol of the quality control of *Ganoderma* materials needs to be established. Since Mn-SOD is present in every stage of growth and development through its life cycle [20,21] and the change in the activity of Mn-SOD was considered to reflect the variation in respiration activity of the tissues [22], Mn-SOD activity may be used as an indication to correlate with a medicinally active component, such as triterpenes. In an effort to test this possibility, cDNA of Mn-SOD from *G. microsporum* has been cloned and sequence of the Mn-SOD gene among genus and several strains of *Ganoderma* have been analyzed [23]. In this study, Mn-SOD was purified and characterized to gain a better understanding of this enzyme.

MATERIALS AND METHODS

Plant material

Ganoderma microsporum was cultured in potato dextrose agar (PDA) medium at 30℃ for 7 days, then the mycelia were transferred to a fresh PDA for another 7 days at 30℃ [24]. Subsequently, mycelia were allowed to grow in a 500 ml-flask containing 200 ml liquid potato dextrose broth at 30℃ for 14 days before harvest. The mycelia were then washed with distilled water, and the fresh weight was measured. The collected sample was frozen at -20℃, vaccum-dried for 36 hr, and then stored at -20℃ until use.

Electrophoresis and SOD activity stain

Analytical polyacrylamide gel electrophoresis (PAGE) was performed according to a modified procedure of Gabriel [25]. Electrophoresis was conducted with a 1.5-mm 12% native acrylamide slab gel in standard Tris-glycine buffer, pH 8.3. An appropriate amount of sample

extract was applied and run at 80 V through stacking gel for 15 min and 120 V through separating gel for 60 min. After electrophoresis, a modified photochemical method of Beauchamp and Fridovich [26] was used to visualize SOD activities. The gel was first soaked in 1.225 mM nitroblue tetrazolium solution for 15 min, briefly washed, then soaked in 100 mM potassium phosphate buffer, pH 7.0, containing 0.028 mM riboflavin and 28 mM TEMED for another 15 min. After the gel was briefly washed, it was illuminated on a light box with intensity of 30 uE s^{-1} m^{-2} for 15 min to initiate the photochemical reaction. To identify CuZn-SOD, 8 mM KCN was included in the riboflavin solution for activity staining. For the identification of Mn-SOD, gels were first soaked in 8 mM hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0, for 30 min, followed by SOD activity staining. All procedures were carried out at room temperature and the two soaking steps were gently shaken.

The protein samples taken from each step of the purification procedure were separated by 12.5% SDS-PAGE to detect the purity or electrophoresed on 5-15% gradient PAGE to detect the SOD activity and to estimate the molecular size of functional forms.

Assay of SOD activity

SOD activity was determined by a method combining electrophoresis and densitometry [27]. Unit of SOD activity was estimated by comparing with that of standard SOD, which was run in the same gel. The unit of standard SOD activity was previously established by measuring in a solution assay. In the solution assay, one unit of SOD activity represented a half-maximal inhibition of the rate of xanthine-xanthine oxidase mediated reduction of nitroblue tetrazolium [28]. Mn-SOD activity was determined by the inclusion of 8 mM H_2O_2 in the activity staining step to inhibit the activity of other SODs.

Heat treatment

The crude extract of the mycelia of *G. microsporum* was incubated at 50, 60, 65, 70 and 75 ℃ for five minutes, respectively, and the remaining SOD activity of each sample was compared in a gel by activity staining after electrophoresis.

Purification of Mn-SOD

Purification steps were carried out at 0-4℃. The mycelia of *G. microsporum* were harvested, frozen in liquid nitrogen, and ground with 5 volumes (ml/g fresh weight) of 0.15 M Tris-HCl, pH 7.2 (buffer A). The homogenate was centrifuged at 12,000*g* for 20 min to remove cell debris and the supernatant was incubated at 70℃ for 5 min. After the heat treatment, the homogenate was centrifuged again at 12,000*g* for 20 min and the supernatant was brought to 50% saturation with solid $(NH_4)_2SO_4$. After stirring for 30 min, the precipitate was removed by centrifugation at 12,000*g* for 30 min. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ and stirred for 60 min. The precipitate was collected by centrifugation at 12,000*g* for 30 min and dissolved in buffer A. Desalting was accomplished by dialysis against buffer A for overnight. The dialyzed sample was applied to a DEAE-52 column (2.6 cm x 35 cm), which had been equilibrated with buffer A, and eluted with buffer A at a flow rate of 3.7 mL/10 min. The active fractions were pooled in a dialyzed bag with a cutoff molecular mass of 1 kD and concentrated with solid polyethylene glycol. The concentrated sample was applied to Sephacryl SH-200 cloumn (2.6 cm

x 85 cm) which had been equilibrated with buffer A, and then eluted with bufferA at a flow rate of 5.8 mL/10 min. The active fractions were pooled, concentrated, then analyzed on SDS-PAGE.

Determination of Mn-SOD molecular mass

The molecular mass of Mn-SOD, after partial purification by anion-exchange cloumn chromatography, was estimated based on the retention time during FPLC on a cloumn (10mm x 30 cm) of Superdex-200, eluted with 50 mM phosphate buffer, pH 6.8 containing 0.15 M NaCl. The protein standards were: ferritin, 440-kD; alcohol dehydrogenase, 158-kD; ovalbumin, 44-kD.

Isoelectric point determination of Mn-SOD

Horizontal isoelectric focusing was performed on 5% SERVALYT Precote ultrathin polyacrylamide gels (0.15 mm x 125 mm) containing SERVALYT carrier ampholytes (pH 3-10). IEF was carried out at 10 ℃ with the Multiphor II system apparatus (LKB Bromma, Sweden). During operation, application of several drops of n-decane under the Precote as a heat exchange liquid was necessary. Samples were applied to paper wicks (4 x 10 mm; Pharmacia LKB) which were placed in the middle of the electrodes. The electrode buffer was applied to the electrode strips. The anode buffer contained 0.33% L-aspartic acid, 0.37% L-glutamic acid and the cathode electrode buffer contained 0.4% arginine, 0.06% L-lysine and 12% ethylenediamine. Focusing was carried out at a constant power of 7W, and a limiting voltage of 1700 volts was carried out for about 5 hr. After isoelectric focusing, the gel was divided into two, one for protein staining and the other for activity staining. The pIs of SOD can be determined by the standard pI proteins separated on the same gel.

Protein determination

Protein was measured by the method of Bradford [29] using a Bio-Rad protein assay reagent, with BSA as the standard.

Amino-terminal sequencing

The purified MnSOD protein was electrophoresed on a 12.5% SDS- PAGE, then blotted on to a polyvinyldifluoride membrane by a Hoefer mini-transblot cell at 0.3A for 1 hr in the presence of 10 mM CAPS (3-(Cyclohexylamino)-1-propanesulfonic acid) in 20% methanol, pH 11. The membrane strip containing the subunit polypeptides of Mn-SOD of was subjected to amino acid sequencing on an Applied Biosystems 476A protein sequencer.

RESULTS AND DISCUSSION

Identification of SOD activies by PAGE

Extracts from mycelia of *G. microsporum* and other eight strains of *G.* species revealed Mn-SOD as the major activity on native PAGE gels, because neither 8mM KCN nor 8 mM H_2O_2 inhibited the enzymatic activity of the slowly migrating SOD (Fig. 1). While CuZn-SOD is present almost in every eukaryotic species [6], little CuZn-SOD activity could be detected in *Ganoderma* species. This Mn-SOD activity which was present as the sole form of SOD was also found in the fruiting body of *Ganoderma tsugae* [20] and in the mycelia of most edible and medicinal fungi [30].

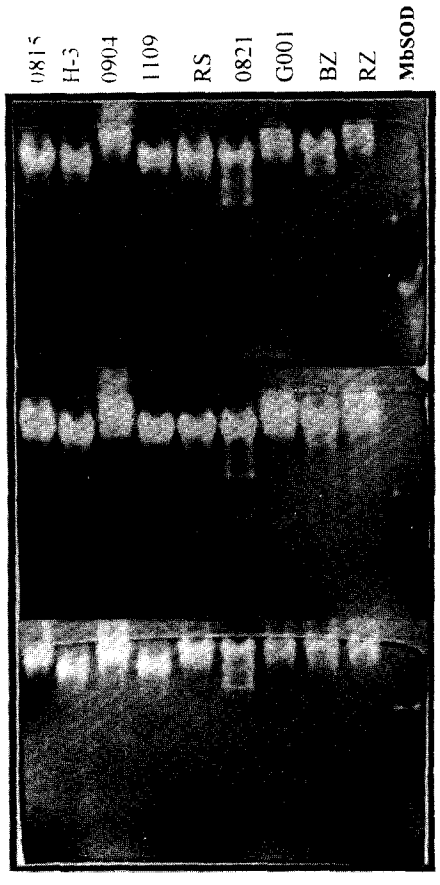

Figure 1. Identification of SOD isozymes from the extract of *G.* species. The upper gel was the control, the middle was the result of treatment with 8 mM H_2O_2, the lower was the result of treatment with 8 mM KCN. 0815 (*G. lucidum*), H-3 (*G. tsugae*), 0904 (*G. fornicatum*), 1109 (*G. tsugae*), RS (*G. boninense*), 0821 (*G. microsporum*), G001 (*G. lucidum*), BZ (*G. sinensis*), RZ (*G. lucidum*), MbSOD (Mungbean SOD).

The nascent polypetide of Mn-SOD could target to localize in the mitochondria [31], peroxisomal matrix [32,33] or other cytosolic subcellular location [34]. The crude extract from 18 strains of the mycelia of *Ganoderma* [21] and 200 strains of edible fungi all showed a similar pattern with the Mn-SOD enzyme being the only SOD form [30]. The high and exclusive Mn-SOD activity in *G.* species imply that cells contain a large amount of mitochondria or the presence of this enzyme in certain subcellular compartments. The latter is consistent with the observation that the derived amino acid sequence based on one Mn-SOD cDNA clone isolated from the mycelia of *G. microsporum* did not indicate the presence of a transit peptide targeting to mitochondria [23].

Heat treatment of SOD

Heat treatments while caused a loss of Mn-SOD activity but increased its specific activity (Table 1). The total Mn-SOD activity was reduced about 57% after preincubation at 75°C for 5 min. However, preincubation at 70°C only caused 33% reduction in activity, but increased specific activity by more than six-fold. Therefore, extracts were incubated at 70°C for 5 min as the first step of purification of Mn-SOD. A heating step was also included in the purification of Mn-SODs from chicken liver [35], bakers yeast [36] and pea [11]. Mn-SOD activity from pea was not as stable as that of the *G. microsporum*; a 5-min incubation at 60°C reduced about 50% of the activity in pea but 67% activity was retained for the *G. microsporum* enzyme after a 5-min incubation at 70°C.

Purification of Mn-SOD from G. microsporum

Ammonium sulfate fractionation of SOD showed that the 50-60% ammonium sulfate saturation was effective in increasing the specific activity, a similar result was observed with the fruiting body of *G. tsugae* [20]. Mn-SOD did not bind to DEAE-52 under the condition described during the first chromatographic step. This property was also reported for the Mn-SODs purified from Scots pine [14]. During gel filtration, Mn-SOD was eluted as a single peak. Purifiaction of Mn-SOD from the mycelia of *G. microsporum* was summarized in Table 2. The recovery of Mn-SOD after gel filtration was 10%, but with an increase of 27-fold purification. This preparation gave only one band of a protein with a molecular mass of 25 kD based on SDS-PAGE (Fig. 2),

Table I. Mn-SOD activity remained after the heat treatment of the extract isolated from the mycelia of *G. microsporum*

Heat treatment (°C)	Total protein (mg)	Mn-SOD activity (x 10^3 U)	Specific activity (U/mg protein)
NO	171	68.1	398
50	89.8	60.4	673
60	35.9	50.5	1407
65	24.3	49.2	2025
70	18.3	45.9	2508
75	17.4	29.1	1672

Table II. Purification of Mn-SOD from mycelia of *G. microsporum*

Purification step	Total protein (mg)	Mn-SOD activity (x 10^{-3} units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Supernatant 12,000*g*	1007	436	433	100.0	1.0
70 ℃, 5 min	122	234	1918	53.6	4.4
$(NH_4)_2SO_4$ 50-60%	28.5	73.9	2593	16.9	6.0
DEAE-52	8.15	50.7	6221	11.6	14.4
Sephacryl SH-200	3.82	44.6	11675	10.2	27.0

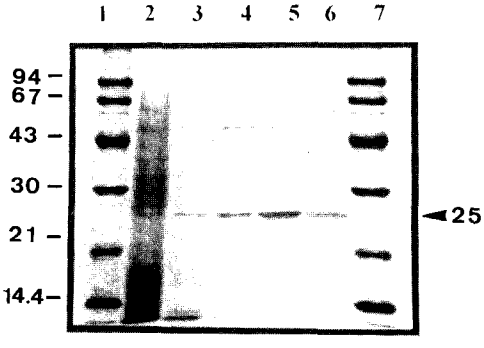

Figure 2. SDS-PAGE of Mn-SOD. Lane 1, 7, Molecular mass markers; lane 2 to 6, the sample was taken from each step of purification: 12,000*g* supernatant, 70℃ treatment, 50-60% sat. $(NH_4)_2SO_4$, DEAE-52, and Sephacryl SH-200. All lanes were stained with CBR reagent.

and this band showed some SOD activity on the denaturation gel (not shown). However, the Mn-SOD activity eluted as a single peak at a position corresponding to a molecular mass of 98 kD by the Superdex-200 filtration. These results suggest that Mn-SOD from *G. microsporum* is a tetramer composed of four subunits of equal size, which are not covalently joined as previously reported [35]. The size of the holoenzyme and the number of subunit in *G. microsporum* agree with a previous report for tetrameric Mn-SODs from other sources [6]. The enzyme activity of purified Mn-SOD from *G. microsporum* had a broad range of pH optima ranging from 5.0 to 10.4 [21].

Isoelectric point determination of Mn-SOD

The purified Mn-SOD was separated into two isoforms on isoelectri focusing, the major activity had a pI 6.34 and the minor one located at pH 5.06. The acidic pI values of the *G.*

Salmonella typhimurium	S	Y	T	L	P	S	L	P	Y	A	Y	D	A	L	E	P	H
Escherichia coli	S	Y	T	L	P	S	L	P	Y	A	Y	D	A	L	E	P	H
Mycobacterium tuberculosis	E	Y	T	L	P	D	L	D	W	D	Y	G	A	L	E	P	H
Saccharomyces cerevision	K	V	T	L	P	D	L	K	W	D	F	G	A	L	E	P	Y
Drosophila melanogaster	K	H	T	L	P	K	L	P	Y	D	Y	A	A	L	E	P	H
Rattus norvegicus	K	H	S	L	P	D	L	P	Y	D	Y	G	A	L	E	P	H
Homo sapiens	K	H	S	L	P	D	L	P	Y	D	Y	G	A	L	E	P	H
Pisum sativum	V	F	T	L	P	D	L	A	Y	D	Y	G	A	L	E	P	V
Maize	T	V	A	L	P	D	L	S	Y	D	F	G	A	L	E	P	V
Pine[a]	S	F	S	L	P	E	L	P	Y	E	Y	S	A	L	E	P	V
G. microsponum (This report)	V	H	T	L	P	A	L	D	Y	P	Y	A	A	L	E	P	H
G. tsugae[b]	V	H	T	L	P	P	L	D	Y	P							
G. lucidum[c]	V	H	T	L	P	P	L	D	Y	P							

Figure 3. Alignment of amino acid sequences from the NH_2-terminus of the subunit of purified Mn-SOD protein from *G. spp.* with the amino acid sequences derived from Mn-SOD cDNA clone of *Salmonella typhimurium* (GenBank accession number U20645), *Escherichia coli* (A24141), *Mycobacterium tuberculosis* (S15205), *Saccharomyces cerevision* (A00521), *Drosophila melanogaster* (L18947), *Rattus norvegicus* (S21661), *Homo sapiens* (S13162), *Pisum sativum* (S18343), Maize (L19461).The amino acid residues common to all sequences are indicated by shadow. a, refer to Steller et al., 1994, b and c, refer to Yeh, 1996.

microsporum SODs are comparable to those described for other plants [14]. Although these two isoforms were not separated by chromatographic steps, sequencing of 17 amino acids from the amino-terminus indicated they are identical. It is possible that Mn-SOD might be encoded by a multigene family in *G. microsporum*. This is consistent with the observation that four distinct Mn-SOD cDNA with high degree of homology were cloned in maize [22]. However, we could not rule out the possibility that the minor activity on IEF might be an artifact generated from partial degradation or denaturation of purified Mn-SOD.

Amino-terminal sequence

The 25-kD polypeptide of purified Mn-SOD from *G. microsporum* had an amino acid sequence of VHTLPALDYPYAALEPH- from the N-terminal end. A homology search of SWISS-PROT databases revealed a certain degree of homology with other known Mn-SOD proteins of many sources (Fig. 3). As expected, species among *Ganoderma* genus shared a high degree of sequence homology but showed a greater variability when compared with angiosperms, a reflection of phylogenetic distance between fungi and angiosperms.

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