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Journal of Chromatography B, 826 (2005) 114–121
JOURNAL OF CHROMATOGRAPHY B
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Purification and partial characterization of recombinant Cu, Zn containing superoxide dismutase of *Cordyceps militaris* in *E. coli*

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Received 4 April 2005; accepted 17 August 2005
Available online 12 September 2005

Abstract

The cDNA of Cu, Zn containing superoxide dismutase from the *Cordyceps militaris* SH (cm-SOD) was overexpressed in *Escherichia coli* BL 21 (DE3) using the pET-21a expression vector. The recombinant cell overexpressed the protein corresponding to 35 ± 3% of total bacterial protein in cytosol. The purification was performed through three steps: DEAE-FF, CM-52, and G-100. After this purification procedure, a specific activity of 27272.7 U/mg of protein was reached, corresponding to 6.1-fold purification with a yield of 85.0%. The purity was homogeneous by SDS–PAGE analysis and 94.2 ± 1.0% by CZE analysis. A subunit molecular mass of the recombinant enzyme was 15704 Da with a Cu and Zn element. In addition, the dimeric and polymeric structures were observed on MALDI-TOF-MS. Isoelectric point value of 7.0 was obtained for the recombinant enzyme that was sensitive to H_2O_2 and KCN. The recombinant enzyme remained 80 ± 2% residual activity at pH 7.8, at 50 °C for 4 h incubation. The properties: N-terminal amino acid sequence (the first 12 amino acid residues), p*I*, subunit molecular mass, thermo-stability of the purified recombinant SOD are similar to that of the native Cu, Zn-SOD from *C. militaris* (N-cm-SOD).

Keywords: *Cordyceps militaris*; Recombinant Cu, Zn containing superoxide dismutase; Purification; Molecular properties

1. Introduction

Cordyceps militaris strain is a broad host range entomopathogenic fungal species recognized as a potential candidate for biological control of pine moth populations [1]. The entomogenous fungal species invade and proliferate within insect larvae, causing a systematic infection that eventually kills the hosts [2]. During host colonization, fungi usually encounter a host resistance response, the generation of an environment around the pathogen rich in reactive oxygen species (ROS) [3]. To cope with this stress condition, fungi present diversity of mechanisms that actively abrogate the oxidative defense responses of hosts by an array of enzymes [3,4].

Superoxide dismutase (SOD)(EC 1.15.1.1), one of this array of enzymes is ubiquitous in all oxygen-respiring organisms, constitutes the first line of defense against oxidative damage by catalyzing the dismutation of superoxide radical to molecular oxygen and hydrogen peroxide [5]. According to the metal ion cofactor identified in their active site, SODs are classified as Mn-SOD [6], Cu, Zn-SOD [7], Fe-SOD [8] and Ni-SOD [9,10]. Fe-SOD is present in the cytosol of prokaryotes as well as in the plastids of some plants [11]. Mn-SOD is found in membrane fractions of prokaryotes and in mitochondria of eukaryotes. Cu, Zn-SOD presented extensively in cytosol of eukaryotic cells is ubiquitous also in mitochondria of the fermentative and respiratory yeasts [12].

Compared with the Fe-SOD and Mn-SOD, the Cu, Zn-SOD suggests a more significant role in the dismutation of extracellularly derived ROS within an animal host [13,14]. In addition, Cu, Zn-SOD gene may play a critical role in organisms' ability to infect, colonize and thrive inside the host [2,3,7]. *C. militaris* belongs to entomopathogenic fungal species, which is potential agent in bio-control [1]. In our previous study, native Cu, Zn-SOD of *C. militaris* SH mycelium (N-cm-SOD) is found as a main SOD that is neutral protein [15]. The aim of the

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doi:10.1016/j.jchromb.2005.08.010

present study was to express the cDNA of the cm-SOD in *Escherichia coli*. The recombinant protein (R-cm-SOD) was purified, and its properties were compared with those of the N-cm-SOD.

2. Materials and methods

2.1. General

C. militaris SH, *E. coli* DH5α, BL21 (DE3), the cloning vector pUC18 and the expression vector pET-21a were stored in our laboratory. PCR primers were synthesized by Sangon Bitotech shanghai (Shanghai, China) in an Applied Biosystems DNA synthesizer 380-A (Perkin-Elmer Co., Boston, USA). T4 DNA ligase, Platinum^{R} Taq DNA polymerase and restriction endonucleases were obtained from Takara (Takara Co. Ltd., Tokyo, Japan). The experiments were performed by using pyrogallol, Coomassie brilliant blue, phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., MO, USA), electrophoresis molecular weight marker kits (MBI Fermentas Inc., Amherst, New England). *O*-diethylaminoethyl-sepharose^{TM} fast flow (DEAE-FF), Carboxy methylcellulose (CM-52) and Sephadex G-100 were bought from Pharmacia (Pharmacia Fine Chemicals, Upsala, Sweden). Bradford protein assay reagent was from Bio-Rad (Bio-Rad, CA, USA). Optical measurements were made with a spectrophotometer (WZF Uv-2100, Unico, Shanghai, China). These and all other chemicals reagents used were of analytical grade.

2.2. RT-PCR amplification and construction of pET-cm-SOD vector

C. militaris SH mycelia RNA were obtained using Trizol Reagent (Sigma, Chemical Co., MO, USA), according to manufacture instructions. The cDNA was prepared using the superscript preamplification system for first-strand cDNA synthesis Kit (Invitrogen Co., OR, USA). Second cDNA fragment corresponding to the open reading frame (ORF) of *C. militaris* SH Cu, Zn-SOD (cm-SOD) was amplified by high fidelity polymerase chain reaction with Cu, Zn-SOD specific primers. The first-strand cDNA was used as the template. The primer1 (5′-TTCATATGGTCAAAGCAGTCTGCGTTC-3′) and primer2 (5′-GAATTCTTAGTTGGCGACGCCAAGTAC-3′) were designed according to the nucleotide sequence of Cu, Zn-SOD gene of *C. militaris* SH (GeneBank accession no AY822477). The primers contained the restriction sites for *Nde*I in the primer1 and *Eco*RI in the primer2, respectively. The amplification conditions for PCR were: 94 °C for 5 min (hot start), followed by 35 cycles of 94 °C for 40 s, 55 °C for 1 min, 72 °C for 1 min and finally 72 °C for 10 min. The PCR product was extracted with S.N.A.P UV-free gel purification kit (Invitrogen Co., OR, USA) and ligated into the *Nde*I–*Eco*RI restriction sites of pET-21a to construct the pET-cm-SOD expression vector that was transformed to competent *E. coli* BL21 (DE3) cells. The insert confirmed by DNA sequencing.

2.3. Overexpression of the cm-SOD

The R-cm-SOD protein was overexpressed in *E. coli* cells by using the strong inducible T7 promoter under control of the presence of isopropyl-β-thiogalactopyranoside (IPTG). Seed culture (at the final concentration of 1%) was inoculated into 500 ml flask containing 200 ml LB medium including 0.4 mM $CuSO_4$, $ZnSO_4$ and 100 μg/ml ampicillin. The cultures were shaken (incubator shaker: Shanghai Xinrui automatic apparatus Co. Shanghai, China) (200 rpm/min) at 37 °C. When the optical density of the cells at 600 nm (OD 600) approached to 0.5, IPTG was simultaneously added to the cultures with the final concentration of 0.4 mM. After 3–4 h of induction, the cells were harvested by centrifugation at 4000 × *g* for 30 min, and washed two times. The harvested cells were resuspended in 20 mM Tris–HCl buffer (pH 8.8), homogenized with a sonic power (300 W, running 5 s, resting 5 s, effective running time of 3 min) and then centrifuged at 12,000 × *g* for 20 min. The supernatant was stored at −20 °C and used for further experiment.

2.4. Culture conditions of C. militaris SH

C. militaris SH strain was maintained on potato dextrose agar (PDA) slants at 4 °C and was grown on PDA at 23 °C for 15 days before inoculation a pieces of culture (5 mm × 5 mm) into 250 ml flasks containing 100 ml potato dextrose broth. The cultures were incubated on a rotary shaker (160 rpm) at 23 °C for 4 days as seeds. Seeds were inoculated at the final concentration of 5% into 500 ml flasks containing 200 ml synthetic medium with following composition: 20% potato broth; 2% glucose; 0.3% yeast extract; 0.1% KH_2PO_4; 0.05% $MgSO_4{\cdot}7H_2O$. Microelements solution including $FeSO_4{\cdot}7H_2O$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4$, and $MnSO_4{\cdot}2H_2O$ (each microelement is contained at the concentration of 4 ppm). The cultures were incubated on a rotary shaker (160 rpm) at 23 °C for 6 days. After cultivation, biomass was filtered through gauze and washed with 20 mM Tris–HCl buffer (pH 8.8) two times. The cultured filtrates were stored at −20 °C.

2.5. Isolation and purification of the R-cm-SOD

Three steps of chromatography were performed in purification of the R-cm-SOD. The supernatant containing R-cm-SOD was loaded directly onto a DEAE Sepharose^{TM} Fast Flow (DEAE-FF) (1.6 cm × 8 cm) column, which was pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.8). The flow-through solution in which high SOD activity found was collected and dialyzed against 10 mM phosphate buffer (pH 5.8) overnight with two changes. The CM-52 column (1.6 cm × 4 cm) equilibrated with 10 mM phosphate buffer (PBS) (pH 5.8) was then applied to purify SOD further. The column was eluted by a NaCl linear gradient of 0–800 mM in the same buffer at 0.3 ml/min flow rate. Fractions of every 3 ml elution were collected. The pooled fractions with SOD activity were condensed by polyethylene glycol 20000 (PEG), and then applied to a Sephadex G-100 (1 cm × 60 cm) column equilibrated previously with 4 mM PBS buffer (pH 7.4). Two milliliters of fraction were collected at a

flow rate of 8 ml/h. The fractions with SOD activity were concentrated by lyophilization and used for further studies.

2.6. Isolation and purification of the N-cm-SOD

Wet mycelia were frozen at −20 °C and thawed overnight at 4 °C and then suspended in 4 volume 20 mM Tris–HCl buffer (pH 8.8) containing 1 mM PMSF. The suspended material was homogenized with a polytron homogenizer. Cell debris was removed by centrifugation at 15,000 × *g*, at 4 °C for 15 min. Crude extract was brought to 50% saturation of ammonium sulfate by gradually adding solid ammonium sulfate and was stirred for 1.5 h. The precipitate was removed by centrifugation at 15,000 × *g* for 15 min. Supernatant fraction was brought to 95% saturation with solid ammonium sulfate and was stirred for 1.5 h. After centrifugation, the pellet was dissolved in 20 mM Tris–HCl buffer (pH 8.8), and dialyzed against the same buffer overnight with two changes. The further purification steps were the same as the procedure for the R-cm-SOD detailed above.

Absorbance at 280 nm, protein content and SOD activity were monitored for each fraction and each purification step.

2.7. SOD activity assay

SOD activity assay system was based on the inhibitory effect of SOD on the spontaneous autoxidation of pyrogallol [16]. One unit was defined as the amount of SOD required to inhibiting the initial rate of pyrogallol autoxidation by 50% and was expressed as U per mg protein (U/mg protein). Protein was determined according to Bradford method, by using bovine serum albumin (BSA) as the standard [17].

2.8. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on Gibco BRL vertical electrophoresis apparatus Mini-V8.10 (Bio-Rad, CA, USA) according to Laemmli method [18]. Gels were stained for proteins with Coomassie brilliant blue R-250. The following proteins were used as SDS–PAGE electrophoresis molecular weight standards: rabbit phosphorylase b (97,400), bovine serum albumin (BSA) (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,000) and hen egg white lysozyme (14,400). Visualization of the SOD activity on native PAGE gels was performed by using the nitroblue tetrazolium (NBT) illumination method described by Beauchamp and Fridovich [19]. Isoelectric points were determined by isoelectric focusing (IEF) on 5% polyacrylamide gels in the presence of 2% ampholines (pH 3–10). The purified enzyme was loaded onto the horizontal gel maintained at 2 °C. The voltage was increased stepwise: 100 V for 15 min, 200 V for 15 min, 450 V for 60 min by using Model 111 Mini IEF Cell (Bio-Rad, CA, USA). The gel was maintained at 2 °C during the run. The p*I* markers (Bio-Rad, CA, USA) ranging from 4.65 to 9.6 were co-electrophoresed to be estimated the p*I* of the protein. After IEF, the proteins were stained with Coomassie brilliant blue R-250.

2.9. Capillary zone electrophoresis (CZE)

CZE experiments were performed on a Beckman P/ACE MDQ CE System equipped with a diode array detector (Beckman Coulter, Fullerton, CA, USA), using a coated fused-silica capillary (Total length 50 cm) (Beckman Coulter, Fullerton, CA, USA) with 50 μm I.D. and an effective length of 40 cm. A computer and MDQ software (5.0 Version) were used for instrument control and for data collection and processing. Before use, the capillary was flushed for 10 min with 0.1 M HCl, water and run buffer, respectively. The run buffer was citrate buffer, pH 3.0 (Methods development kit/proteins, Beckman Coulter, Fullerton, CA, USA). Between injections, the capillary was rinsed with run buffer (5 min). The separation voltage was 20 kV and the temperature was set to 25 °C. Commonly, samples were hydrodynamically injected at 0.5 psi for 5 s, and detection was carried out at 214 nm. Each sample (1 mg/ml, protein) was analyzed in triplicate.

2.10. Automatic amino acid sequence analysis

The purified enzyme (10 μg) was loaded on each track of SDS–PAGE gel (15% polyacrylamide). After electrophoresis, the gel was electrophoretically transferred onto PVDF membrane cartridge. N-terminal amino acid sequence analysis was performed using an applied Biosystems sequencer (ABI491A, PE Co., Boston, USA).

2.11. Mass spectrometric analysis and spectrometric characterization

Mass spectrum of the purified R-cm-SOD and the N-cm-SOD were obtained by Matrix-assisted laser desorption ionization time of flight mass spectrum (MALDI-TOF-MS) (Beckman Co., Reflex-III, Bruker, Germany). The sample (100 pmol) was dissolved in 0.1% (v/v) TFA, and applied to the target. Trypsinogen (23,982 Da) and bovine serum albumin (66,430 Da) were used for mass scale calibration. Ultraviolet absorption spectrum of the purified SOD ranging from 250 to 500 nm was recorded on a Beckman DU-600 spectrophotometer (Beckman Co., Bruker, Germany).

2.12. Determination of metal content

Metal content of the enzyme was determined by ICP-AES absorption (Varian, Spectr AA-300 PLUS, CA, USA) after the enzyme was dialyzed extensively against 10 mM phosphate buffer (pH 7.4), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and followed by buffer lacking EDTA.

2.13. The characters of the R-cm-SOD

Thermal stabilities of the purified enzyme were determined in 20 mM PBS buffer (pH 7.8), at 50 and 60 °C in incubation for 0–4 h, respectively. The residual activity was measured every 30 min. The effects of H_2O_2 and KCN treatment on SOD activity were investigated. The enzyme solution containing

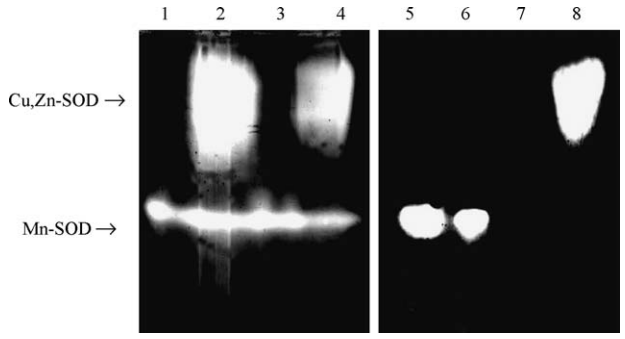

Fig. 1. Identification of Cu, Zn-SOD activity in different extracts of *E. coli* cultured in LB medium and purified R-cm-SOD by 8% native-PAGE gel staining with NBT: lane 1, total protein extract from control cells; lane 2, soluble proteins of recombinant cells; lane 3, soluble proteins of control cells; lane 4, total proteins of recombinant cells; lane 5, soluble proteins of recombinant cells with 5 mM H_2O_2; lane 6, soluble proteins of control cells with 5 mM KCN (lane 1–6 had about 20 μg protein loaded); lane 7, the purified R-cm-SOD after G100 with 5 mM KCN (H_2O_2 and KCN were added into crude extracts before electrophoresis); lane 8, the purified R-cm-SOD after G100 (lanes 7 and 8 had about 5 μg protein loaded).

each compound at different concentration levels was incubated in 20 mM PBS buffer (pH 7.8), at 25 °C for 30 min, and then assayed under the standard conditions. All purified enzyme solutions used were 400 U/ml enzyme (0.02 mg/ml, protein).

3. Results and discussion

3.1. Overexpression

The activity of R-cm-SOD protein in crude extracts and soluble fractions of *E. coli* cells with (recombinant cells) or without the cm-SOD gene (control cells) was analyzed by using native PAGE gels for superoxide dismutase. As presented in Fig. 1, PAGE of *E. coli* extracts showed that the Mn- and Cu, Zn-SOD which were classified by their sensitivity to KCN and H_2O_2. The Mn-SOD was identified in both crude extract and soluble fraction of both two cells (lane 1–6), and it was insensitive to H_2O_2 or KCN (lanes 5 and 6), which is the characteristic of Mn-SOD [20]. This result suggested Mn-SOD was intrinsic SOD. However, the Cu, Zn-SOD protein was detected only in crude extract or soluble fraction of the recombinant cells (lanes 2 and 4). Its activity was inhibited by H_2O_2 or KCN (lanes 5 and 7), which is the characteristic of Cu, Zn-SOD [21]. Intrinsic Cu, Zn-SOD of *E. coli* has been found within the periplasmic space in *E. coli* [21], but it is too instable to be detected on activity stain gels [22]. Fe-SOD was insentive to KCN and sentive to H_2O_2 [21]. Therefore, the intensity of the overexpressed Cu, Zn-SOD only in recombinant cells indicated that it was recombinant Cu, Zn-SOD (namely R-cm-SOD) and was much more abundant than Mn-SOD. SDS–PAGE analysis revealed the R-cm-SOD produced corresponded to 35 ± 3% of total bacterial protein as determined by densitomeric gel scanning (Fig. 2(A), lane 1).

The content of cm-SOD in *C. miltiraris* mycelia was so low that a significant band could not be observed on SDS–PAGE in corresponding position (Fig. 2(B), lane 5); however, a large amount of cm-SOD was overexpressed in *E. coli* (Fig. 2(A), lane 1). This is ideal method to obtain a large amount of aimed protein for further study.

3.2. Purification step

The procedures for isolating the R-cm-SOD included anion exchange chromatography, cation exchange chromatography and size exclusion chromatography. A summary of the purification procedures is presented in Table 1. The DEAE-FF column used did not absorb the Cu, Zn-SOD at pH 8.8, but high efficiency was accomplished by removal of the majority of contaminants (Fig. 2(A), lane 4). This is consistent with the observation in Q-Sepharose Fast Flow column by Osatomia et al. [23] and Liu et al. [24]. An 88.4% of SOD activity was recovered in the flow-through solution with a 3.3-fold increase in specific activity after this step. The CM-52 cation exchange chromatography was used to purify the enzyme further. An 87.9% of SOD activity was recovered in the pooled fraction which was collected at range of concentration of NaCl from 0.20 to 0.35 mM with a 5.2-fold increase in specific activity. Then the size exclusion chromatographic step was used as a desalting step and removed some of the impurities. After the last step, the specific activity

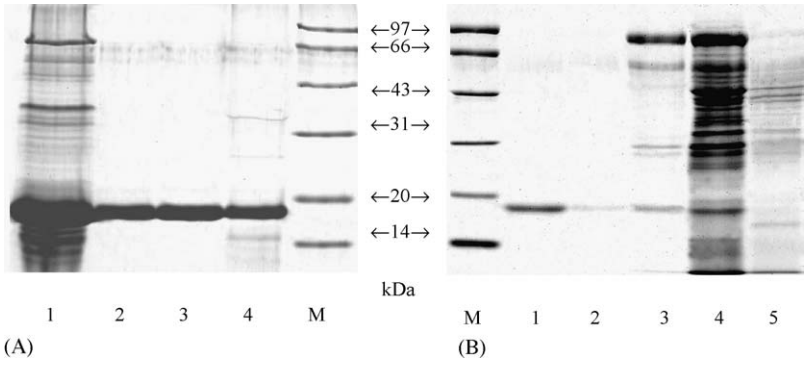

Fig. 2. The purifications of the R-cm-SOD (A) and the N-cm-SOD protein (B) were done by ion-exchange chromatography and gel filtration. The protein samples were run on 13.5% SDS–PAGE gel stained with Coomassie blue. (A) Lane 1, crude supernatant fraction (40 μg protein); lane 2, G-100 purified fraction (10 μg protein); lane 3, CM-52 purified fraction (15 μg protein); lane 4, DEAE-Fast Flow purified fraction (20 μg protein). (B) Lane 1, enzyme solution after G-100 column (5 μg protein); lane 2, enzyme solution after CM-52 column (10 μg protein); lane 3, enzyme solution after DEAE-FF column (20 μg protein); lane 4, ammonium sulfate precipitation (40 μg protein); lane 5, crude extracts (40 μg protein); lane M, molecular weight markers (in kDa).

Table 1
Purification of the recombinant-Cu, Zn-SOD in *E. coli*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude extract	46.4	208362.6	4488.6	100.0	1.0
DEAE-FF	12.4	184285.4	14873.7	88.4	3.3
CM-52	7.8	183200.3	23457.2	87.9	5.2
Sephadex G-100	6.5	177000.1	27272.7	85.0	6.1

Table 2
Purification of the native Cu, Zn-SOD from *C. militaris* mycelia

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude extract	255.2	16443.5	64.4	100.0	1.0
$(NH_4)_2SO_4$ Precipitation	89.7	8363.3	93.2	50.9	1.5
DEAE-FF	19.8	7478.8	377.7	45.9	5.9
CM-52	3.7	3680.5	13631.6	22.4	211.6
Sephadex G-100	0.2	2900.2	17060.2	17.6	264.9

of the purified R-cm-SOD was 6.1-fold increase and was determined to be 27272.7 U/mg of protein that was higher than that of the purified N-cm-SOD (17060.2 U/mg of protein) (Table 2) and the other source SODs [7,22]. Native-PAGE analysis showed one band for the purified R-cm-SOD (Fig. 1, lane 8) and the activity of the purified R-cm-SOD was inhibited by KCN (Fig. 1, lane 7). SDS–PAGE analysis demonstrated the homogeneity of the R-cm-SOD (Fig. 2(A), lane 2). The purified R-cm-SOD presented a purity of 94.2 ± 1.0% by capillary zone electrophoresis (Fig. 3(A)). In addition, the MALDI-TOF-MS spectrum of the protein gave no indications of any major impurities (Fig. 4(A)).

The purified N-cm-SOD was obtained through four steps: ammonium sulfate precipitation, anion exchange chromatography, cation exchange chromatography (the pooled fraction was collected at range of concentration of NaCl from 0.20 to 0.25 mM in eluting buffer), and gel filtrate. A summary of the purification procedures is presented in Table 2. After above-mentioned steps, 17.6% of SOD activity was recovered with a 264.9-fold increase in specific activity determined to be 17060.2 U/mg of protein. This yield and activity were higher than that reported by Wang [15]. SDS–PAGE analysis demonstrated the purification to homogeneity of the purified enzyme (Fig. 2(B), lane 1) for which a purity of 92.1 ± 1.0% was obtained by capillary zone electrophoresis and it was confirmed by MALDI-TOF-MS (Fig. 4(B)).

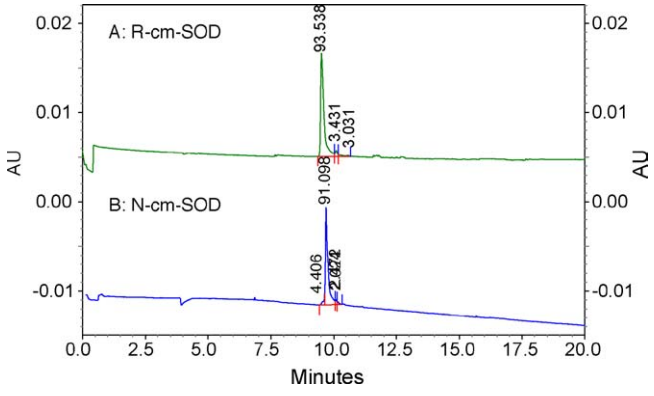

Fig. 3. Capillary zone electrophoresis of the purified cm-SOD. Experimental conditions: carrier electrolyte, citrate buffer; pH 3.0 (Methods development kit/proteins); separation voltage, 20 kV; temperature, 25 °C; UV detection, 214 nm; pressure injection, 0.5 psi for 5 s; column size, 50 cm (an effective length of 40 cm) × 50 μm I.D.; sample, 1 mg/ml.

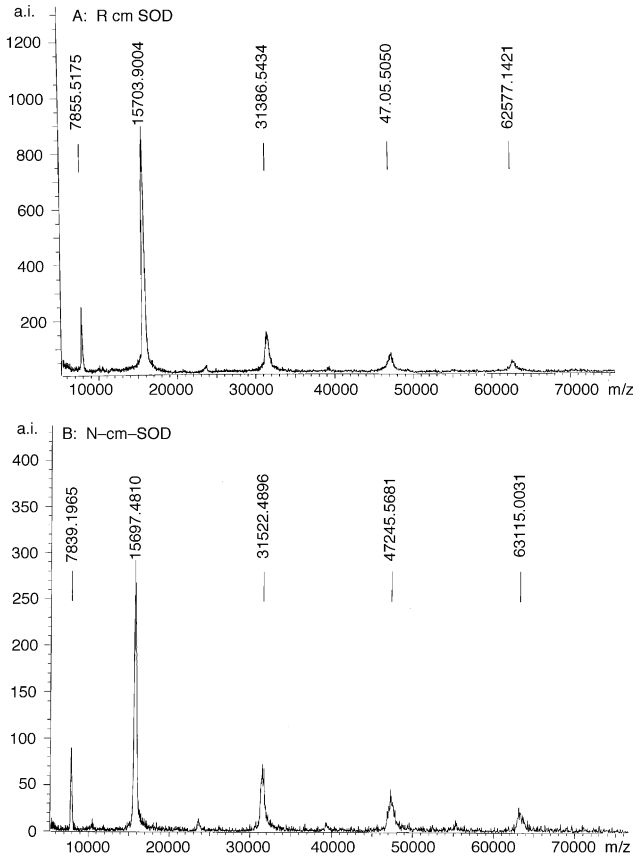

Fig. 4. Matrix-assisted laser desorption ionization time of flight mass spectrum (MALDI-TOF-MS) of the functional unit of the R-cm-SOD ((A) 100 pmol) and N-cm-SOD ((B) 100 pmol). The samples were dissolved in 0.1% TFA and applied onto the target. Solution of trypsinogen (23982 Da) and bovine serum albumin (66430 Da) were used to calibrate the mass scale.

Table 3
Comparison of N-terminal amino acid sequences of the purified enzyme with other Cu, Zn-SODs of fungi and *E. coli*

Cu, Zn-SOD sources	Amino acid sequence												Similarity[a] (%) (total no. of amino acids)
	1	2	3	4	5	6	7	8	9	10	11	12	
R-cm-SOD	V	K	A	V	C	V	L	R	G	D	A	K	This study
C. militaris [15]	V	K	A	V	C	V	L	R	G	D	A	K	100
C. militaris #	V	K	A	V	C	V	L	R	G	D	A	K	100
C. sinensis ##	V	K	A	V	C	V	L	R	G	D	S	K	98
H. lutea 110 [25]	V	K	A	V	A	V	L	R	G	D	S	K	83
S. cerevisiae [26]	V	Q	A	V	A	V	L	K	G	D	A	G	75
A. fumigatus [27]	–	V	A	V	A	V	L	R	G	D	S	K	73
E. coli [22]	A	S	E	K	V	E	M	N	L	V	T	S	–

Genebank, Accession No. (#) AY822477; Genebank, Accession No (##) AY438328; (–) no homology.
[a] Percent sequence similarity in amino acid overlap.

3.3. Compared N-terminal amino acid sequences of the purified enzyme with other source Cu, Zn-SODs

The deduced amino acid sequence of the cm-SOD (GeneBank accession no. AY822477) is 154. Compared it with the complete amino acid sequences of Cu, Zn-SODs from *C. militaris* (GeneBank accession no. AY195841), *C. tenuipes* (GeneBank accession no. AY176060) and *C. sinensis* (GeneBank accession no. AY438328) revealed 100, 94 and 77% homology, respectively. The N-terminal amino acid sequence of the purified R-cm-SOD was determined for the first 12 amino acid residues, and the comparison of the amino acid sequence with other known sequences showed extensive homology (73–100%) with Cu, Zn-SODs from fungi (Table 3). It is noted that N-terminal methionine in SOD is initiating amino acid which is removed after its synthesis (Table 3). The purified R-cm-SOD sequences demonstrated 100% identity with native Cu, Zn-SOD of *C. militars*, 98% identity with *C. sinensis* SOD, which had one amino acid difference, but no homology with bacterium *E. coli* Cu, Zn-SOD. Furthermore, this confirmed the R-cm-SOD was heterologous Cu, Zn-SOD.

3.4. Molecular properties of the R-cm-SOD

The molecular mass was measured by several methods. On SDS–PAGE, both the R-cm-SOD and N-cm-SOD (Fig. 2) were estimated a subunit molecular mass of 17,000 Da. However, the subunit molecular mass of the R-cm-SOD was 15704 Da (the highest mass peak) assessed by MALDI-TOF-MS (Fig. 4(A)), which was excellent agreement with the theoretical subunit molecular mass 15708 Da (15579 Da for apo-SOD+ 129 Da for Cu plus Zn) (GeneBank accession no.: AY822477) and 15697 Da of the N-cm-SOD mass (Fig. 4(B)). In addition, the double electric charge peak of the R-cm-SOD was presented as 7856 Da. Its dimeric and polymeric structures were also detected on MALDI-TOF-MS (Fig. 4(A)). The similar result was observed for the N-cm-SOD (Fig. 4(B)). Furthermore, no significant different was observed between both cm-SODs on MALDI-TOF-MS. SODs are classified mainly as Mn-SOD [6], Cu, Zn-SOD [7], and Fe-SOD [8] by the metal ion cofactor identified in their active site. The mitochondrial Mn-SOD is a tetrametic protein with 22 kDa subunits [6]; Fe-SOD is a dimeric protein [28] or a tetrametic protein with 22 kDa subunits [29]. Most of Cu, Zn-SODs are a dimeric protein with a 16 kDa per subunits [7,25,30,31]. However, the Cu, Zn-SOD of *Phanerochaete crysosporium* is a homodimer with identical molecular mass of 22 kDa [32]; Cu, Zn-SODs from *Aspergillus* spp. are tetrameric or pentameric structures [27,33], whereas the extracellular Cu, Zn-SOD is a tetrameric glycoprotein composed of four 30 kDa subunits [34]. In its subunit molecular mass, the cm-SOD resembles many Cu, Zn-SODs analyzed by MALDI-TOF-MS from other fungi [25,30,31]. However, the mechanism of high specific activity for cm-SOD was not clear, and this might be related to its unique polymeric structure.

Isoelectric point value of 7.0 for the R-cm-SOD was in concordance with the N-cm-SOD (Fig. 5). In contrast, the p*I* values between the recombinant SOD and native SOD from Peiking duck are different [24]. Furthermore, this neutral p*I* value of the cm-SOD is also different from p*I* values (5.0–6.0) of Cu, Zn-SODs from other fungi [7,27,33]. The p*I* value of Cu, Zn-SOD from *E. coli* is 5.3 [22]. The amount of Cu, Zn ions in the purified recombinant SOD is affected by the amount of Cu,

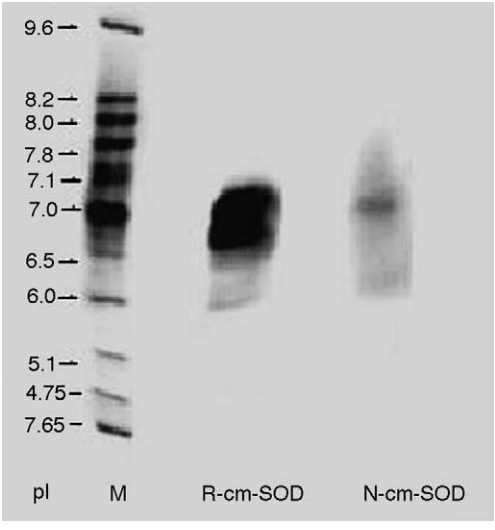

Fig. 5. Isoelectric focusing of the purified enzyme with 5% polyacrylamide gel in the presence of 2% ampholines (pH 3–10). M, standard protein makers; R-cm-SOD, the recombinant Cu, Zn-SOD (50 μg protein); N-cm-SOD, the native Cu, Zn-SOD (10 μg protein).

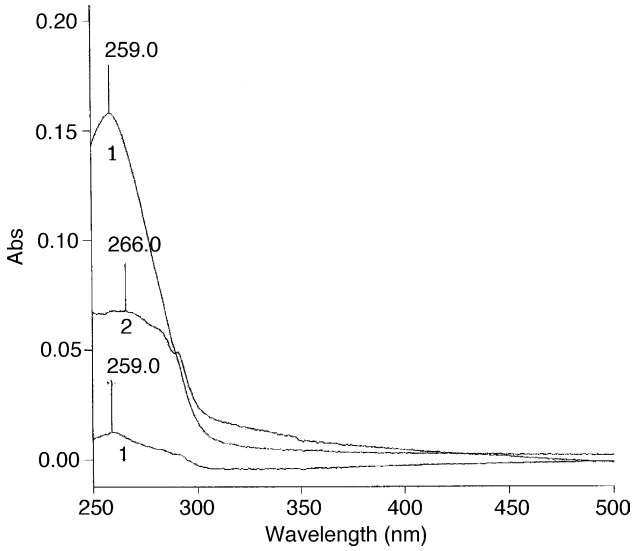

Fig. 6. Optical absorption spectra of Cu, Zn-SOD. Line 1, the recombinant cm-SOD (1 or 0.1 mg/ml protein); line 2, the native cm-SOD (0.5 mg/ml protein).

Zn ions in cell cultured medium and low Cu ion causes that most of the Cu sites are occupied by Zn ions in LB medium [34]. Despite the equal amount of copper and zinc ion in the cell cultured media, ICP-AES spectroscopy revealed the R-cm-SOD contained 0.90 ± 0.02 atom of copper and 0.51 ± 0.01 zinc per subunit. This result suggested that other factors might affect the metal copper and zinc absorbing or distributing in cells. In addition, the N-cm-SOD contained 0.86 ± 0.02 atom of copper and 1.01 ± 0.03 zinc per subunit.

Compared the UV profiles of the R-cm-SOD and the N-cm-SOD (Fig. 6), they are representative for the Cu, Zn-SOD enzyme family which lack of tyrosine and tryptophan residues do not exhibited a peak at 280 nm [25]. The R-cm-SOD exhibited a deep peak at 259 nm that was slightly different from a blunt peak at 259–660 nm for the N-cm-SOD, which may result from their different conformations. The similar phenomenon has been observed in recombinant human Cu, Zn-SOD by Hartman [34]. The UV spectrum of Human Cu, Zn-SOD from recombinant *E. coli* cultured in LB medium is 279 nm, but it is 265 nm for reconstituted Human Cu, Zn-SOD, apo-enzyme, and obtaining from recombinant *E. coli* cultured copper-supplemented casein hydrolysate medium [34]. The UV spectrum of the cm-SOD was also distinguished from the UV spectrum (275 nm) of Cu, Zn-SOD of *E. coli* [22]. In its UV spectrum, the cm-SOD resembles to Cu, Zn-SOD rather than Mn- or Fe-SOD that presents a maximum of UV spectrum at 280 nm.

3.5. Thermo-stability variations of enzyme activity

The thermo-stability variations were investigated by pre-incubating the R-cm-SOD and the N-cm-SOD in 20 mM PBS buffer, pH 7.8, at 50 and 60 °C with respect to time, respectively. The remaining activity values were determined under the standard assay conditions. As can be seen in Fig. 7, no differences were observed between the R-cm-SOD and the N-cm-SOD at either 50 or 60 °C. The residual activities were maintained at

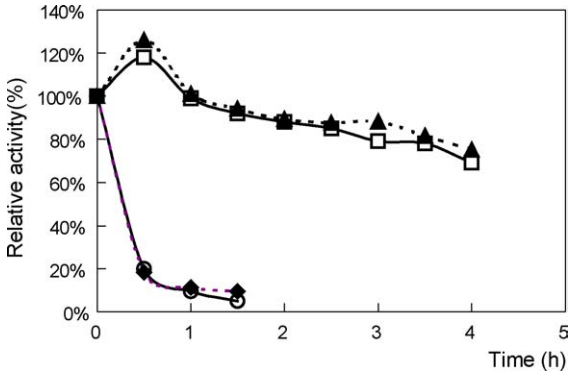

Fig. 7. Temperature stability variations for incubation of the recombinant cm-SOD and the native cm-SOD at pH 7.8 with respect to time (0–4 h): (□) the native cm-SOD incubation at 50 °C; (◯) the native cm-SOD incubation at 60 °C; (▲) the recombinant cm-SOD incubation at 50 °C; (◆) the recombinant cm-SOD incubation at 60 °C.

about $80 \pm 2\%$, at 50 °C for 4 h, but apparent inactivation was observed for both the R-cm-SOD and the N-cm-SOD at 60 °C for 30 min. These results are consistent with that of many fungus source Cu, Zn-SODs which are low thermo-stability [33], and indicates that the thermo-stable character of the products of the cm-SOD gene expressed in *E. coli* is not changed. In contrast, Cu, Zn-SODs from *Aspergillus fumigtus*, bovine and human retain 100, 70, and 40% activity after pretreatment 1 h at 70 °C, respectively [27,35]. Three cysteine residues, Cys6, Cys58, and Cys147 are present in the deduced amino acid sequence of cm-SOD of *C. militaris* (GeneBank accession no AY822477). The low thermo-stability of the cm-SOD might be related with its free Cys6 [35]. Site-directed mutagenesis studies suggest that free cysteine residues might be the major molecular determinants for different protein stabilities among various Cu, Zn-SODs [35–39]. Some investigators confirmed that human SOD exhibited higher thermo-stability after removal of its free Cys6 and Cys111 [36,38].

3.6. The effect of H_2O_2 and KCN treatment on SOD activity

Hydrogen peroxide and KCN are known inhibitors of Cu, Zn-SOD activity and are widely used to distinguish between different SOD types. Hydrogen peroxide and KCN inactivate Cu, Zn-SOD, whereas the Mn-SOD is resistant to hydrogen peroxide and KCN mediated inhibition. Fe-SOD is sensitive to hydrogen peroxide and insensitive to KCN [40]. The R-cm-SOD and the N-cm-SOD were assayed after incubating the protein samples at increasing concentrations of hydrogen peroxide and KCN. As shown in Fig. 8, the SOD activity of the recombinant protein declined as the concentration of hydrogen peroxide and KCN were increased in incubation for 30 min. This hydrogen peroxide and KCN inhibition of SOD activity of the purified recombinant protein confirmed further it to be a Cu, Zn-SOD. The same results were observed for the N-cm-SOD (Fig. 8).

The present study described overexpression of the cm-SOD gene from *C. militaris* in cytoplasm of *E. coli*, purification and molecular characters of recombinant protein. The recombinant enzyme was sensitive to KCN and H_2O_2, contained Cu and Zn

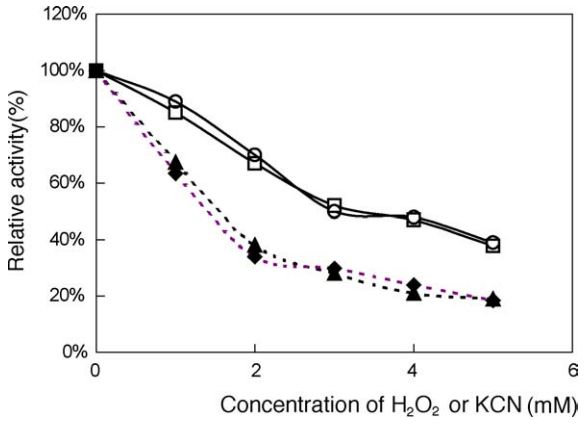

Fig. 8. The effect of hydrogen peroxide (dot lines) and KCN (solid lines) treatment on the SOD activity. (□) The native cm-SOD treated with KCN; (◯) the recombinant cm-SOD treated with KCN; (▲) the native cm-SOD treated with H_2O_2; (♦) the recombinant cm-SOD treated with H_2O_2.

ions, represented a maximum spectrum at 259 nm, and had a subunit molecular mass of 15704 Da. These proprieties are similar to those of most known Cu, Zn-SODs that indicates little evolutionary divergence has taken place in Cu, Zn-SOD. The properties: p*I*, molecular mass, and thermo-stability of the purified recombinant SOD are similar to that of the native Cu, Zn-SOD from *C. militaris*. But the mechanism of high specific activity for cm-SOD was not clear. Further studies are performing for the correlation between its high structure, the low thermo-stability and the high specific activity.

Acknowledgements

The authors acknowledge the additional support for IEF and MALDI-TOF-MS by Research Center for Proteome Analysis, Key Lab of Proteomics, Institute of Biochemistry and Cell Biology, Shanghai, Institutes for Biological Sciences Chinese Academy of Sciences. The authors are grateful to Ping Shi (post doctor in State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology) for CZE technique support.

References

[1] S. Alicja, Towards an integrated management of *Dendrolimus pini* L. Proceedings, USDA forest service general technical report NE- 247, 1998, p. 129.
[2] J.M. Clarkson, A.K. Charnley, Trends Microbiol. 4 (1996) 197.
[3] A.M. Mayer, R.C. Staples, N.L. Gil-ad, Phytochemistry 58 (2001) 33.
[4] F.O. Perkins, J. Shellfish Res. 15 (1995) 67.
[5] J.M. McCord, I. Fridovich, J. Biol. Chem. 244 (1969) 6049.
[6] I. Fridovich, Annu. Rev. Biochem. 44 (1975) 147.
[7] S.E. Bittencourt, L.A. Castro, S.E. Farias, Res. Microbiol. 155 (2004) 681.
[8] F.J. Yost, I. Fridovich, J. Biol. Chem. 248 (1973) 4905.
[9] E.J. Kim, H.J. Chung, B. Suh, Y.C. Hah, J.H. Roe, J. Bacteriol. 180 (1998) 2014.
[10] H.D. Youn, E.J. Kim, J.H. Roe, Y.C. Hah, S.O. Kang, Biochem. J. 318 (1996) 889.
[11] C. Bowler, M. Van Montagu, D. Inze, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 83.
[12] T.S. Nedeva, V.Y. Petrova, D.R. Zamfirova, E.V. Stephanova, A.V. Kujumdzieva, FEMS Microbiol. Lett. 230 (2004) 19.
[13] M. Lynch, H. Kuramitsu, Microbes Infect. 2 (2000) 1245.
[14] L.T. Benov, I. Fridovich, Arch. Biochem. Biophys. 327 (1996) 249.
[15] Z.S. Wang, Z.J. He, S.X. Li, Q.S. Yuan, Enzyme Microb. Technol. 36 (2005) 862.
[16] S. Marklund, G. Marklund, Eur. J. Biochem. 47 (1974) 248.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] U.K. Laemmli, Nature 227 (1970) 680.
[19] C. Beauchamp, I. Fridovich, Anal. Biochem. 44 (1971) 276.
[20] A. Carlioz, D. Touati, EMBO J. 5 (1984) 623.
[21] A. Battistoni, G. Rotilio, FEBS Lett. 374 (1995) 199.
[22] L.T. Benov, W.F. Beyer, J.R.R.D. Stevens, I. Fridovich, Free Radic. Biol. Med. 21 (1996) 117.
[23] K. Osatomi, Y.K. Masuda, K. Hara, T. Ishihara, Comp. Biochem. Physiol. Part B 128 (2001) 751.
[24] W. Liu, R.H. Zhu, G.P. Li, D.C. Wang, Protein Exp. Purif. 25 (2002) 379.
[25] P. Dolashka-Angelova, M. Angelova, L. Genova, S. Staoeva, W. Voelter, Spectrochim. Acta Part A 55 (1999) 2249.
[26] O. Bermingham-McDonogh, E.B. Gralla, J.S. Valentine, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 4789.
[27] M.D. Holdom, R.J. Hay, A.J. Hamilton, Free Radic. Res. 22 (1995) 519.
[28] M. Kasunose, K. Ichihara, Y. Noda, J. Biochem. 80 (1976) 1343.
[29] K. Asada, K. Yoshikawa, Takahashim, J. Biol. Chem. 250 (1975) 2801.
[30] P. Dolashka-Angelova, S. Stevanovic, A. Dolashki, M. Angelova, J. Serkedjieva, E. Krumova, S. Pashova, S. Zacharieva, W. Voelter, Biochem. Biophys. Res. Commun. 317 (2004) 1006.
[31] M. Angelova, P. Dolashka-Angelova, E. Ivanova, J. Serkedjieva, L. Slokoska, S. Pashova, R. Toshkova, S. Vassilev, I. Simeonov, H.J. Hartmann, S. Stoeva, U. Weser, W. Voelter, Microbiology 147 (2001) 1641.
[32] R. Ozturk, L.A. Bozkaya, E. Atav, N. Saglam, L. Tarhan, Enzyme Microbial. Technol. 25 (1999) 392.
[33] M.D. Holdom, R.J. Hay, A.J. Hamilton, Infect. Immun. 64 (1996) 3326.
[34] J.R. Hartman, T. Geller, Z. Yavin, D. Bartfeld, D. Kanner, H. Aviv, M. Gorecki, Proc. Natl. Acad. Sci. U.S.A. 83 (1986) 7142.
[35] R.A. Hallewell, K.C. Imlay, P. Lee, N.M. Fong, C. Gallegos, E.D. Getzo, J.A. Tainer, D.E. Cabelli, P. Tekamp-Olson, G.T. Mullenbach, L.S. Cousens, Biochem. Biophys. Res. Commun. 181 (1991) 474.
[36] D.F. McRee, S.M. Redford, E.D. Getzo, J.R. Lepock, R.A. Hallewell, J.A. Tainer, J. Biol. Chem. 265 (1990) 14234.
[37] H.E. Parge, R.A. Hallewell, J.A. Tainer, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 6109.
[38] J.R. Lepock, H.E. Frey, R.A. Hallewell, J. Biol. Chem. 265 (1990) 21612.
[39] M.C. Bonaccorsi di Patti, M.T. Carri, R. Gabbianelli, R. Da Gai, C. Volpe, A. Giartosio, G. Rotilio, A. Battistoni, Arch. Biochem. Biophys. 377 (2000) 284.
[40] G. Regelsberger, W. Atzenhofer, F. Ruker, G.A. Peschek, C. Jakopitsch, M. Paumann, P.G. Furtmuller, C. Obinger, J. Biol. Chem. 277 (2002) 43615.