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cDNA cloning, high-level expression, purification, and characterization of an avian Cu,Zn superoxide dismutase from Peking duck

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Abstract

As a special species of avian, Peking duck is often used as a model for exploring effective factors against cardio-cerebrovascular diseases, and therefore investigations of antioxidant enzymes including superoxide dismutase are intriguing. By using 3′-RACE with a gene-specific primer, a cDNA encoding duck Cu,Zn SOD was amplified from the total RNA extracted from Peking duck liver. Three free cysteine residues are found in the deduced amino acid sequence of duck SOD, among which Cys153 at the carbonyl-terminal is a distinctive feature. Production with a high yield of recombinant duck Cu,Zn SOD was achieved in *Escherichia coli* after the reconstituted expression vector pET-3a-dSOD was transformed into the bacterial strain BL21(DE3)pLysS. After two steps of anion exchange chromatography, a great quantity of the purified enzyme (100 mg/L fermented culture) with an enzymatic activity comparable to that of native duck and bovine SOD was finally obtained. Duck SOD is a homodimer with 153 residues for each subunit. The molecular mass of the recombinant enzyme is 15,540.0 Da measured by mass spectrum, which well coincides with the estimated size of the sequence but significantly differs from that of the native counterpart. Five charge isomers were observed on isoelectricfocusing (IEF). The most interesting observation is that the thermal stability of duck SOD is much lower than that of the bovine enzyme as revealed by irreversible heat inactivation at 70 °C. These properties are discussed in relation to the distinctive free Cys residues in duck Cu,Zn SOD.

Keywords: Superoxide dismutase; Peking duck; Overexpression; Cysteine; Thermal stability

Superoxide dismutases (SOD; EC 1.15.1.1) are ubiquitous metalloenzymes in aerobic organisms, which play a crucial role in protecting organisms against the toxic effects caused by reactive oxygen species (ROS), in particular the superoxide radicals (O_2^-) [1,2]. SODs catalyze a dismutation reaction that converts the superoxide radicals into molecular oxygen and hydrogen peroxide [3], which otherwise may initiate a radical chain reaction to give rise to other various ROS and in turn cause a number of pathologic processes [4]. Thus, SOD is regarded as an antioxidant and a very important member of cellular defensive system as well. Three isoenzymatic categories with different metal cofactors are known to date. Copper and zinc superoxide dismutases (Cu,Zn SODs) are widely distributed in eukaryotes, whereas manganese and iron containing enzymes are predominantly found in mitochondria or prokaryotes [5]. Each molecule of the eukaryotic Cu,Zn SODs is a dimer of identical subunits with approximately 153 amino acids. Homologs from various species display a high degree of conservation in both nucleotide and amino acid sequence [6].

A multitude of genes encoding Cu,Zn SOD from diverse organisms have been cloned, some of which were expressed in various systems such as *Escherichia coli* [7–10], *Lactococcus lactis* [11], yeast [12–14], *Drosophila melanogaster* [15], and even transgenic mice [16]. Nevertheless, few literatures about avian SODs are available as yet. One of the few cases is the chicken enzyme whose nucleotide sequence of cDNA was published in 1996 [17]

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although it was firstly isolated from chicken liver as early as in 1973 [18]. The authors of these two papers both noticed some distinctive properties that obviously belong to avain dismutase such as containing one or two more reactive thiol groups compared with the bovine and human homologs, but the expression of the chicken enzyme has not been reported later, and hence characterization of recombinant avian SOD is unavailable yet.

Peking duck (*Anas platyrhynchos domestica*) is a domestic species from *Anas platyrhynchos*. For getting specially delicious meat, Peking ducks compulsively consume much more than they physiologically need in feeding, thereby making themselves excessively obese compared with other duck species. Nevertheless, Peking ducks rarely suffer from cardiovascular and cerebrovascular diseases [19]. By virtue of this characteristic, Peking duck was used as a model to explore effective factors against cardio-cerebrovascular diseases like atherosclerosis [20]. In view of the close relationship between lipid peroxidation and atherosclerosis [21] as well as aging [22], investigations of Peking duck superoxide dismutase become interesting. In this paper the cDNA cloning, the overexpression, and characterization of duck SOD (dSOD) will be reported. Some interesting features such as discrepancies of the molecular size and the p*I* value between the recombinant protein and its native counterpart, as well as the lower thermal stability of dSOD will be described and discussed.

Materials and methods

Animals, vectors, strains, and materials

Five-day-aged Peking ducks (*Anas platyrhynchos domestica*) used for extraction of the total RNA were purchased from Beijing Goldenstar duck-culture center.

The cloning vector pBlueScript-KS(+) was purchased from Stratagene and the expression vector pET-3a was purchased from Novagen. Host strains DH5α and BL21(DE3)pLysS were stored in our laboratory.

Tryptone and yeast extract were purchased from Merck. PCR primers were synthesized in an Applied Biosystems DNA synthesizer 380-A (Perkin–Elmer).

3′-RACE and PCR amplification

The total RNA was extracted from 100 mg of fresh duck liver by using TRIZOL RNA extraction kit and 3′-rapid amplification of cDNA ends (3′-RACE) was performed by using the 3′-RACE kit. Both kits were provided by Life Technologies (USA). The sense amplification primer, i.e., the gene-specific primer (GSP), was designed as 5′-CG**GAATTC**ATGGCGACGCTGAAGGCC-3′, corresponding to the conserved region of the 5′-terminal coding sequence of chicken Cu,Zn SOD [17]. In addition, an *Eco*RI site (bold) was juxtaposed to the ATG start codon.

Taq DNA ploymerase was applied in PCR amplification for the sake of getting an additional 3′-A overhang on each strand of the product. PCR were performed in 100 μl of reaction solution containing 2 μg of total cDNA just synthesized as the template. After an incubation at 94 °C for 5 min, 30 cycles were carried out with 1 min of denaturation at 94 °C, 1 min of annealing at 53 °C, and 1 min of elongation at 72 °C, followed by a final extension at 72 °C for 7 min. The PCR product was analyzed using 1% agarose gel electrophoresis and ethidium bromide staining, whereafter purified by using Glassmilk DNA Purification Kit (BioDev).

Cloning and sequencing of the amplification products

The purified PCR product was cloned into vector pBlueScript-KS(+) using TA cloning technique, and then transformed into *E. coli* host strain DH5α. Once the correct recombinants were further confirmed by colony PCR or double restriction enzyme digestion, sequencing would be done. All DNA sequencing reactions were performed on an ABI PRISM 377XL DNA Sequencer.

Construction of the expression vector

A pair of primers was synthesized for subcloning the open reading frame (ORF) encoding duck Cu,Zn SOD into the expression vector. Primer 1, 5′-CC**CATATG** GCGACGCTGAAGGCC -3′, was designed to provide an *Nde*I restriction site (bold) at the start codon; primer 2, 5′-GCC**GAATCC**ATTAGCACTTGGCTATTCCG AT-3′, was designed to generate a *Bam*HI restriction site (bold) adjacent to the stop codon. Besides, the stop codon was changed from the original TGA in dSOD gene to TAAT (underlined) so as to ensure the proper termination of protein synthesis in *E. coli*. PCR amplification was performed under the same conditions as described above. Later on, the purified PCR product was reinserted into pBlueScript-KS(+) also using TA cloning method. The correct recombinants confirmed by DNA sequencing were digested with *Nde*I and *Bam*HI before being ligated into the corresponding restriction sites of the expression vector pET-3a (Novagen). The final constructed plasmid pET-3a-dSOD was transformed into the bacterial strain BL21(DE3)pLysS.

Overxpression of dSOD

The recombinant enzyme was overexpressed in *E. coli* cells by using the strong inducible T7 promoter under control of the presence of isopropyl-β-thiogalactopyranoside (IPTG). Five ml of starter culture was inoculated to 500 ml Luria broth medium containing 50 μg/ml

carbenicillin and 34 μg/ml chloramphenicol before the cultures were vigorously shaken at 37 °C until the OD_{600} approached to 0.5. They were immediately shifted to 25 °C for at least 15 min prior to induction. After that, $CuSO_4$, $ZnSO_4$, and IPTG were simultaneously added to the cultures with the final concentration of 1.2, 0.25, and 0.4 mM, respectively. After 5–6 h of induction, the induced cells were harvested by centrifugation at 4000*g* for 20 min.

Cell lysis

BugBuster Protein Extraction Reagent (Novagen) was used to lyse the bacterial cells, with all procedures performed in conformity to the instructions presented by the manufacturer before centrifugation of the lysate at 16,000*g* for 20 min. Both the supernatant and the insoluble cell debris were later resolved in 1% SDS and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to check up whether the expressed product was soluble or aggregated to form inclusion bodies.

Purification of recombinant dSOD

Two steps of anion exchange chromatography were performed in purification of recombinant dSOD (rdSOD). Both columns utilized in downstream purification were connected to an ÄKTA purifier (Pharmacia). The supernatant containing rdSOD was loaded directly onto a HiLoad column (1.6 × 30 cm) packed with Sepharose Q Fast Flow (Pharmacia) and pre-equilibrated with buffer A (20 mM Tris–HCl, pH 7.8). The UV-absorbency was measured at 280 nm and the flow rate was set at 2 ml/min. After elution with the same buffer for two column volumes, a linear sodium chloride gradient ranging from 0 to 0.3 M was developed on this column. Fractions with the highest superoxide dismutase activity were collected and concentrated to a desired degree by ultra filtration using centrifugation concentration tubes (Pall) with a nominal 10 kDa cut-off. The concentrated sample with a visible blue color was immediately applied to a Mono Q HR 5/5 column (Pharmacia). At this step, buffer B (50 mM Tris, pH 8.0) was used to equilibrate the column and to wash out the unbounded sample, and meanwhile the absorbency was measured at 257 nm where dSOD is expected to have the maximal absorbance. Similar NaCl gradient as above was developed and the peak containing the recombinant enzyme was pooled, concentrated, and desalted with HiTrap desalting columns (Pharmacia), and finally lyophilized.

Purification of native dSOD

Native dSOD was isolated from Peking duck erythrocytes by means of classical extraction method with minor modifications as described by Li et al. [23]. The acetone-precipitated sample was dissolved in 20 mM Tris–HCl, pH 7.5 before applied to a HiTrap Sepharose Q High Performance column (5 ml) (Pharmacia), which was pre-equilibrated with the same buffer. Duck SOD was eluted with 0.2 M NaCl at a step-wise gradient. The enzyme was further fractionated by size-exclusion chromatography using a HiLoad Superdex 75 column (1.6 × 70 cm) (Pharmacia) and eluted with 0.05 M NH_4HCO_3. The identity and the purity of enzyme were verified by SOD activity assays, SDS–PAGE and mass spectrometric analyses.

Gel electrophoresis

SDS–PAGE was performed with Laemmli's Tris–glycine buffer system [24]. According to the standard procedures for discontinuous SDS–PAGE, the stacking and separating gel were prepared at 3% T and 15% T, respectively. Isoelectricfocusing (IEF) was done on 5% polyacrylamide gels containing 3% Ampholine with pH range 3–9.5 (Pharmacia). Bands of protein were stained with Coomassie brilliant blue on both SDS–PAGE and IEF.

Mass spectrometric analysis and spectroscopic characterization

The purity and molecular weight of the purified rdSOD was analyzed by a Finigan LCQ ion trap mass spectrometer (ThermoQuest) equipped with an electrospray ionization source; spray voltage was 4.50 kV. Calculation was executed and elaborated using the program provided by the manufacturer.

Ultraviolet absorption spectrum of dSOD ranging from 220 to 300 nm was recorded on a Beckman DU-600 spectrophotometer. X-band EPR spectrum was measured on a Varian E-109 spectrometer operating at 9.1 GHz with 100 kHz field modulation. Far-UV circular dichroism analysis in the region of 190–260 nm was performed on a JASCO J-720 spectropolarimeter using a 0.1-mm path length CD cuvette and the final spectrum was obtained on the average of four scans.

Superoxide dismutase activity assays and protein concentration determination

SOD activity was assayed by the pyrogallol autoxidation method [25]. One unit (IU) of activity was defined as the amount of enzyme required catalyzing the inhibition of pyrogallol auto-oxidation by 50% per minute in 1 ml of reagent. Bovine Cu,Zn SOD purchased from Sigma was used as the standard control.

Protein concentration was determined by Bradford's method [26] with bovine serum albumin to give a standard curve.

Thermal inactivation assay

The lyophilized dSOD sample was dissolved in 50 mM potassium phosphate buffer, pH 7.8 to prepare 1 ml of solution containing 200 μg/ml enzyme, which was incubated continuously in a water bath at 70 °C. A 50-μl aliquot from the heated solution was transferred to a fresh tube and stored on ice every 10 or 20 min. After all aliquots were taken in 180 min, their residual SOD activity was measured as described above. Subsequently, a far-UV CD spectrum for each aliquot sample was recorded to investigate the enzyme conformational stability during heating procedure.

Results

cDNA cloning of duck Cu,Zn SOD

From the total RNA of duck liver, a PCR product with length of 0.7 kb as judged by agarose gel electrophoresis was amplified using 3′-RACE. The sequencing result (Fig. 1) shows that the inset is composed of 664 bp. The ORF is 462 bp long and ends with the stop codon TGA. The non-coding region following the stop codon is 199 bp in length, much longer than that of human SOD (hSOD) [27]. In consistence with human and chicken SOD genes, the signal for processing nuclease and poly(A) polymerase, the AATAAA hexanucleotide, which is commonly found upstream of the 3′-poly(A) tail in most eukaryotic cellular mRNAs, is replaced by ATTAAA in dSOD cDNA.

```
1- ATG GCG ACG CTG AAG GCC GTG TGC GTG ATG AAG GGG GAC GGC CCC
   Met Ala Thr Leu Lys Ala Val Cys Val Met Lys Gly Asp Gly Pro
   GTG GAG GGC GTC ATC CAC TTC CAG CAG CAG GGT AAC GGA CCA GTA
   Val Glu Gly Val Ile His Phe Gln Gln Gln Gly Asn Gly Pro Val
   AAA GTT ACT GGA AGA ATC AGT GGC TTG TCT GAT GGA GAT CAT GGC
   Lys Val Thr Gly Arg Ile Ser Gly Leu Ser Asp Gly Asp His Gly
   TTC CAT GTC CAC GAA TTT GGG GAC AAC ACA AAT GGA TGT ACC AGT
   Phe His Val His Glu Phe Gly Asp Asn Thr Asn Gly Cys Thr Ser
   GCA GGT GCT CAC TTC AAT CCT GAA GGC AAG AAG CAC GGT GGA CCA
   Ala Gly Ala His Phe Asn Pro Glu Gly Lys Lys His Gly Gly Pro
   AAG GAT GCA GAG AGG CAC GTG GGT GAC CTC GGC AAC GTG ACT GCT
   Lys Asp Ala Glu Arg His Val Gly Asp Leu Gly Asn Val Thr Ala
   AAA GGA GGA GTA GCA GAT GTG GAA ATA GAA GAT TCC GTC ATC TCT
   Lys Gly Gly Val Ala Asp Val Glu Ile Glu Asp Ser Val Ile Ser
   CTG ACT GGA CCA CAC TGC ATC ATT GGG CGC ACC ATG GTG GTC CAT
   Leu Thr Gly Pro His Cys Ile Ile Gly Arg Thr Met Val Val His
   GCA AAA AGC GAC GAC CTG GGC AAA GGG GGA GAC AAC GAG AGC ACG
   Ala Lys Ser Asp Asp Leu Gly Lys Gly Gly Asp Asn Glu Ser Thr
   CTA ACT GGG AAT GCC GGG CCT CGC CTG GCC TGT GGT GTC ATC GGA
   Leu Thr Gly Asn Ala Gly Pro Arg Leu Ala Cys Gly Val Ile Gly
   ATA GCC AAG TGC TGA GTG GTG TGC CTG AAG TGC TCG AGG TGA CAT
   Ile Ala Lys Cys END
   TGA GAA ACG GGC CGG TGT TCA TCG TGG TCA CCG TAC TTG CTC TTC
   CTC GTT CAA GCA ACA GAT TTC AGT TAA TCT CAT TAC TAC TCT GCA
   TTC TTG AGT ATC AGT TAA ACT GGT GAA GAC TGA CTT AAT TTT GTA
   ACG TGT ATG TTG CAA TTA AAG TGA TGT TGA TGG A - 664
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Fig. 1. The nucleotide and the deduced amino acid sequences of the cDNA encoding duck Cu,Zn SOD. The nucleotide sequence from 1 to 664 and the deduced amino acid sequence corresponding to the entire mature protein numbered 1–154 are shown in bold and italic, respectively.

Overexpresstion of duck Cu,Zn SOD in E. coli

The coding sequence of duck Cu,Zn SOD was subcloned into the expression vector pET-3a between *Nde*I and

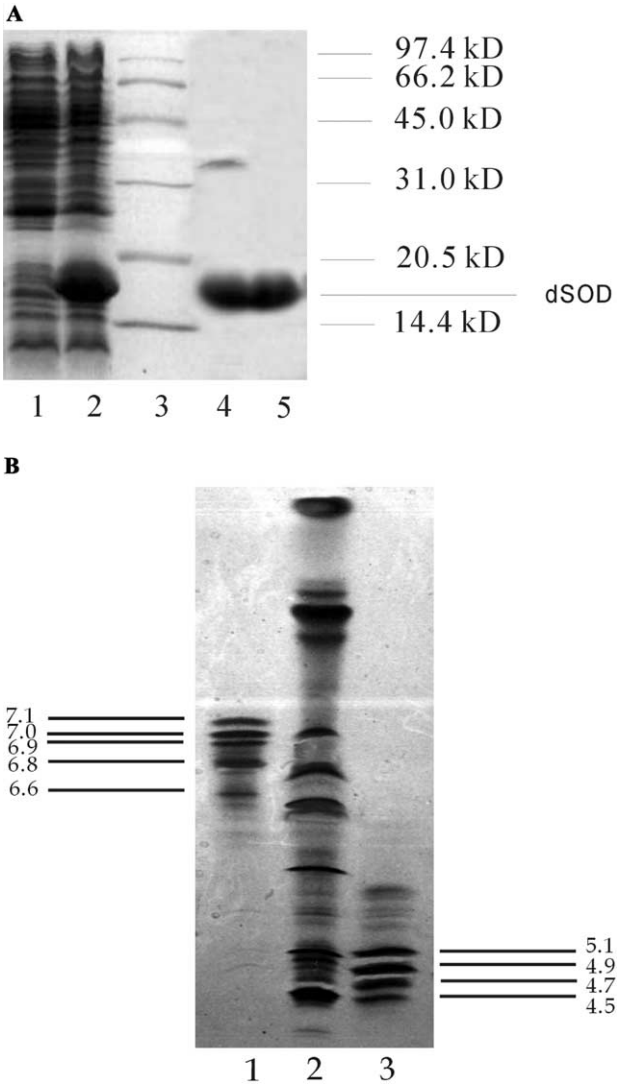

Fig. 2. (A) SDS–PAGE of duck Cu,Zn SOD preparations. Lanes: 1, total cell protein before induction; 2, total cell protein 6 h after induction at 25 °C; 3, protein size markers (in kDa) (Bio-Rad); 4, rdSOD purified by using Sepharose Q Fast Flow column (1.6 × 30 cm); 5, rdSOD further purified by using Mono Q column (0.5 × 5 cm). (B) The purified dSOD in isoelectric focusing (IEF) showing its charge isomers. Lanes: 1, recombinant dSOD; 2, protein p*I* standards (Bio-Rad); 3, native dSOD. The IEF was performed on 5% polyacrylamide gels, containing 3% Ampholine with pH range 3–9.5 (Pharmacia). All protein bands were stained with Coomassie brilliant blue.

*Bam*HI sites, thereby under control of the strong bacteriophage T7 promoter. In comparison with the non-induced culture, a 16 kDa protein was expressed at a tremendously high-level within a few hours after induction with IPTG (Fig. 2A). The apparent molecular mass of the expressed protein determined by SDS–PAGE agrees with the theoretic subunit size calculated from the amino acid sequence of dSOD. As is estimated from gel scanning, the synthesized product comprises more than 50% of the total cell proteins. To obtain soluble and active enzyme as much as desired, a series trial had been made. Finally we found that prolonged induction (6 h) at 25 °C was optimal. Under this condition, the bulk of the recombinant protein was expressed as a soluble form. Like other reconstituted SODs produced in *E. coli*, [9,10] however, the enzymatic activity of supernatant in bacterial lysate was much lower than expected in spite of the presence of a huge sum of the expressed enzyme. Attempts to add Cu^{2+} to the bacterial growth medium suggested that the intracellular concentration of Cu^{2+} was insufficient to saturate the large amount of nascent duck SOD produced so rapidly. Actually, only when Cu^{2+} concentration reaches above 1 mM does the lysate have maximal SOD activity. Therefore, appropriate quantities of Cu^{2+} and Zn^{2+} (see Materials and methods) were simultaneously added with IPTG to produce fully active rdSOD.

Purification of recombinant duck Cu,Zn SOD

The purified recombinant enzyme was obtained through two steps of anion exchange chromatography, which made the purification profile simple and efficient. The second peak in flowthough fraction corresponded to the highest catalytic activity, which indicated that rdSOD could not be absorbed in Sepharose Q Fast Flow column at pH 7.8 (Fig. 3A). Nevertheless, high efficiency was accomplished by removal of the majority of impurities at this step (Fig. 2A). It was notable that another peak eluted with increasing sodium chloride gradient (peak 3 in Fig. 3A) also showed somewhat of SOD activity despite much lower than that of the former fraction. It seemed likely that the bacterial superoxide dismutase might be responsible for that peak because the catalytic activity of this fraction kept almost constant whether sufficient Cu^{2+} or nothing was added to the growth medium during induction. A Mono Q column was utilized for polishing purification. At this step the absorbency was measured at 257 nm instead of 280 nm due to the fact that dSOD contains merely phenylalanine residues but no tyrosine or tryptophan (Fig. 1). The fraction with enzymatic activity was pooled and lyophilized (Fig. 3B). The final yield of recombinant duck Cu,Zn SOD reached 100 mg/L of *E. coli* growth culture.

The homogeneity of the purified product was examined by SDS–PAGE (Fig. 2A). The purity was above 95% as estimated from gel scanning and displayed as a single peak on mass spectrum (Fig. 4A).

SOD activity and chemical characterization

The enzymatic activities in parallel with the purity of rdSOD are listed in Table 1. The rough size of rdSOD subunit determined by SDS–PAGE was 16 kDa, which agrees with that of the native enzyme (Fig. 2A). The exact molecular mass as measured by electrospray mass spectrometry was 15,540.0 Da (Fig. 4A), being well coincident with the estimated mass value of 15541.8 Da from the amino acid sequence deduced from dSOD gene (Fig. 1). Besides, mass spectrometric analysis confirmed the removal of the initial methionine and no acetylation at the N-terminal of mature peptides, which coincides with the cases of other SODs produced in *E. coli* [7,9]. The native duck enzyme was also analyzed by MALDI-TOF mass spectrometry. Two peaks, respectively, corresponding to 15902.7 and 16199.4 Da were detected on the spectrum (Fig. 4B), both of which displayed remarkable discrepancy from the molecular weight of the recombinant enzyme. The dimeric form of either dSODs has a

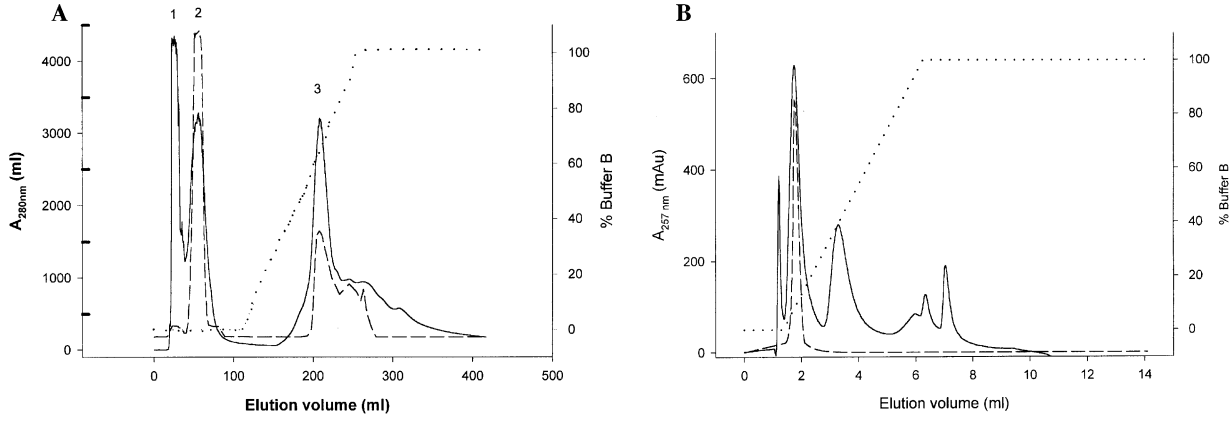

Fig. 3. The chromatography scheme in purification of recombinant duck Cu,Zn SOD from Sepharose Q Fast Flow column (A) and Mono Q column (B). Protein absorbance, SOD activity and the elution gradient are denoted by the solid, dashed, and dotted lines, respectively.

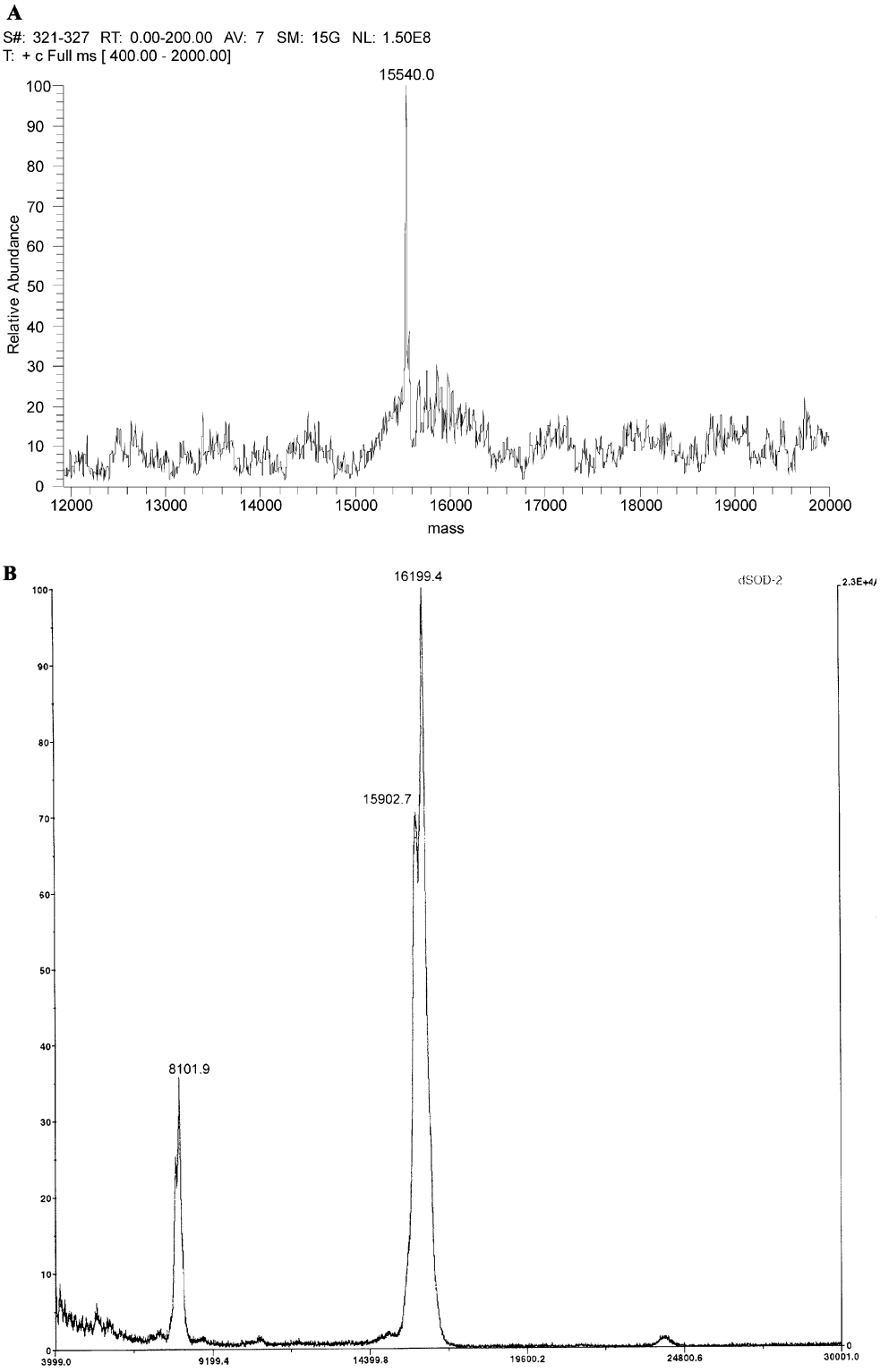

Fig. 4. Electrospray mass spectra of recombinant duck Cu,Zn SOD (A) and native enzyme (B).

size of approximately 32 kDa as determined by gel filtration chromatography on Superdex 75 (data not shown).

The presence of charge isomers in various eukaryotic Cu,Zn SODs has been extensively reported in a wealth of literatures. For example, three to eight isomers were discovered in the human [28], chicken [29,30], and rat [31,32] enzymes. Even though most investigations referred charge heterogeneity of SOD mainly in relation to the native enzymes, isoelectric variants were also

Table 1
Enzymatic activity of recombinant duck Cu,Zn SOD preparations in comparison with that of bovine SOD

SOD preparations	Crude lysate	rdSOD[a]	rdSOD[b]	dSOD[c]	Bovine SOD[d]
Purity of rdSOD	≈50%	⩾ 85%	⩾ 95%	—	—
Activity (U/mg)	2132.1	3867.8	4121.8	4145.5	3887.2

[a] After purification by using Sepharose Q Fast Flow column (1.6 × 30 cm).
[b] After final purification by using Mono Q column (0.5 × 5 cm).
[c] Purified native duck SOD.
[d] Purchased from Sigma.

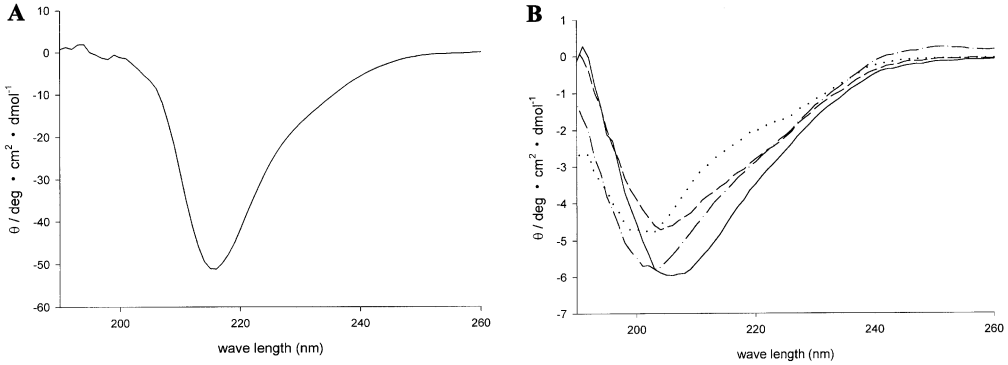

Fig. 5. Far-UV circular dichroism spectrum of duck Cu,Zn SOD in Tris–HCl buffer at pH 7.5 (A) and during irreversible thermal inactivation (B). The solid, dashed, dash-dotted, and dotted lines in (B), respectively, represent the CD spectra recorded at 0, 50, 100, and 165 min after incubation at 70 °C.

presented in recombinant proteins, such as human SOD expressed in *E. coli* [33]. To the end of exploring the situations of dSOD, we examined both the recombinant and the native enzymes with isoeletrofocussing electrophoresis. As the result revealed in Fig. 2B, five and four isomers were observed in rdSOD and the native counterpart, respectively. The measured p*I* values of these isomers are 7.1, 7.0, 6.9, 6.8, and 6.6 for the recombinant protein, whereas 5.1, 4.9, 4.7, and 4.5 for the native.

Spectroscopic characterization

The optical absorption spectrum of rdSOD in the UV region is similar to that of recombinant human SOD except that the maximum absorbance of duck SOD is at 258 nm, slightly different from that of the reconstituted hSOD (265 nm) [9]. The copper EPR spectrum does not show any remarkable difference from that of recombinant hSOD [33]. The circular dichroism spectrum of rdSOD shows a maximal negative peak at 215 nm, (Fig. 5A) which clearly indicates that the duck enzyme is a typical β-protein. All spectroscopic characteristics of rdSOD are identical to those of native dSOD.

Thermal stability

The thermal stability of dSOD was detected with irreversible thermal denaturation. During continuous incubation at 70 °C, the residual enzymatic activity was assayed at fixed intervals by means of the pyrogallol method. The result plotted in Fig. 6 reveals that duck SOD, either the recombinant or the native, has much lower heat-stability than the bovine enzyme. Data for both duck and bovine SOD fit the most linear relationship assuming a zero-order kinetic rather than a first-order.

To inspect the conformational stability of the duck enzyme in consecutive heating, the circular dichroism spectra were recorded at the same intervals when aliquots were taken to assay residual activity. The spectra and the corresponding secondary structures are

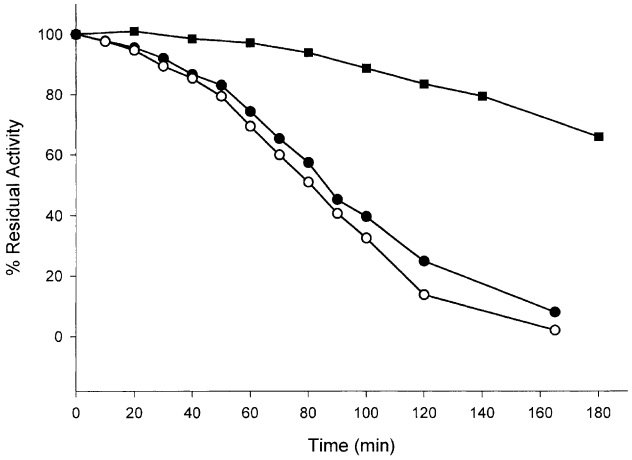

Fig. 6. Examination of the thermal stability of duck SOD (circle) by irreversible heating inactivation at 70 °C in comparison with that of the bovine enzyme (square). Two independent experiments were repeated with similar results. The solid and the hollow circles represent recombinant and native dSOD, respectively.

Table 2
Secondary structure of rdSOD during thermal inactivation at 70 °C estimated from CD spectra

Incubation time (min)	0	10	20	30	40	50	60	70	80	90	100	120	165
α-Helix (%)	6.3	5.5	4.9	5.6	4.4	5.1	3.0	2.2	2.4	2.3	1.4	2.2	1.5
β-Sheet (%)	55.4	54.8	53.8	49.7	52.2	51.1	53.6	54.8	53.5	53.3	54.4	51.6	49.5
β-Turn (%)	5.4	4.3	5.0	7.6	6.3	6.1	5.0	4.7	5.5	6.0	5.1	6.5	8.6
Random (%)	33.0	35.4	36.4	37.1	37.1	37.7	38.4	38.3	38.6	38.3	39.0	39.7	40.5

given in Fig. 5B and Table 2, respectively. Though the random coil increases steadily along with the incubation time at 70 °C, the β-sheet keeps almost constant.

Discussion

Sequence comparison

The deduced amino acid sequence from the cDNA of dSOD is compared with those of the chicken (cSOD) [30], human (hSOD) [27], bovine (bSOD) [6], and *Xenopus laevis* (XSODA) [34] counterparts using Clustal W program (Fig. 7). The alignment reveals that duck SOD shares about 94%, 76%, 74%, and 66% homology with cSOD, hSOD, bSOD, and XSODA, respectively. The highest homolog with cSOD demonstrates a close evolutional relationship between these two poultries. All the residues involved in metal binding and activity center or essential

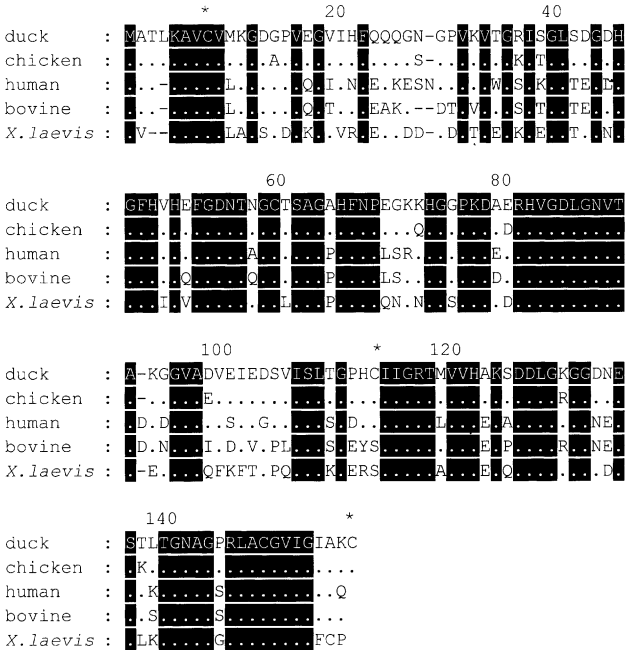

Fig. 7. Sequence comparisons of duck Cu,Zn SOD with the chicken, human, bovine, and *Xenopus laevis* homologs (XSODA). The alignment program Clustal W automatically introduces gaps (dash) to maximize similarity among the primary structures of these SODs. All amino acid residues identical to corresponding ones of duck SOD are represented with dot and the invariant residues in all five sequences are highlighted with a black background. The three extra free cysteine residues of the duck enzyme are labeled with asterisk.

for maintaining the β-barrel scaffold are conserved in dSOD. Nevertheless, it is worthy to note that three extra cysteine residues, Cys7, Cys110, and Cys153, are present in the sequences of both dSOD and cSOD. All these three Cys residues have free reactive thiols. Cys7 corresponds to Cys6 of hSOD and bSOD and Cys110 corresponds to Cys111 of hSOD, while Cys153 located at the carbonyl-terminal is distinct for both of the poultry enzymes. At present, it is not clear whether Cys153 exists ubiquitously in all avian enzymes. Unlike Cys7, which buries its side chain toward the interior of the β-barrel [13,35,36], both Cys110 and Cys153 are solvent accessible assuming that dSOD adopts the same molecular scaffold as hSOD [27]. Compared with those enzymes containing only one extra solvent-exposed Cys residue such as hSOD and XSODA, much fewer eukaryotic SODs present two or even more such residues. In addition to some fruit fly enzymes (data from GenBank and Swissport), only duck and chicken SODs have been observed containing two exposed cysteine residues to date.

Charge heterogeneity

There are plenty of evidences in the literatures that Cys residues with free thiol are related to charge heterogeneity of SODs [30,33]. Schinina et al. [30] purified chicken SOD and identified the S-thiolation of exposed Cys residues with different number of glutathiones. In disagreement with that result, the single peak on mass spectrum (Fig. 4A) demonstrates that recombinant duck SOD expressed in *E. coli* is homogeneous in molecular mass, and furthermore, the measured subunit weight being identical to that calculated value in terms of amino acid sequence rules out the possibility of covalently binding with glutathione or any other molecules like that. However, the notable difference between molecular sizes of the recombinant and the native enzymes (Figs. 4A and B) should not be ignored. In addition, the apparent discrepancy observed in the p*I* values of charge isomers between the two dSODs (Fig. 2B) should also be taken into consideration. S-thiolations on free cysteines of the native enzyme may reasonably account for the above results. The differences of the molecular mass between the two fractions of the native enzyme (Fig. 4B) and the recombinant counterpart are 361.7 and 658.4 Da, respectively. In view of the fact that the molecular size of glutathione is 305.3 Da [30], these discrepancies are very likely to imply that native dSOD

takes one or two glutathiones by chemical modification. In this case, the obvious variation perceived in isoelectricfocusing can also be rationally understood.

Similar to the recombinant human dimutase produced in *E. coli* [33], the reconstituted duck enzyme consists multiple charge components notwithstanding no S-thiolation occur on the exposed Cys residues of the protein. Nevertheless, free cysteines are inferred to be involved in charge heterogeneity of recombinant SODs. As dSOD has one more solvent-accessible cysteine than hSOD and Cys153 at the C-terminal is even more reactive than Cys110 [36], dSOD contains more variants is expectable. Taken together of these results, we suppose that the thiol modification on free Cys residues of natural superoxide dismutase may commonly occur in diverse organisms as a cellular response to oxidative stress [37,38]. Since that charge heterogeneity was observed in both the native and the recombinant enzymes whether or not being thiol-modified, this reaction seems less likely to be chiefly responsible for inhomogeneity, at variance with the conclusion drawn by Schinina et al. [30]. Certainly, it requires more experimental data to identify this assumption.

Lower thermal stability

Probably the most striking feature of duck SOD is its obvious lower thermal stability than that of the bovine enzyme. As shown in Fig. 6, either native or recombinant dSOD lost its catalytic activity far more rapidly than bSOD during continuously incubation at 70 °C. Site-directed mutagenesis studies indicated that free cysteine residues might be the major molecular determinants for different protein stabilities among various Cu,Zn SODs [12,13,35,39,40]. despite some exceptions such as the enzymes from yeast or wheat germ [41–43]. Some investigators confirmed that human SOD exhibited higher instability than bSOD during heat denaturation, whereas mutant hSOD Cys111Ser displayed almost the same steadiness as bSOD. These results definitely clarified that Cys111 in hSOD is answerable for the decrease of its thermal stability. In this manner, Cys110 in dSOD, corresponding to Cys111 in hSOD, is very likely related to its higher thermal lability. Recently, the discrepancy of conformational stability between two isoenzymes from *Xenopus laevis*: XSODA and XSODB, was explored [40]. The results showed that the residue Cys150 in XSODA is quite sensitive to its heat resistance. Considering Cys153 in dSOD is located in an analogous position of Cys150 in XSODA, Cys153 may be another possible candidate besides Cys110 for contribution to the lower thermal stability of the duck enzyme.

Despite the great loss of catalytic activity during incubation at 70 °C, the secondary structure of dSOD keeps nearly constant as shown by CD spectra (Fig. 5B). This result indicates that the β-barrel scaffold is not undergoing a radical transformation during the heat treatment (Table 2). The close packing of hydrophobic interfaces in dimeric protein is generally believed to contribute to enzymatic stability [36]. Therefore, the residues involved in the dimmer interface seem to be very important. In a recent paper Liu et al. [44] identified Cys111 in hSOD as a new Cu^{2+} binding site and proposed that two Cys111 residues on opposite subunits of the dimeric enzyme may provide a docking location for initial metal insertion during biosynthesis of wild-type Cu,Zn SOD in vivo. According to this hypothesis, Cys110 and Cys153 of dSOD could probably be involved in the formation of metal binding site for they are both close to the dimeric interface. It is of great interest to investigate this possibility and to test if the presence of an exposed copper-binding site could affect the lower thermal stability of dSOD. From a comprehensive view drawn by above discussion, Cys110 and the distinct Cys153 residues in duck SOD seem to be very likely candidates responsible for its special properties.

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