JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Copper/Zinc-Superoxide Dismutase from Lemon cDNA and Enzyme Stability

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A full-length cDNA clone of 744 bp encoding a putative copper/zinc-superoxide dismutase (Cu/Zn-SOD) from lemon (*Citrus limon*) was cloned by PCR approach. Nucleotide sequence analysis of this cDNA clone revealed that it comprised an open reading frame coding for 152 amino acid residues. The deduced amino acid sequences showed high identity (65−84%) with the sequences of the Cu/Zn-SODs from other plant species. Computer analysis of the residues required for coordinating copper (His-45, -47, -62, and -119) and zinc (His-62, -70, and -79 and Asp-82), as well as the two cysteines (56 and 145) that form a single disulfide bond, showed they were well-conserved among all reported Cu/Zn-SOD sequences in the present study. To further characterize the lemon Cu/Zn-SOD, the coding region was subcloned into an expression vector, pET-20b(+), and transformed into *Escherichia coli* BL21(DE3). Expression of the Cu/Zn-SOD was confirmed by enzyme activity staining on a native gel and purified by Ni^{2+}−nitrilotriacetic acid Sepharose superflow. The purified enzyme showed two active forms (70% monomer and 30% dimer) in equilibrium, and the specific activity was 7 456 units/mg. The activity of the dimer was 65% higher than that of the monomer. The thermal inactivation rate constant K_d value calculated for the dimer at 90 °C was -7.0×10^{-3} min^{-1}, and the half-life for inactivation was 99 min. Both activity and forms of the enzyme were affected very little by acidic pH, basic pH, or 4% SDS. The dimeric structure was more resistant to heat and proteolytic attack with trypsin or chymotrypsin compared to the monomeric structure. Imidazole caused the dimer to dissociate into monomers. These studies suggested subunit interaction might be important for enzyme stability.

KEYWORDS: Lemon; *Citrus limon*; SOD; expression; PCR; pET-20b(+)

INTRODUCTION

Superoxide dismutases (SODs) are metalloproteins and can be classified into three types, Cu/Zn-, Mn-, and Fe-SODs, depending on the metal found in the active site (*1*−*3*). Cu/Zn-SOD is predominantly associated with the cytosolic fraction in eukaryotes and is very sensitive to cyanide and hydrogen peroxide. Mn-SOD is associated with mitochondria and insensitive to cyanide and hydrogen peroxide. Fe-SOD is found in prokaryotes and is not sensitive to cyanide but is inhibited by hydrogen peroxide.

The role of SOD is to catalyze the dismutation of the superoxide ion (O_2^-) to hydrogen peroxide and molecular oxygen during oxidative energy processes. It is considered to be an important enzyme in neutralizing oxygen radical-mediated toxicity. The reported medical applications of SOD include cosmetics, anti-inflammation, prevention of oncogenesis and tumor promotion, and protection against reperfusion damages of ischemic tissue (*4*, *5*). Recently, yeast-overexpressed human SOD1 has been reported to fight environmental oxidative stress (*6*) and to be resistant to freeze−thaw stress (*7*). These results emphasize the many potential applications of SODs.

Lemon is a high-quality and economically valuable commodity in Taiwan. Many women use lemons as a natural method to eliminate melanin-filled cells on the skin's surface to clarify complexion. This natural elimination of melanin-filled cells may be attributed to SODs. Almost all reported SODs are labile in an acidic environment. Because lemons are so acidic, are they a stable environment for SOD? This is one question that leads us to study lemon SOD. The other question arose from the fact that lemon Cu/Zn-SOD cDNA was not reported. Recently, we have cloned full-length Cu/Zn-SOD cDNA clones from papaya (*8*, *9*) and pineapple (*10*). Both cDNAs were introduced into an expression vector; we obtained pure enzymes from papaya to study their properties, but in acidic pH, the enzyme was less stable, having dissociated into monomer units. Here we report the cDNA sequence and the deduced amino acid sequence from a lemon Cu/Zn-SOD cDNA clone. In addition, the coding region of Cu/Zn-SOD cDNA was introduced into an expression vector, pET-20b(+), and transformed into *Escherichia coli* BL21(DE3). This Cu/Zn-SOD cDNA clone can overexpress Cu/Zn-SOD

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10.1021/jf0207070 CCC: $22.00
Published on Web 11/09/2002

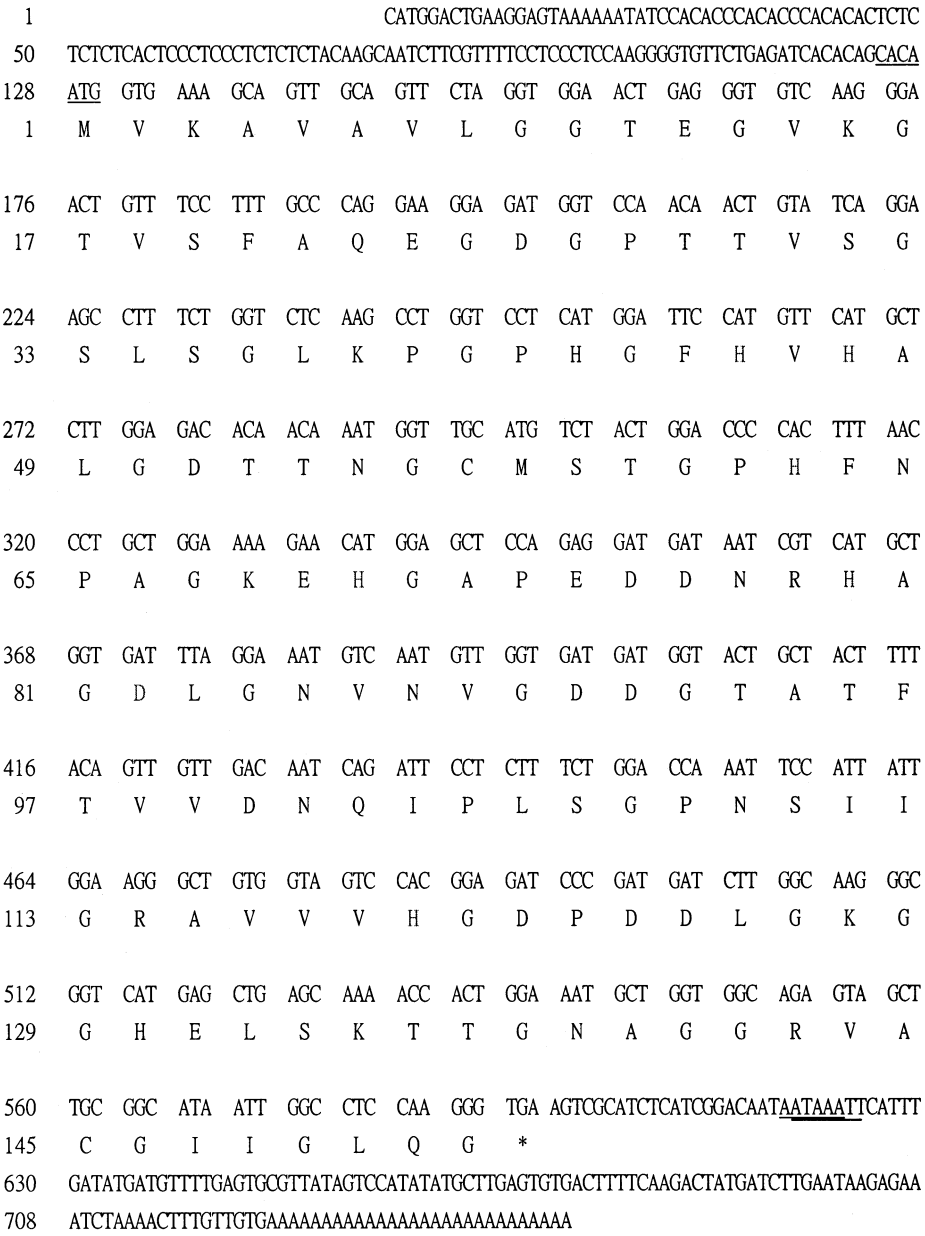

Figure 1. Nucleotide sequence of the lemon Cu/Zn-SOD cDNA and the deduced amino acid sequence. Numbers to the left refer to nucleotide and its deduced amino acid residues. Consensus sequence of the translation start site is underlined, and the polyadenylation signal is marked by a double underline. The asterisk denotes the translation stop signal.

enzyme in *E. coli*. A more stable enzyme than papaya was obtained for biochemical analysis.

MATERIALS AND METHODS

Sample. A fresh lemon weighing 15 g was obtained from a local market.

Total RNA Preparation and Single-Strand cDNA Synthesis. Fresh lemon (2.3 g) was put into liquid nitrogen and ground to powder in a ceramic mortar. The sample was dissolved in 20 mL of TRIzol reagent (GIBCO, Frederick, MD). The mixture was shaken vigorously for 15 s, incubated at 4 °C for 5 min, at which point 3 mL of chloroform was added, and then centrifuged at 12000*g* for 15 min at 4 °C. The aqueous phase was transferred to a new tube, 10 mL of isopropyl alcohol was added, it was then incubated at 4 °C for 15 min and then centrifuged at 12000*g* for 30 min at 4 °C. The pellet was then washed with 0.8 mL of 70% ethanol and centrifuged twice at 12000*g* for 5 min at 4 °C. The total RNA obtained was 83 μg. Single-strand cDNA was synthesized using a kit (GeneRacer) from Invitrogen (Grand Island, NY).

Subcloning, DNA Sequence Analysis, and Transformation. According to the cDNA sequence of the sweet potato Cu/Zn-SOD (EMBL accession no. X73139), one primer (Ta-NR: 5′ GAT CCC GAT GAT CTT GGT AA 3′) was synthesized. Eight picomoles of GeneRacer 3′ primer (from GeneRacer kit), 10 pmol of Ta-NR, and 0.05 μg of single-strand cDNA used as template were added for Polymerase Chain Reaction (PCR). One 0.26 kb cDNA was amplified by the PCR technique (25 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s). The fragment obtained by PCR was subcloned into a pCR2.1 cloning vector (Invitrogen) using *E. coli* TOPO 10 as a host. The nucleotide sequence of the insert was determined in both directions by auto-sequencing (ABI PRISM 377-96 DNA Sequencer, Perkin-Elmer, Cypress, CA). On the basis of this cDNA sequence containing poly-(A^+), a LeCu-3 primer (5′ CTG CCA CCA GCA TTT CCA GTG 3′), which was antisense sequence for 5′-RACE, was synthesized. To a 0.5 mL microtube containing 0.05 μg of the single-strand cDNA from lemon as template was added 8 pmol of GeneRacer 5′ primer (from GeneRacer kit) and 10 pmol of LeCu-3 primer. One 0.55 kb cDNA (5′-RACE) was amplified by the PCR technique (25 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s). The 0.55 kb cDNA fragment

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Atsodc
Pssodc
Slsodc
Sosodp  MAAHTILASA PSHTTFSLIS PFSSTPTNAL SSSLQSSSFN GLSFKLSPTT          50
Slsodp  ....S.FTTT S--..N.FLY .I..SS---- ..PNIN...L .V.LNVNAKF          44
Pssodp  ..SQ.LVSP- ---------. .L..H----- --..LRT.FS .V.V..AP--          31

Clsodc                        MVKAVAVLG GTEGVKGTVS FAQEGDGPTT          29
Ibsodc                        ........S SS...S..IF .S........          29
Sosodc                        .G...V..S SS...S...Y ..........          29
Zmsodc                        ........A G.D-....IF .S........          28
Ossodc                        ........A SS......IF .S.......S          29
Atsodc                        .A.G....N SS...T..IF .T.....V..          29
Pssodc                        ........S NSNE.S..IN .S...N....          29
Slsodc                        ........N SS...S..YL .T.V.VA...          29
Sosodp  QSLSLSTSAA S--KPLTIVA ATK......K ..SN.E.V.T LT..D.....          98
Slsodp  -GQ..TLY.V TTP....VF. .TK......K .NSN.E.V.T LS.DD.....          93
Pssodp  ---QF..L.T .NF....V.. .A....S..K ..SA.E.V.T LT.DDE....          78

Clsodc  VSGSLSGLKP GPHGFHVHAL GDTTNGCMST GPHFNPAGKE HGAPEDDNRH          79
Ibsodc  .T.NV..... .L........ .......... .......... ....G.....          79
Sosodc  .T.NV..... .L........ .......... ...Y..N... .......V..          79
Zmsodc  .T..I..... .L........ .......... ......V... ......ED..          78
Ossodc  .T..V..... .L........ .......... ......T... ....Q.E...          79
Atsodc  ...TV..... .L........ .......... ......D..T ......A...          79
Pssodc  .T.T.A.... .L....I... .......I.. ......N... ......ET..          79
Slsodc  .N.NI..... .L........ .......... ...Y...... ......EV..          79
Sosodp  .NVRI...A. .K....L.EF .......... ......DK.T ......EV..         148
Slsodp  .NVRIT..A. .L....L.EY .......... .A....NKLT ....G.EI..         143
Pssodp  .NVRIT..T. .L....L.EY .......I.. ......NKLT ......EI..         128

Clsodc  AGDLGNVNVG DDGTATFTVV DNQIPLSGPN SIIGRAVVVH GDPDDLGKGG          129
Ibsodc  ......IT.. E....S..IT .K....T.A. .V........ ..........          129
Sosodc  ......IT.. ........II .S........ ..V....... AE.....R..          129
Zmsodc  .......TA. E..VVNVNIT .S....A..H .......... A.........          128
Ossodc  ......ITA. A..V.NVN.S .S....T.A. .......... A.........          129
Atsodc  ......IT.. ........IT .C....T... ..V....... A.........          129
Pssodc  ......I... ....VS..IT ..H...T.T. .......... A.........          129
Slsodc  ......IT.. E....S..IT .K....T..Q .......... A.........          129
Sosodp  ......IVAN T..V.EA.I. ......T... .VV...L... ELE.......          198
Slsodp  ......IVAN A..V.EV.L. ......T... .VV...L... ELE.......          193
Pssodp  ......IVAN AE.V.EA.I. ......T... .VV...L... ELQ.......          178

Clsodc  HELSKTTGNA GGRVACGIIG LQG                                      152
Ibsodc  .....S.... .......... ...                                      152
Sosodc  .......... .......... ...                                      152
Zmsodc  .....S.... .......... ...                                      151
Ossodc  .......... .......... ...                                      152
Atsodc  ....LA.... .......... ...                                      152
Pssodc  .......... .......... ...                                      152
Slsodc  .....S.... ...I...... ...                                      152
Sosodp  ....P..... ...L...VV. .TPV                                     222
Slsodp  ....L..... ...L...VV. .TPI                                     217
Pssodp  ....LS.... ...L...VV. .TPV                                     202
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Figure 2. Optimal alignment of Cu/Zn-SOD among several plant species. The alignment of amino acid sequences translated from cDNA sequences used the PILEUP program in the Wisconsin Sequence Analyze package. The accession numbers in EMBL and the names for these sequences are as follows: Clsodc, lemon; Ibsodc, sweet potato (X73 139); Sosodc, spinach (X53 872); Zmsodc, mazine (M15 175); Ossodc, rice (D01 000); Atsodc, *Arabidopsis* (X60 935); Pssodc, pea (M63 003); Slsodc, tomato (X14 040); Sosodp, spinach (D10 244); Slsodp, tomato (X14 041); Pssodp, pea (J04 087). Numbers refer to amino acid residues of each species. A dot refers to identities with lemon, and a dash denotes deletion. Residues coordinating copper and zinc are indicated with dark squares. The two cysteines that form a disulfide bridge are underlined.

was subcloned into a pCR2.1 cloning vector using TOPO 10 as a host. The nucleotide sequence of the insert was determined in both directions according to the dideoxy technique using autosequencing. Sequence analysis revealed that 3′-RACE and 5′-RACE cover the full-length of Cu/Zn-SOD cDNA (744 bp). On the basis of 5′-RACE and 3′-RACE sequences, using 0.05 μg of single-strand cDNA as template, one full-length cDNA of Cu/Zn-SOD was created by the PCR technique (EMBL accession no. AF318938). The coding region of this full-length cDNA

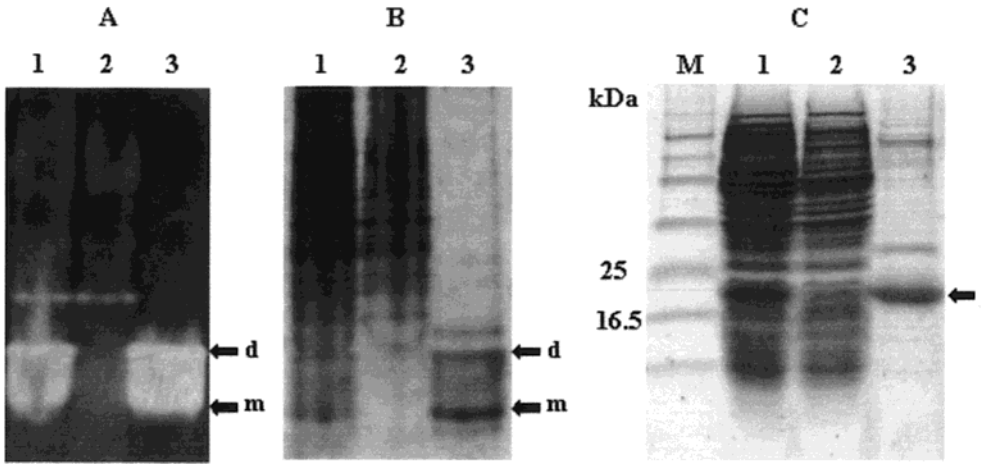

Figure 3. Total soluble protein profile of IPTG induced and one-step purification. Induced *E. coli* BL21(DE3) containing the pET-SOD clone. Fifteen microliters of induced crude extract (lane 1, 3.37 μg/uL), passthrough (lane 2, 1.54 μg/uL), and dialyzed sample (lane 3, 0.34 μg/μL) were subjected to a 10% native-PAGE or a 15% SDS-PAGE: (A) activity staining; (B) native-PAGE with Coomassie blue staining ("d" denotes dimer, "m" denotes monomer); (C) SDS-PAGE with Coomassie blue staining, an arrow denotes Cu/Zn-SOD protein. The molecular weights of protein markers are 175, 83, 62, 47.5, 32.5, 25, and 16.5 kDa.

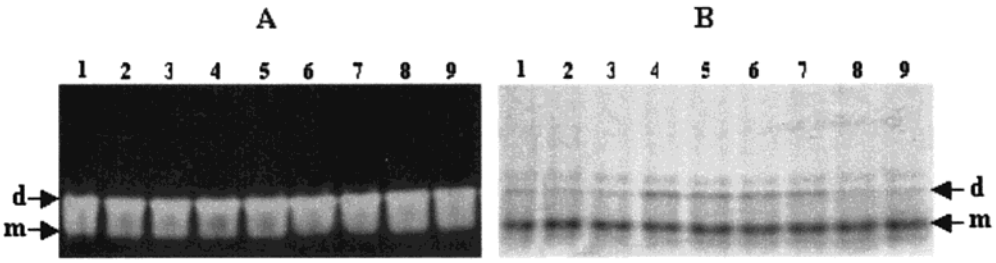

Figure 4. Effect of pH on enzyme stability. The enzyme samples were incubated in buffers with different pH values at 37 °C for 1 h and then subjected to a 10% native- PAGE: (A) activity staining (1.5 μg/lane); (B) Coomassie blue staining (1.9 μg/lane), lanes 1–9, pH 2.3, 3.0, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0, and or 11.0, respectively. The total areas of activity measured by densitometer were 1293.0 ± 10.1 (pH 2.3), 1410.3 ± 11.5 (pH 3.0), 1381.3 ± 22.7 (pH 4.0), 1456.0 ± 68.0 (pH 5.0), 1500.3 ± 106.5 (pH 7.0), 1460.7 ± 104.1 (pH 8.0), 1444.3 ± 70.9 (pH 9.0), 1576.0 ± 117.2 (pH 10.0), and 1521.3 ± 57.5 (pH 11.0). "d" denotes dimer, "m" denotes monomer. Triplicate experiments were done.

Table 1. Comparison (Percent Identity, Percent Similarity) of Amino Acid Sequences for Cu/Zn-SOD of Lemon and Other Organisms

type	source	common plant name	EMBL no.	organ	cultivar	% identity	% similarity
cytoplasmic	*Citrus limon*	lemon	AF318938	fruit			
	Ipomoea batatas	sweet potato	X73139	tuber root	Tainong 57	84	92
	Spinacia oleracea L.	spinach	X53872	leaf	King of Denmark	84	91
	Zea mays	maize	M15175			82	90
	Oryza sativa	rice	D01000	developing seed	Nipponhare	82	89
	Arabidopsis thaliana	thale cress	X60935	leaf, stem	Columbia	82	87
	Pisum sativum	pea	M63003	seedling leaf	Little Marvel	81	90
	Solanum lycopersicum	tomato	X14040	seedling leaf	Sherry-type	80	88
plastidic	*Spinacia oleracea* L.	spinach	D10244	seedling leaf		70	80
	Solanum lycopersicum	tomato	X14041	seedling leaf	Sherry-type	66	78
	Pisum sativum	pea	J04087	seedling leaf	Progress No. 9	65	79

could encode for 152 amino acid residues. Using the program of the University of Wisconsin Genitics Computer Group, this amino acid sequence was compared with other organism species.

According to the 744 bp cDNA sequence, a 5′ upstream primer (5′ GAA TTC GAT GGT GAA AGC AGT TGC AGT T 3′) and a 3′ downstream primer (5′ CTC GAG CCC TTG GAG GCC AAT TAT GCC 3′) were synthesized. Using 0.05 μg of lemon single-strand cDNA as template, 10 pmol of each 5′ upstream and 3′ downstream primer was added. A 0.45 kb fragment was then amplified by PCR, ligated with pCR2.1, and transformed into *E. coli* TOPO 10 as host. A positive clone was selected by hybridization (*11*) with ^{32}P-labeled Cu/Zn-SOD cDNA as probe, and plasmid (recombinant DNA) was prepared. Appropriate plasmid DNA was digested with *Eco*RI and *Xho*I and then electrophoresed on an 0.8% agarose gel. A 0.45 kb insert DNA containing *Eco*RI and *Xho*I sites was recovered and ligated with pET-20b(+) (pretreated with *Eco*RI and *Xho*I) from Novagen (Madison, WI) and then transformed into BL21(DE3). A transformed clone was selected by hybridization with ^{32}P-labeled Cu/Zn-SOD cDNA as probe.

Culture and Enzyme Purification. The transformed *E. coli* cells were grown at 37 °C in 500 mL of Luria−Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 0.9. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 37 °C for 4 h at 150 rpm, and then the bacterial cells were harvested by centrifugation at 7000*g* for 5 min. The cells were suspended in 4 mL of 10 mM Tris buffer (pH 8.0) containing 0.1% glycerol and 1 g of glass beads, then vortexed for 3 min and centrifuged at 10000*g* for 5 min. The extraction procedure was repeated two times, and the supernatants were pooled together. The final crude enzyme (16 mL) was loaded on a Ni^{+}−nitrilotriacetic acid Sepharose superflow (Qiagen) column (bed volume = 4 mL), and then the column was

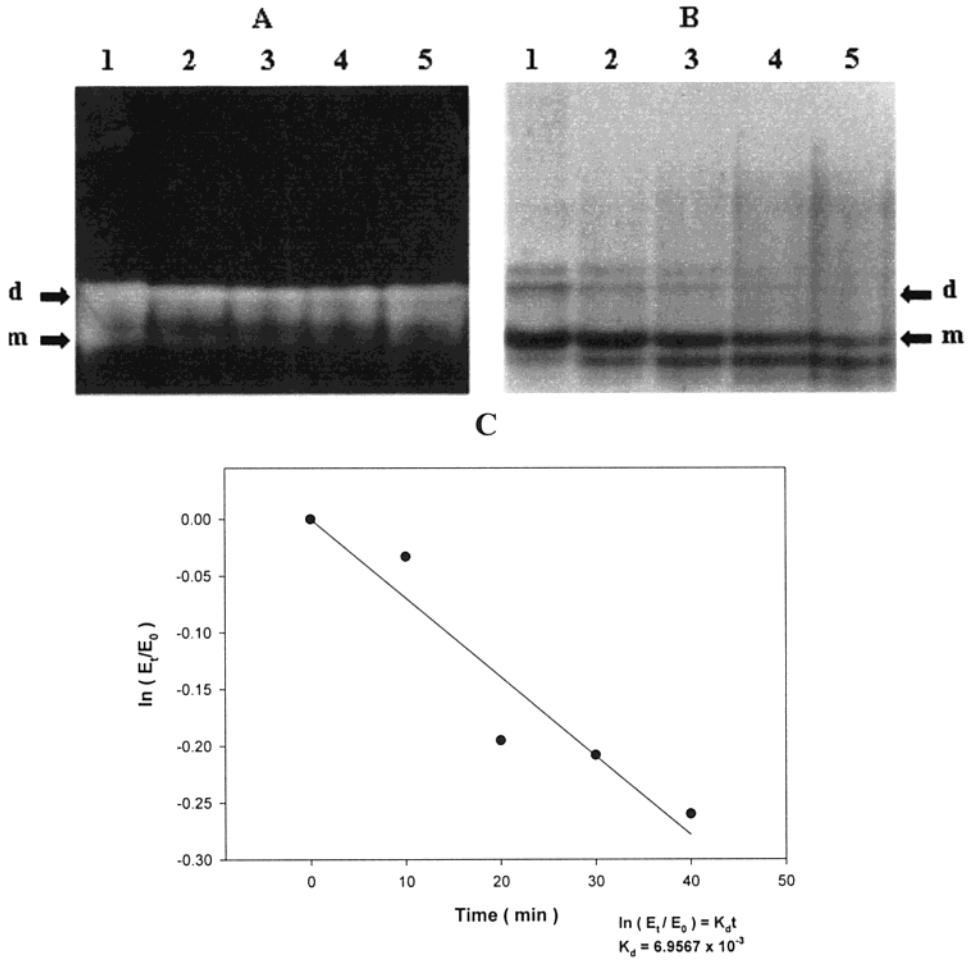

Figure 5. Effect of temperature on enzyme stability. Enzyme samples heated at 90 °C for various times were subjected to a 10% native-PAGE: (A) staining for activity (3.4 μg/lane); (B) Coomassie blue staining (3.4 μg/lane), lanes 1–5, control, 10, 20, 30, and 40 min, respectively; (C) plot of thermal inactivation kinetics. PAGE data were quantitated by a densitometer for calculation. E_0 and E_t are original activity and activity after being heated for time t, respectively. ●, dimeric form. "d" denotes dimer, "m" denotes monomer. Triplicate experiments were done.

washed with 20 mL of PBS buffer containing 20 mM imidazole. The enzyme was eluted with PBS containing 250 mM imidazole (flow rate = 0.4 mL/min, 4 mL/fraction, at room temperature). The purified enzyme (8 mL) that was dialyzed against 300 mL of PBS containing 0.1% glycerol at 4 °C for 4 h 2 times was either used for immediate analysis or stored at −20 °C for further analysis.

Protein Concentration Measurement. Protein concentration was determined by Bio-Rad protein assay kit (Richmond, CA) using bovine serum albumin as a reference standard.

Enzyme Assay in Solution. The SOD activity was measured by using a RANSOD kit (RANDOX, Ardmore, U.K.). One milliliter of the assay solution contained 40 mM CAPS at pH 10.2, 0.94 mM EDTA, 0.05 mM xanthine, 0.025 mM 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT), and 0.01 unit of xanthine oxidase. The amount of SOD added was adjusted so as to obtain the rate of INT reduction at 25 °C over the first 3 min time interval, measured as the absorbance at 505 nm, and falling within the percentage of inhibition that could be transformed into units of SOD by referring to a standard curve according to manual instructions.

Enzyme Assay by Activity Staining on a Native Gel. Samples of the enzyme were electrophoresed on a 10% native gel for 2.5 h at 100 V. The slab acrylamide gel was then cut into parts. One part was assayed, as previously described (*12*), for Cu/Zn-SOD activity staining: The gel was soaked in 0.6 mg/mL nitro blue tetrazolium (NBT) solution for 15 min in the dark with gentle shaking, followed by an immersion with illuminating in a solution containing 0.45% tetramethylenediamine and 10 μg/mL riboflavin. During illumination, the gel became uniformly blue except at positions containing SOD, which showed achromatic zones revealing insolubility of the blue reduction product of NBT by superoxide anion. The other part was stained with Coomassie blue.

The area and intensity of activity and protein bands were measured by a computing densitometer (Molecular Dynamics, Modesto, CA).

Enzyme Characterization. Each enzyme sample was 6.8 μg/20 μL for the following tests. After treatments as shown below, each sample was divided into two parts, and then each part of the sample was electrophoresed onto a 10% native polyacrylamide gel to determine the changes of activity and protein.

(1) pH Stability. Enzyme sample was amended with a half-volume of buffer in different pH values: 0.2 M citrate buffer (pH 2.2, 3.0, 4.0, or 5.0), 0.2 M Tris-HCl buffer (pH 7.0 or 9.0), or 0.2 M glycine−NaOH buffer (pH 10.0 or 11.0). Each sample was incubated at 37 °C for 1 h.

(2) Thermal Stability. The enzyme samples were heated at 90 °C for 10, 20, 30, or 40 min.

(3) SDS Effect. SDS was added into the enzyme sample to the level of 1.0, 2.0, 3.0, or 4.0% and then incubated at 37 °C for 1 h. Both SDS and imidazole are protein-denaturing reagents.

(4) Imidazole Effect. Imidazole was added into the enzyme to the level of 0.25, 0.5 or 1.0 M and then incubated at 37 °C for 1 h.

(5) Proteolytic Susceptibility. The enzyme was incubated with $^1/_{20}$ in weight of trypsin or chymotrypsin at pH 8.8 and 37 °C for up to 1, 2, 3, or 4 h. In the chymotrypsin digestion, $CaCl_2$ was added to 20 mM. Aliquots were removed from time to time and analyzed.

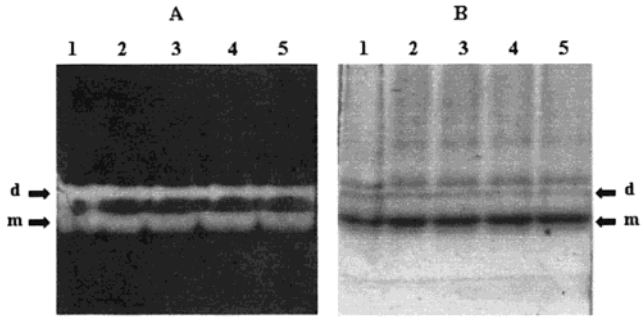

Figure 6. Effect of SDS on enzyme stability. Enzyme samples were incubated in various SDS concentrations at 37 °C for 1 h and subjected to a 10% native-PAGE: (A) activity staining (3.4 μg/lane); (B) Coomassie blue staining (3.4 μg/lane), lanes 1–5, control, 1, 2, 3, or 4%, respectively. The dimer areas of activity measured by densitometer were 604.0 ± 27.6 (control), 501.1 ± 12.5 (1%), 434.6 ± 20.9 (2%), 450.2 ± 18.5 (3%), and 390.0 ± 10.6 (4%). The monomer areas of activity measured by densitometer were 466.1 ± 50.0 (control), 502.5 ± 45.5 (1%), 450.0 ± 44.3 (2%), 502.6 ± 47.9 (3%), and 453.8 ± 22.0 (4%). "d" denotes dimer, "m" denotes monomer. Triplicate experiments were done.

RESULTS AND DISCUSSION

Cloning and Characterization of a cDNA Coding for Lemon Cu/Zn-SOD. Figure 1 shows the nucleotide and deduced amino acid sequence of one lemon Cu/Zn-SOD clone. Sequence analysis indicated that the cDNA was full-length, comprising a complete open reading frame coding for 152 amino acid residues. The DNA sequence translation start site (CACAATGG) matches the consensus sequence (AACAATGG) reported (*13*).

Table 1 shows higher identity with the amino acid sequence of the cytoplasmic Cu/Zn-SOD from other plant species (80−84%) than with the plastidic Cu/Zn-SOD sequence from other plants (65−70%).

Figure 2 shows that seven residues coordinating copper (His-45, -47, -62, and -119) and zinc (His-62, -70, and -79 and Asp-82), as well as the two cysteines (56 and 145) that form a disulfide bridge, are conserved as they are all reported Cu/Zn-SOD sequences (*2*).

One goal of this study was to clone and express the lemon Cu/Zn-SOD coding sequence in *E. coli*. Using single-strand cDNA as the template and two specific primers corresponding to the translation initiation and termination sequences, respectively, the 0.45 kb DNA fragment coding for the lemon Cu/Zn-SOD was amplified by PCR and successfully subcloned into the expression vector pET-20b(+). Positive clones were verified by DNA sequence analysis.

The transformants were induced with IPTG, and their total cellular proteins were analyzed by a 10% native-PAGE with activity staining or protein staining (**Figure 3**).

Purification of Lemon Cu/Zn-SOD. The lemon Cu/Zn-SOD was fused in the pET-20b(+)−6His-tag vector and expressed in *E. coli* BL21(DE3). The enzyme containing the His-tag in the C terminus was purified by affinity chromatography with nickel-chelating Sephrose (Qiagen) according to the instruction manual. The yield was 2.7 mg from 0.5 L of culture. The specific activity was 7456 units/mg. The purified enzyme showed two active enzymatic forms (30% dimer and 70% monomer in equilibrium, **Figure 3B**, lane 3) on a 10% native-PAGE. From lane 3 of **Figure 3A**, the activity of dimer was 65% higher than that of monomer. In lane 3 of **Figure 3C**, an arrow indicates the lemon SOD was dissociated to a single protein band on SDS-PAGE.

Characterization of the Purified Lemon Cu/Zn-SOD. As shown in **Figure 4** (lanes 1−9), lemon SOD was very stable in a broad pH range, from pH 2.3 to 11. Quantitation of activity (**Figure 4A**) and protein (**Figure 4B**) by a densitometer revealed that the lemon SOD retained its integrity in both acidic pH (2.2−4.0) and alkaline pH (10−11). This indicates that this enzyme is much more stable than that from papaya (*9*).

The enzyme inactivation kinetics at 90 °C fit the first-order inactivation rate equation $\ln(E_t/E_0) = k_d t$, where E_0 represents the original activity and E_t the residual activity that remained after heating for time t. The thermal inactivation rate constant k_d value calculated for the enzyme at 90 °C was 7.0×10^{-3} min^{-1}, and the half-life for inactivation was 99 min (**Figure 5C**). This showed the enzyme was not only much more thermally stable than that of papaya (*9*), or that from teleost fish skin, which when heated at 70 °C was completely inactivated (*14*), but also dimer rather than monomer as shown in lanes 2−5 of **Figure 5A**. As shown in lanes 2−5 of **Figure 5B**, protein bands decrease depending on heating time, whereas additional denaturing bands lower than monomer were observed under high temperature.

The enzyme activity showed only a 20% decrease even in a high concentration of SDS up to 4% (**Figure 6A**, line 5), but SDS did not affect the protein integrity of either dimer or monomer as shown in lanes 2−5 of **Figure 6B**. With another denaturing reagent, a significant decrease of SOD activity was observed in a high concentration of imidazole to the level of 0.5 M (data not shown). The enzyme (especially the dimeric form) was resistant to digestion by chymotrypsin and trypsin (data not shown) even at a high enzyme/substrate (w/w) ratio of 1/20. These results indicated the enzyme was highly stable compared to other species under extreme pH, high temperature, and high concentration of denaturing reagents or proteases. These stable properties are beneficial for various applications: as an agent against inflammatory reactions associated with chemical irritation and acne (*15*); in cosmetics with porphyrin (*16*) or even melanin (*17*) for the protection of the skin, hair, and/or mucosae against the harmful and/or unesthetic effects caused by oxygen-containing free radicals; in liposomes administrated orally to prevent inflammation (*18*); and for the removal free radicals in the gastrointestinal tract to protect against diarrhea.

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Received for review June 25, 2002. Revised manuscript received September 24, 2002. Accepted September 24, 2002. This work was partially supported by the National Science Council of the Republic of China under Grant NSC 90-2313-B-019-050 to C.-T.L. and by the Council of Agriculture, Executive Yuan under Grant 90-BT-2.1-FID-F1 (3) to C.-T.L.

JF0207070