

# Molecular Cloning of a cDNA Coding for Copper/Zinc Superoxide Dismutase from Zebrafish and Its Expression in *Escherichia coli*

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A full-length complementary DNA (cDNA) clone encoding a putative copper/zinc superoxide dismutase (Cu/Zn-SOD) was amplified by a polymerase chain reaction (PCR)-based technique from cDNA synthesized from zebrafish (*Danio rerio*) mRNA. Nucleotide sequence analysis of this cDNA clone revealed that it comprised a complete open reading frame coding for 154 amino acid residues. The deduced amino acid sequence showed higher identity (73.5–74.3%) with swordfish and shark Cu/Zn-SOD than with Cu/Zn-SOD from mammals (69.6–70.9%) and plants (55.8–56.2%). The amino acid residues required for coordinating copper and zinc are conserved, as they are present in all reported Cu/Zn-SOD sequences. It lacks a targeting sequence, which suggests that the zebrafish cDNA clone encodes a cytosolic Cu/Zn-SOD. Furthermore, the coding region of Cu/Zn-SOD from zebrafish was introduced into an expression vector, pET-23a(+)-thioredoxin, and transformed into *Escherichia coli* AD494(DE3)pLysS. A predominant achromatic zone was detected by activity staining of native PAGE, and the expression pattern was shown by Coomassie blue staining of SDS-PAGE. This indicates that the Cu/Zn-SOD cDNA clone can be expressed in *E. coli*.

**Keywords:** Zebrafish; *Danio rerio*; molecular cloning; cDNA; copper/zinc superoxide dismutase; expression; *Escherichia coli*; PCR; pET-23a(+)-thioredoxin

## INTRODUCTION

Superoxide dismutases (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) are enzymes that scavenge molecular oxygen radicals and thus prevent the harmful effects of reactive oxygen in aerobic organisms. SODs are metalloproteins that can be classified into three types (Mn-, Fe-, and Cu/Zn-SOD) depending on the metal found in the active site (Brock et al., 1980; Harris et al., 1980). Previously, we have cloned and sequenced a Cu/Zn-SOD from sweet potato tuberous root (Lin et al., 1993). The gene structure was determined (Lin et al., 1995a), and we also established that subunit interaction enhanced the enzyme activity and stability of the recombinant Cu/Zn-SOD (Lin et al., 1995b).

In fish, the oxidative stress occurs as a result of the effect of the heavy metals or chemical stimuli and thus disturbs the natural antioxidant enzyme system (Dimitrova et al., 1994; Mather-Mihaich and Diguilio, 1986; Nicholls et al., 1989; Radi et al., 1988; Vinikour et al., 1980). There have been a few studies on fish SODs: (1) Cu/Zn-SOD isolated from swordfish liver was composed of two identical subunits of 16 kDa and appeared to dissociate more readily into subunits (Bannister et al., 1977). (2) Nakano's study suggested that the distribu-

tion of higher SOD activity in the dark parts of the skin of two fish species (plaice and coho salmon) might have been related to melanization (Nakano et al., 1993). (3) A unique Cu/Zn-SOD containing four identical subunits of 16 kDa was isolated from plaice skin, but the unique enzyme was completely inactivated at 70 °C (Nakano et al., 1995). (4) Primary structures of fish Cu/Zn-SOD are known by protein sequence analysis of the enzymes from swordfish and shark (Calabrese et al., 1989). However, none of the fish Cu/Zn-SOD cDNA sequences were reported. Recently, we have obtained full-length Cu/Zn-SOD cDNA clones from zebrafish and black progy *Acanthopagrus schlegelii* (unpublished results). We report in this paper the cDNA sequence and deduced amino acid sequence of a zebrafish Cu/Zn-SOD cDNA clone. In addition, the coding region of Cu/Zn-SOD cDNA from zebrafish was introduced into an expression vector, pET-23a(+)-thioredoxin, and transformed into *Escherichia coli* AD494(DE3)pLysS. This Cu/Zn-SOD cDNA clone can express Cu/Zn-SOD enzyme in *E. coli*.

## MATERIALS AND METHODS

Zebrafish (*Danio rerio*) provided by Dr. Jen-Leih Wu (Institute of Zoology, Academia Sinica, Taiwan) was raised in freshwater for 2 weeks before use for cloning experiments.

**Preparation of mRNA and cDNA Synthesis.** One male zebrafish weighing 1 g was put into liquid nitrogen and ground to powder in a ceramic mortar. The sample was dissolved in 10 mL of TRIzol reagent (GIBCO BRL, Gaithersburg, MD). After the sample had been incubated for 5 min at room temperature, 2 mL of chloroform was added, and the mixture was shaken vigorously for 15 s and incubated at room temperature for 2–3 min. The sample was centrifuged at

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TCGAGCGGCCCGCCCGGGCAGGTGTCAGC

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29  ATG GTG AAC AAG GCC GTT TGT GTG CTT AAA GGC ACC GGT GAA GTG ACC GGC
   1  M  V  N  K  A  V  C  V  L  K  G  T  G  E  V  T  G

80  ACC GTC TAT TTC AAT CAA GAG GGT GAA AAG AAG CCA GTG AAG GTG ACT GGT
  18  T  V  Y  F  N  Q  E  G  E  K  K  P  V  K  V  T  G

131 GAA ATT ACT GGC CTT ACT CCA GGA AAA CAT GGT TTC CAC GTC CAT GCC TTT
  35  E  I  T  G  L  T  P  G  K  H  G  F  H  V  H  A  F

182 GGT GAC AAC ACA AAC GGC TGC ATC AGT GCA GGT CCG CAC TTC AAC CCT CAT
  52  G  D  N  T  N  G  C  I  S  A  G  P  H  F  N  P  H

233 GAC AAA ACT CAT GGT GGG CCA ACC GAT AGT GTT AGA CAC GTC GGA GAC CTG
  69  D  K  T  H  G  G  P  T  D  S  V  R  H  V  G  D  L

284 GGT AAT GTG ACC GCT GAT GCC AGT GGT GTT GCA AAA ATT GAA ATC GAG GAT
  86  G  N  V  T  A  D  A  S  G  V  A  K  I  E  I  E  D

335 GCA ATG CTG ACT CTG TCA GGC CAA CAT TCT ATT ATT GGG AGG ACC ATG GTG
  103 A  M  L  T  L  S  G  Q  H  S  I  I  G  R  T  M  V

386 ATT CAT GAG AAG GAG GAT GAC TTG GGG AAG GGT GGC AAT GAG GAA AGT CTA
  120 I  H  E  K  E  D  D  L  G  K  G  G  N  E  E  S  L

437 AAA ACT GGC AAC GCT GGT GGT CGT CTG GCT TGT GGA GTG ATC GGC ATC ACT
  137 K  T  G  N  A  G  G  R  L  A  C  G  V  I  G  I  T

488 CAG TGAATCTGCTCTAATGGAAGAGCCGGTTGAAATATTGGTGACCAATGTGGATGCCTCTGAAGCA
  154 Q  *

CTAGCCCGCTGACATTACATCTCTATTTTTGTTAGGTAACGTTGAAAGAATTGTACTTTAGCCTTGTTAAGCT
GTTTTTCTGTGCATTTCTATGTGGGCAACCTTTTATTTGTTATCTGCTTAATTCATTAAACATTTGAGCAGACT
GGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

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

12000g for 15 min at 4 °C, and the aqueous phase was then transferred to a new tube. Five milliliters of isopropyl alcohol was added and the sample incubated at 4 °C for 10 min and then centrifuged at 12000g for 10 min at 4 °C. The total mRNA pellet was washed once with 75% ethanol. The poly-(A)<sup>+</sup> RNA was isolated by oligo-(dT) affinity chromatography. Double-strand blunted cDNA was synthesized using a kit (cDNA synthesis module RNP 1256) from Amersham (Little Chalfont, Buckinghamshire, England).

**Subcloning and DNA Sequence Analysis.** One microgram of blunted cDNA was ligated with 30 pmol of Marathon cDNA adaptor (Clontech, Palo Alto, CA) at 4 °C for 16 h. According to the amino acid sequence (DED RHVGD, DDLGRG-GN) of the swordfish (Calabrese et al., 1989) Cu/Zn-SOD, two degenerate primers were synthesized. Using 0.05 g of the ligated cDNA as a template, 10 pmol of each of the two degenerate primers was added. One 0.15 kbp cDNA was amplified by the polymerase chain reaction (PCR) technique (25 cycles of 94 °C for 30 s, 46 °C for 1 min, and 68 °C for 1.5 min). The 0.15 kbp cDNA was subcloned into the pGEM-T

cloning vector (Promega, Madison, WI) using *E. coli* JM109 as the host. Nucleotide sequence was determined in both directions according to the dideoxy technique using a Taq Track sequencing system kit (Promega). According to the 0.15 kbp cDNA sequence, an FCu-4R primer (5' CCA TGG TCC TCC CAA TAA TAG AAT 3') and an FCu-4 primer (5' ATT CTA TTA TTG GGA CCA TGG 3') were synthesized. Using 0.05 g of the ligated cDNA as a template, for 5' end extension, 10 pmol of Clontech adaptor primer 1 (AP1) and 10 pmol of FCu-4R primer were added to perform PCR (25 cycles of 94 °C for 30 s, 46 °C for 1 min, 72 °C for 2 min). Again using 0.05 g of the ligated cDNA as a template, for 3' end extension, 10 pmol of AP1 primer and 10 pmol of FCu-4 primer were added for PCR under the same conditions as for 5' end extension. One 0.3 kbp cDNA (5'-RACE: 5'-cDNA end) and one 0.4 kbp cDNA (3'-RACE: 3'-cDNA end) were amplified by the PCR technique. Both 0.3 and 0.4 kbp cDNA fragments were subcloned into pGEM-T using *E. coli* JM109 as a host. Sequence analysis revealed that 5'-RACE and 3'-RACE cover the full-length Cu/Zn-SOD cDNA (0.7 kbp). Using the 5'-RACE and 3'-RACE as

	1				41	
zebrafish	M	VNKAVCVLKG	TGEVTGTVYF	NQEGEKPKVK	VTGEITGLTP	
swordfish		.L.....R.	A..T.....	E...NANA.G	KGIILK....	
shark		M.....	.....L.	E.-AADG..T	LK.S.....	
mouse	.	AM.....	D.P.Q..IH.	E.KASGE..V	LS.Q....E	
human	.	AT.....	D.P.Q.IIN.	E.KESNG... .	W.S.K...E	
xenopus		V.....A.	S.D.K.V.R.	E.Q.DDGD.T	.E.K.E...D	
<i>C.elegans</i>	.	S.R.A..R.	E.T...IWI	T.KS.NDQAV	IE...K....	
drosophila	.	.V.....IN.	DAK--...F.	E..SSGT..K	.S.VC..AK	
yeast	.	.Q...A....	DAG.S.V.K.	E.AS.SE.TT	.SY..A.NS.	
tomato	A	TK...A....	NSN.E.V.TL	S.D.DDG.TT	.NVR....A.	
sweet potato	.	-....A..SS	SEG.S..IF.	S...DG-.TT	...NVS..K.	
pea	.	-....A..SN	SN..S..IN.	S...NG..TT	...TLA..K.	
	42	* *	o	* * * *	91	
zebrafish	GK	HGFHVHAF	GDNTNGCISA	GPHFNPHDKT	HGGPTDSVRH	VGDLGNVTAD
swordfish		.E-H.....G.	.....	.....AS.K	.A..K.ED..	.....
shark		..-H.....	.....	...Y..FS.N	...D.EE..	.....E.N
mouse		.Q-H.....QY	...Q..T..	.....S.K	...A.EE..	.....G
human		.L-H.....E.	...A..T..	.....LSRK	...K.EE..	.....
xenopus		.N-H...I.VF	.....L..	.....QN.N	..S.K.AD..	.....E
<i>C.elegans</i>		.L-H.....QY	..S.....	.....FG..	...KSEI..	.....E.G
drosophila		.L-H.....E.	.....M.S	.....YG.E	..A.V.EN..	L.....IE.T
yeast		NAER...I.E.	..A...V..	.....FK..	..A...E... .	M...KT.
tomato		.L-H...L.EY	..T...M.T	.A...NKL.	..A.G.EI..	A.....IV.N
sweet potato		.L-H.....L	..T...M.T	.....AG.E	..A.G.DN..	A.....I.VG
pea		.L-H...I..L	..T.....T	.....NG.E	..A.E.ET..	A.....INVG
	92			*	138	
zebrafish	ASGVAKIEIE	DAMLTLSGQH	SIIGRTMVIH	EKEDDLGKGG	N---EESLKT	
swordfish	.N....D.T	.K-IS.T.PY	.....	..A...R..	---.....	
shark	GN...EF..K	.RQ.H...ER	.....L.V.	.....	D---...R.	
mouse	KD...NVS..	.RVIS...E.	.....V.	..Q.....	---...T..	
human	KD...DVS..	.SVIS...E.D	C.....L.V.	..A.....	---...T..	
xenopus	G...QFKFT	.PQIS.K.E.R	.....A.V.	..Q.....	D---D....	
<i>C.elegans</i>	.D...KLT	.TLV..Y.PN	TVV..S..V.	AGQ...E.V	GDKA...K..	
drosophila	GDCPT.VN.T	.SKI..F.AD	.....V.V.	ADA...Q..	H---.L.KS.	
yeast	EN...GSFK	.SLIK.I.PT	.VV..SV... .	AGQ.....-	--DT.....	
tomato	.D...EVLV	.NQIP.T.PN	.VV..AL.V.	.L.....	H---.L..T.	
sweet potato	ED.T.SFT.T	.KQIP.T.AN	.V...AV.V.	GDP.....	H---.L.KS.	
pea	DD.TVSFT.T	.NHIP.T.TN	....AV.V.	ADP.....	H---.L.KT.	
	139	o	154			
zebrafish	GNAGGRLACG	VIGITQ				
swordfish	....S.....	...TE				
shark	....S.....	...AKD				
mouse	....S.....	...A.				
human	....S.....	...A.				
xenopus	.....	...FCP				
<i>C.elegans</i>	....A.A...	..ALAAPQ				
drosophila	....A.IG..	...AKV				
yeast	....P.P...	...L.N				
tomato	.....	.V.L.PI				
sweet potato	.....V...	I..LQG				
pea	.....V...	I..LQG				

**Figure 2.** Optimal alignment of Cu/Zn-SOD among several species: zebrafish, this study (EMBL Y12236); shark and swordfish (Calabrese et al., 1989); mouse (X06683); human (X02317); African clawed frog (X16585); *C. elegans* (L20135); yeast (J03279); fruit fly (Y00367); tomato (X14041); sweet potato (X73139); pea (M63003). Numbers refer to amino acid residues of zebrafish. A dot refers to identities with zebrafish. A dash denotes deletion. Residues coordinating copper and zinc are indicated with asterisks. The two cysteines that form a disulfide bridge are circled.

templates, one fused full-length cDNA of Cu/Zn-SOD was created by the PCR technique (EMBL Accession No. Y12236).

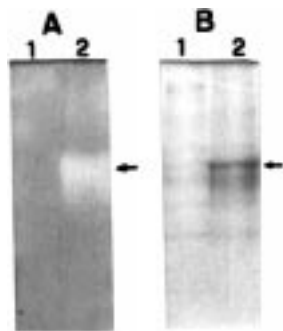
**Recombinant DNA Preparation and Transformation.** According to the 0.7 kbp cDNA sequence, a 5' upstream primer (5' GGG GTA CCC ATG GTG AAC AAG GCC GTT TG 3') and a 3' downstream primer (5' CCG CTC GAG CTG AGT GAT GCC GAT CAC TCC 3') were synthesized. Using 0.1  $\mu$ g of zebrafish blunted cDNA as a template, 10 pmol of each 5' upstream and 3' downstream primer was added; a 0.45 kbp

fragment was amplified by PCR and ligated with pGEM-T and then transformed into *E. coli* JM109 host. A positive clone was selected by hybridization with  $^{32}$ P-labeled Cu/Zn-SOD cDNA as probe, and the plasmid DNA was prepared. A suitable amount of the plasmid DNA was digested with *KpnI* and *XhoI* and then run on 0.8% agarose gel. A 0.45 kbp insert DNA containing *KpnI* and *XhoI* sites was recovered and ligated with pET-23a(+)-thioredoxin expression vector (pretreated with *KpnI* and *XhoI*) provided by Dr. Shye-Jye Tang (Institute

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

organism	EMBL no.	genus species	identity (%)	similarity (%)
zebrafish	Y12236	<i>Danio rerio</i>		
swordfish <sup>a</sup>		<i>Xiphias gladius</i>	77.612	82.090
shark <sup>a</sup>		<i>Prionace glauca</i>	77.612	81.343
mouse	X06683	<i>Mus musculus</i>	72.794	79.412
human	X02317	<i>Homo sapiens</i>	69.853	74.265
African clawed frog	X16585	<i>Xenopus laevis</i>	68.310	74.648
<i>C. elegans</i>	L20135	<i>Caenorhabditis elegans</i>	64.444	73.333
yeast	J03279	<i>Saccharomyces cerevisiae</i>	61.111	68.056
fruit fly	Y00367	<i>Drosophila melanogaster</i>	59.701	67.164
tomato	X14041	<i>Solanum lycopersicum</i>	57.931	67.586
sweet potato	X73139	<i>Ipomoea batatas</i>	56.250	63.194
pea	M63003	<i>Pisum sativum</i>	56.250	61.806

<sup>a</sup> Calabrese et al. (1989).



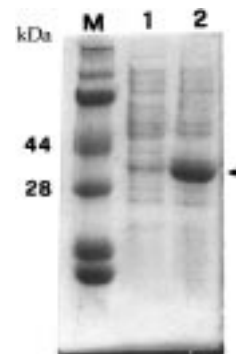
**Figure 3.** Activity staining and Coomassie blue staining of the enzyme extract of recombinant zebrafish Cu/Zn-SOD cDNA. Cells obtained from 3.0 mL induced cultures were prepared for the enzyme extract as described under Materials and Methods. Two aliquots of 10  $\mu$ L of enzyme extract containing 5.2  $\mu$ g of protein were subjected to a 10% native PAGE followed by activity staining (panel A) or Coomassie blue staining (panel B), respectively. Lane 1, AD494(DE3)pLysS carrying pET-23a(+)-thioredoxin as control; lane 2, AD494-(DE3)pLysS carrying recombinant Cu/Zn-SOD cDNA. An arrow denotes Cu/Zn-SOD activity (panel A) and Cu/Zn-SOD protein (panel B).

of Marine Biotechnology, National Taiwan Ocean University, Taiwan). The recombinant DNA was then transformed into AD494(DE3)pLysS (Novagen, Madison, WI) as a host. A transformed clone was selected by hybridization with <sup>32</sup>P-labeled Cu/Zn-SOD cDNA as probe.

**Culture and Enzyme Extraction.** The transformed *E. coli* cells were grown at 37 °C in 3.0 mL of Luria Bertani medium containing 50  $\mu$ g/mL ampicillin, 30  $\mu$ g/mL kanamycin, and 34  $\mu$ g/mL chloramphenicol until *A*<sub>600</sub> reached 0.9. Isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 37 °C for 3 h at 120 rpm, and the bacterial cells were then harvested by centrifugation at 6000g for 5 min. The cells were suspended in 0.2 mL of 10 mM Tris-HCl buffer (pH 8.0) containing 0.1% glycerol and 0.01 g of glass beads, then vortexed for 5 min, and centrifuged at 13000g for 5 min. The enzyme extract contained active Cu/Zn-SOD.

**Enzyme Assay by Activity Staining on Native PAGE.** An aliquot of 10  $\mu$ L of sample containing 5.2  $\mu$ g of protein of the extracted enzyme was electrophoresed on a 10% native PAGE gel for 2.5 h at 100 V. The slab polyacrylamide gel was then cut into two parts: One was assayed for Cu/Zn-SOD activity by a staining method as described previously (Beauchamp and Fridovich, 1971), and the other was stained with Coomassie brilliant blue (Smith, 1984).

**SDS-PAGE Analysis.** An aliquot of 15  $\mu$ L of sample containing 7.8  $\mu$ g of protein of the extracted enzyme with the buffer dye was heated for 5 min and then subjected to electrophoresis on a 10% polyacrylamide gel with SDS (Laemmli, 1970).



**Figure 4.** SDS-PAGE of the enzyme extract of recombinant zebrafish Cu/Zn-SOD cDNA with Coomassie blue staining. Two aliquots of 15  $\mu$ L of enzyme extract containing 7.8  $\mu$ g of protein with the buffer dye were heated for 5 min and then subjected to a 10% SDS-PAGE followed by Coomassie blue staining. M, protein molecular weight marker; lane 1, AD494-(DE3)pLysS carrying pET-23a(+)-thioredoxin as control; lane 2, AD494-(DE3)pLysS carrying recombinant Cu/Zn-SOD cDNA. An arrow denotes the expression protein of zebrafish Cu/Zn-SOD.

**Protein Concentration Measurement.** Protein concentration was determined by a kit (Bio-Rad protein assay) from Bio-Rad (Richmond, CA).

## RESULTS AND DISCUSSION

Figure 1 shows the nucleotide and deduced amino acid sequences of one zebrafish Cu/Zn-SOD clone. Sequence analysis revealed that the cDNA was of full length, comprising a complete open reading frame coding for 154 amino acid residues. The translation start site (CAGCATGG) matches the consensus sequence (AACAAATGG) as reported (Lütcke et al., 1987).

Table 1 shows higher identity with the amino acid sequence of the Cu/Zn-SOD from swordfish and shark (73.5–74.3%) than with the Cu/Zn-SOD sequences from other organisms including the mammals (mouse, 70.9%; human, 69.6%), African clawed frog (65.5%), and sweet potato (56.2%). These comparisons were done by the PILEUP program developed by the University of Wisconsin Genetics Computer Group. In addition, the amino acid sequence was compared with other organisms containing targeting sequence (Bordo et al., 1994). This suggests that zebrafish cDNA clone encoded a cytosolic Cu/Zn-SOD.

Figure 2 shows that seven residues coordinating copper (His-47, -49, -64, and -121) and zinc (His-64, -72, and -81 and Asp-84), as well as the two cysteines (58 and 147) that form a disulfide bridge, are conserved, as



they are present in all reported Cu/Zn-SOD sequences (Fridovich, 1986). There is another cysteine between Val-6 and Val-8, which is not found in *C. elegans* or all known plant species. It would be of great interest to determine the ability of Cys-7 to compete with Cys-58 or Cys-147 to form a disulfide bridge. Further studies are currently under way to gain deeper insight into the structure–function relationship of this enzyme.

As shown in Figure 3, an achromatic zone (panel A, lane 2) denoted the activity of recombinant Cu/Zn-SOD from crude enzyme extract matching the predominant protein by Coomassie blue staining (panel B, lane 2). This indicates that this recombinant Cu/Zn-SOD cDNA can be expressed as an active Cu/Zn-SOD enzyme in *E. coli* AD494(DE3)pLysS.

As shown in Figure 4, a predominant protein band was denoted (lane 2) in the SDS–PAGE. This also confirmed the expression of zebrafish recombinant Cu/Zn-SOD enzyme in *E. coli* AD494(DE3)pLysS.

Zebrafish is a model fish; the Cu/Zn-SOD cDNA of the fish is essential for studying the role of SOD in fish genetics and development and possibly for increasing the resistance of the fish to environmental stress by transgenic manipulation. Furthermore, the successful expression of the cDNA in an *E. coli* system provides an easy method for obtaining large amounts of active enzyme for further enzymatic or application studies.

#### CONCLUSION

A full-length cDNA encoding a putative Cu/Zn-SOD from zebrafish was amplified by the PCR technique. This clone comprises a complete open reading frame coding for 154 amino acid residues. The coding region was introduced into an expression vector, pET-23a(+)-thioredoxin, and transformed into *E. coli* AD494(DE3)-pLysS. The expression of the recombinant Cu/Zn-SOD cDNA clone was confirmed by enzyme activity staining on the native polyacrylamide gel.

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