Cloning and Characterization of a cDNA for Manganese Superoxide Dismutase from Callus of Sweet Potato

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A full-length complementary DNA (cDNA) clone encoding a putative manganese superoxide dismutase (Mn-SOD) from the callus of sweet potato was amplified by polymerase chain reaction technique from cDNAs synthesized from callus mRNA. Nucleotide sequence analysis of this cDNA clone revealed that it comprises a complete open reading frame coding for 233 amino acid residues and a 29 amino acid transit peptide at the N terminus. The deduced amino acid sequence showed greater identity (67−75%) with plant mitochondrial Mn-SOD than with Mn-SOD from mammalian mitochondria (53%), yeast (47%), or bacteria (44%). The residues required to coordinate the single tervalent manganese ion and the 11 residues putatively involved in the active center are conserved as they are among all reported Mn-SOD sequences. These suggest that the sweet potato callus cDNA clone encodes a mitochondrial Mn-SOD. In addition, the coding region of Mn-SOD cDNA from sweet potato callus was introduced into an expression vector, pET-28a(+) and transformed into *Escherichia coli* BL21(DE3). A predominant protein band was detected by Coomassie blue staining of native PAGE, and activity staining confirmed the result of Coomassie blue staining. These indicate that this Mn-SOD cDNA clone can express Mn-SOD enzyme in *E. coli*.

Keywords: *Manganese superoxide dismutase; Mn-SOD; callus; sweet potato (Ipomoea batata L.)*

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 and are classified into three types (Mn-, Fe-, and Cu/Zn-SOD) depending on the metal found in the active site (Brock et al., 1980; Fridovich, 1986; Harris et al., 1980). In higher plants, the activity of SOD increases in response to a variety of environmental and chemical stimuli (Fridovich, 1986; Perl-Treves et al., 1991). The most prominent plant SODs are Cu/Zn isoenzymes found in the cytosol and plastids (Sakamoto et al., 1992). Mn-SOD is less abundant and is primarily located in mitochondria. Many plant Cu/Zn-SOD complementary DNAs (cDNAs) from leaves or seedlings have been sequenced and compared, but only a few plant Mn-SOD sequences including tobacco, rubber tree, rice, pea, and maize have been reported (Bowler et al., 1989; Miao et al., 1993; Sakamoto et al., 1993; Wong-Vega et al., 1991; Zhu and Scandalios, 1993), with no reports of Mn-SOD cDNA from callus tissue. 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). Here we report the cDNA sequence and deduced amino acid sequence of a Mn-SOD cDNA clone from sweet potato leaf callus. Furthermore, the coding region of Mn-SOD cDNA was introduced into an expression vector, pET-28a(+), and transformed into *Escherichia coli* BL21-(DE3). The enzyme activity was detected by activity staining.

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MATERIALS AND METHODS

Materials. Callus was induced from the leaf of sweet potato (*Ipomoea batatas* L. Lam. cv. Tainong 57) and cultured on Murashige-Skoog (MS) medium containing 3 ppm of kinetin, 8% sucrose, and 0.8% agar in the dark at 25 °C for 10 days. Growing callus (27.5 g) was obtained from Mr. Heng-Long Hwang of the Institute of Biological Chemistry, Academia Sinica, Taiwan.

mRNA Preparation and cDNA Synthesis. Callus was frozen in liquid nitrogen and ground to powder in a ceramic mortar. Total RNA was prepared according to the guanidium−HCl procedure (Chirgwin et al., 1979). The poly(A)$^+$ RNA was isolated according to oligo-(dT) affinity chromatography. Double-strand blunted cDNAs were synthesized using a kit (cDNA synthesis module RPN 1256) from Amersham (Little Chalfont, Buckinghamshire, England).

Subcloning and DNA Sequence Analysis. One microgram of blunted cDNAs was ligated with 30 pmol of Marathon cDNA adaptor (Clontech, Palo Alto, CA) at 22 °C for 4 h. A Tm-6 primer (5′ CAT CTT TTG TAT CAG AGC ATC 3′) was synthesized according to a 3′-partial sequence of Mn-SOD clone from sweet potato root tuber (Accession No. L36676) that we previously cloned and sequenced from a cDNA library constructed in the cloning vector in λgt11. Using 0.05 μg of the ligated cDNA as a template, 10 pmol of Clontech adaptor primer and 10 pmol of Tm-6 primer were added, and one 0.5 kbp cDNA (5′-RACE: 5′-cDNA end) was amplified by polymerase chain reaction (PCR) technique. The 0.5 kbp cDNA was subcloned into pGEM-T using JM109 as a host. Nucleotide sequence was determined in both directions according to the dideoxy technique using Sequenase (United States Biochemical, Cleveland, OH). According to the 0.5 kbp cDNA sequence, a 5′ upstream primer (5′ CTC ACA CTT TCT CGA

S0021-8561(96)00484-0 CCC: $14.00

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   1                CTCACACTTTCTCGACTGCTCGATATATAGTAACAGAATCTCGAAGAAGCAGGAAGAGTATAACA

  66  ATG GCA CTT CGA AAC CTA GCC ACC CGA AAA ACC CTT TCC GCC ACC GGC AAG CGT TCC GGC
   1    M   A   L   R   N   L   A   T   R   K   T   L   S   A   T   G   K   R   S   G

 126  AGC ACG TGC CGG CTT GCA GAC GCC ACG CTT CCT GAT CTT CCC TAT GAC TAC GCG CCC TGG
  21    S   T   C   R   L   A   D   A   T   L   P   D   L   P   Y   D   Y   A   P   W

 186  AGC GCC ATT AGC GGA GAG ATC ATG CAG CTC CAT CAC CAG AAG CAC CAC CAG GCC TAC GTC
  41    S   A   I   S   G   E   I   M   Q   L   H   H   Q   K   H   H   Q   A   Y   V

 246  ACC AAC TAC AAC AAG GCT CTC GAG CAG CTC CAC GAG GCC ATC AGC AAG GGC GAT GCT TCC
  61    T   N   Y   N   K   A   L   E   Q   L   H   E   A   I   S   K   G   D   A   S

 306  GCC GCC GTC AAA TTG CAG AGC GCC ATC AAG TTC AAC GGC GGA GGC CAT ATA AAC CAC TCA
  81    A   A   V   K   L   Q   S   A   I   K   F   N   G   G   G   H   I   N   H   S

 366  ATT TTC TGG AAG AAC CTT GCC CCT ACT CGG GAA GGA GGT GGC GAG CCT CCG AAG AGT TCT
 101    I   F   W   K   N   L   A   P   T   R   E   G   G   G   E   P   P   K   S   S

 426  TTG GGC TGG GAG ATT GAC AAT CAC TTT GGC TCT TTA GAT GCT CTG ATA CAA AAG ATG TCT
 121    L   G   W   E   I   D   N   H   F   G   S   L   D   A   L   I   Q   K   M   S

 486  GCA GAA GGT GCT GCT GTA CAA GGT TCT GGC TGG GTG TGG CTG GGT TTG GAC AAA GAG CTG
 141    A   E   G   A   A   V   Q   G   S   G   W   V   W   L   G   L   D   K   E   L

 546  AAG CAC CTT GTG GTT GAA ACC ACC CCA AAT CAG GAC CCT TTG GTT ACT AAA AAT CCA AAC
 161    K   H   L   V   V   E   T   T   P   N   Q   D   P   L   V   T   K   N   P   N

 606  AAG GTT CCT CTA CTG GGC ATA GAT GTT TGG GAA CAT GCG TAC TAC TTA CAG TAC AAG AAT
 181    K   V   P   L   L   G   I   D   V   W   E   H   A   Y   Y   L   Q   Y   K   N

 666  GTG AGG CCG GAT TAC TTG AAG AAT ATA TGG AAA GTT GTG AAC TGG AAA TAT GCA TGT GAA
 201    V   R   P   D   Y   L   K   N   I   W   K   V   V   N   W   K   Y   A   C   E

 726  GTT TAC CAA CAA TTT ACA CCA TTG CCT GCA AGC AGA GAC TGATCAGACCAGGTGATCCAACACTCA
 221    V   Y   Q   Q   F   T   P   L   P   A   S   R   D   *

 792  TCAAGGTGCCCGAGTTTCTTTAATCTCGTGGGATAAAATAATGCTAAGTTAACTTTGCTTCGGCTTTCTGCTGAACTGT

 871  TGTTGCTTCTGTTTTCTGTGTAGTGGTAAATAAATTGTTGTATTTGTGAATTTTGTTGCTTTTAGCACACTGTCACTGT

 950  GATGTGAAACTTATCACTTCAACAGAGTTAAAATTGAAAAATGCAGGAGTTTTTCTGCT  1008
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Figure 1. Nucleotide sequence of a sweet potato callus SOD (SW-MnSOD) cDNA and the deduced amino acid sequence. Numbers to the left refer to nucleotide and amino acid residues. Consensus sequence of the translation start site and polyadenylation signal are underlined. The asterisk denotes the stop signal.

CTG CTC 3′) was synthesized; a 3′ downstream primer (5′ AGC AGA AAA ACT CCT GCA TTT T 3′) next to the polyadenylated sequence at the 3′ end was also synthesized according to a 3′ partial sequence of Mn-SOD clone described formerly; again using 0.05 μg of callus-blunted cDNA ligated with Marathon adaptor as a template, a full-length cDNA (1.0 kbp) of sweet potato Mn-SOD was amplified by PCR technique. The 1.0 kbp Mn-cDNA was subcloned into pGEM-T (Promega, Madison, WI) using JM109 as a host, and nucleotide sequence was determined in both directions by the dideoxy technique using Taq Track Sequencing Systems kit (Promega).

Recombinant DNA Preparation and Transformation. With 50 ng of Mn-SOD cDNA as a template, 10 pmol of each upstream primer and downstream primer was added (upstream primer, 5′ CCA TAT GCT TGC AGA CGC CAC GCT TCC TGA TCT TC 3′; downstream primer, 5′ CGG GAT CCT CAG TCT CTG CTT GCA GGC AAT GG 3′). A 0.6 kbp DNA fragment (not including transit peptide) amplified by PCR was ligated with pGEM-T (Promega) and transformed into *E. coli* JM109 host. A positive clone was selected by hybridization with ^{32}P-labeled Mn-SOD cDNA as probe and plasmid DNA was prepared; appropriate plasmid DNA was digested with *Nde*I and *Bam*HI and then run on 0.8% agarose. A 0.6 kbp insert DNA containing *Nde*I and *Bam*HI sites was recovered and ligated with pET-28a(+) (pretreated with *Nde*I and *Bam*HI) from Novagen (Madison, WI). Transformed clone was selected by hybridization with ^{32}P-labeled Mn-SOD cDNA as probe.

Culture and Enzyme Extraction. The transformed *E. coli* were grown at 37 °C in 20 mL of Luria Bertani medium containing 37.5 μg/mL kanamycin until A_{600} reached 0.9. The culture temperature was reduced to 30 °C, and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 30 °C for 5 h at 120 rpm, and then the bacterial cells were harvested by centrifugation at 6000*g* for 5 min. The cells were suspended in 0.15 mL of 10 mM Tris buffer (pH 6.8) containing 0.5% glycerol and 0.1 g of glass beads, then vortexed for 5 min, and centrifuged at 13000*g* for 5 min (the extraction was repeated three times). The enzyme extraction contained active Mn-SOD.

Enzyme Assay by Activity Staining on Native PAGE. Two 27 μL samples of the enzyme extraction were electrophoresed on 10% native gel for 3 h at 120 V; the slab acrylamide gel was then cut into two parts: one was assayed for Mn-SOD activity staining as described previously (Beauchamp and Fridovich, 1971) and the other was stained with Coomassie blue.

RESULTS AND DISCUSSION

Figure 1 shows the nucleotide and deduced amino acid sequences of one Mn-cDNA clone. Sequence analysis found that the cDNA was full length, comprising a complete open reading frame coding for 233 amino acid residues. There is a putative mitochondrial transit peptide (derived amino acids 1−29), and Leu-30 is presumably the amino terminus of the mature protein. The DNA sequence translation start site fully matches the consensus sequence (AACAATGG) reported in plants (Lütcke et al., 1987).

Table 1 shows higher identity with the amino acid sequence of the mitochondrial Mn-SOD from other plant species (tobacco, 75%; maize, 67.4%) than with the Mn-SOD sequences from other organisms including mam-

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   SW-MnSOD  MALRNLATRK TLS----ATG KRSGSTC--- ----R-LADA TLPDLPYDYA PWSA-ISGEI  47
     pssodr  ..A.T.LC.. ...SVLRNDA .PI.AAIAAA STQS.G.HVF .+...A...G ALEPV.....  60
    npmnsod  ....T.VS.R ..-------- ----A.GLGF RQQL.G.QTF S........G ALEPA...D.  48
    hevsodmn ....S.V... N.PSAFK--- ---AA.GLG- --QL.G.QTF S........G ALEPA.....  51
   ricsodaoa ....T..S.. ..----A.AA LPLAAAAAA- -----.GVTTV A........G ALEPA.....  51
  mzemtmsoda ....T..SKN A..FALGGAA RP.AAS---- ---A.GVTTV A....S..FG ALEPV.....  53
    hsmnsod             M..RAVCG.S RQLAPALGYL GSRQKH---- S........G ALEPH.NAQ.  46
    mmnsodr             M.CRAACS.. R.L.PVAVAA GSRHKH---- S........G ALEPH.NAQ.  46
    scsodmng            MFAKTAA.NL TKK.GLSLLS TTARRT--KV .....KW.FG ALEPY...Q.  48
      ecsod                                         MSY ...S...A.D ALEPHFDKQT  23
     bacsoda                                        MPF E..A...P.D ALEPH.DK.T  23
         tth                                      MPYPF K....G.P.E ALEPH.DAKT  25

   SW-MnSOD  MQLHHQKHHQ AYVTNYNKAL EQLHE----- --AISKGDAS AAVKLQSAIK FNGGGHINHS 100
     pssodr  ..I.......  T.I.......  ....D-----  --.VA.A.T. .T....N... .......... 113
    npmnsod  ......N...  T.........  ....D-----  --.......P .VA..H.... .......... 101
    hevsodmn ..........  T.I.......  ...ND-----  --..E...SA TV........ ......V... 104
   ricsodaoa .R.......A  T..A......  ...DA-----  --.VA....P TI.H...... ......V... 104
  mzemtmsoda .R.......A  T..V......  ...DA-----  --VVV..... .V.Q..G... ......F... 106
    hsmnsod  .....S...A  ...N.L.VTE  .KYQ.-----  --.LA...VT .QTA..P.L. .......... 99
    mmnsodr  .....S...A  ...N.L.ATE  .KY..-----  --.LA...VT TQ.A..P.L. .........T 99
    scsodmng NE..YT....  T..NGF.T.V  D.FQ.LSDLL AKEP.PAN.R KMIAI.QN.. .H...FT..C 108
      ecsod  .EI..T....  T..N.A.A..  .S.P.FANLP VEELITKLDQ LPADKKTVLR N.A...A... 83
     bacsoda .NI..T...N  T....L.A..  .GHPDLQNKS LEELLSNLEA LPESIRT.VR N.....A... 83
         tth .EI......G  .....L.A..  .KYPYLHGVE VEVLLRHL.A LPQDI.T.VR N.....L... 85

   SW-MnSOD  IFWKNLAPTR EGGGEPPKSS LGWEIDNHFG SLDALIQKMS AEGAAVQGSG WVWLGLD-KE 159
     pssodr  ........V.  ........E.  ...A..TN..  ..E.....IN .....L.A.. .......-.D 172
    npmnsod  ........V.  ........G.  ...A..TN..  .LE..V...N .....L.... .....V.-.. 160
    hevsodmn ........V.  .....L.HG.  ...A..AD..  .LEK...L.N .....L.... ....A..-.. 163
   ricsodaoa ...N..K.IS  ....D..HAK  ...A..ED..  .FE..VK... .....L.... ....A..-.. 163
  mzemtmsoda ...E..K.I.  .-.....HGK  ...A..ED..  .FE..VKR.N .....L.... ....A..-.. 164
    hsmnsod  ...T..S.--  -N..GE..GE  .LEA.KRD..  .F.KFKE.LT .ASVG..... .G...FN-.. 155
    mmnsodr  ...T..S.--  -K..GE..GE  .LEA.KRD..  .FEKFKE.LT .MSVG..... .G...FN-.. 155
    scsodmng L..E....E.  Q......TGA  .AKA..EQ..  ...E..KLTN TKL.G..... .AFIVKNLSN 168
      ecsod  L...G.----  -KK.TTLQGD  .KAA.ERD..  .V.NFKAEFE KAA.SRF... .A..V.K--- 135
     bacsoda L..TI.S.--  -N..GE.TGE  .ADA.NKK..  .FT.FKDEF. KAA.GRF... .A..VVN--- 137
         tth L..RL.T.--  -..AKE.VGE  .KKA..EQ..  GFQ..KE.LT QAAMGRF... .A..VK.--P 140

   SW-MnSOD  LKHLVVETTP NQDPLV---- TKNPNKVPLL GIDVWEHAYY LQYKNVRPDY LKNIWKVVNW 215
     pssodr  ..R.V....A  ......----  ..GASL....  W.........  ..........  .......I.. 228
    npmnsod  ..R..I...A  ......----  ..GA......  ..........  ..........  .S.....M.. 216
    hevsodmn ..K......A  ......----  ..G.T.....  ..........  ..........  .......M.. 219
   ricsodaoa A.K.S....A  ......----  S.GA......  ..........  ..........  .......M.. 219
  mzemtmsoda P.K.S....A  ......----  ..GASL....  ..........  ..........  .N.....M.. 220
    hsmnsod  RG..QIAAC.  .....Q----  G-TTGLI...  ..........  ..........  ..A..N.I.. 210
    mmnsodr  QGR.QIAACS  .....Q----  G-TTGLI...  ..........  ..........  ..A..N.I.. 210
    scsodmng GGK.D.VQ.Y  ...TV-----  --TGPL...V  A..A......  ...Q.KKA..  F.A..N.... 221
      ecsod  GDK.A.VS.A  ...SPLMGEA  ISGASGF.IM  .L........  .KFQ.R....  I.EF.N.... 195
     bacsoda NGE.EITS..  ...SPIMEGK  .------.I.  .L........  .K.Q.R.PE.  IAAF.N.... 191
         tth FGK.H.LS..  ...NPVMEGF  .------.IV  .I........  .K.Q.R.A..  .QA..N.L.. 194

   SW-MnSOD  KYACEVYQQF TPLPASRD  233
     pssodr  .H.S...EKE SS        240
    ricsodaoa ...G...ENA .A       228
    hevsodmn ...S...AKE C.SS      233
    npmnsod  ...N...EKE C.        231
   mzemtmsoda ...G...ENV LA       232
    hsmnsod  ENVT.R.MAC KK        222
    mmnsodr  ENVT.R.TAC KK        222
    scsodmng .E.SRRFDAG KI        233
      ecsod  DE.AARFAAK K         206
     bacsoda DEVAKR.SEA KAK       204
         tth DV.E.FFKKA           204
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Figure 2. Optimal alignment of SW-MnSOD and other organisms. SW-MnSOD, sweet potato callus Mn-SOD (this study); pssodr, pea SOD (Wong-Vega et al., 1991); npmnsod, tobacco SOD (Bowler et al., 1989); hevsodmn, rubber tree SOD (Miao et al., 1993); ricsodaoa, rice SOD (Sakamoto et al., 1993); mzemtmsoda, maize SOD (Zhu and Scandalios, 1993); hsmnsod, human SOD; mmnsodr, mouse SOD; scsodmng, yeast SOD; ecsod, *E. coli* SOD; bacsoda, *Bacillus stearothermophilus* SOD; tth, *T. thermophillus* SOD (Stallings et al., 1985). A dot refers to identities with SW-MnSOD. A dash denotes deletion. The amino-terminal residues of each sequence are underlined. A circle refers to residues requiring binding the single tervalent manganese or involving the enzyme active center.

malian mitochondria (human, 52.8%), yeast (47%), and bacteria (44%). These comparisons were done by the program of the University of Wisconsin Genetics Computer Group.

Figure 2 shows that four residues (H-22, H-70, D-159, and H-163 in this mature enzyme), which are putatively required to coordinate the single tervalent manganese, are conserved as they are among all reported Mn-SOD (Brock et al., 1980; Fridovich et al., 1986; Harris et al., 1980; Stallings et al., 1985; White and Scandalios, 1988; Wong-Vega et al., 1991; Zhu and Scandalios, 1993). Residues H-22 through Y-30 containing four histidines, as well as the helix (residues P-154 through Y-169 which contain H-163, a patch of four aromatic and two acidic residues) are conserved and presumably form a tervalent manganese binding region. In addition, the putative active center involving 11 residues (H-22, H-26, Y-30, H-70, F-73, W-74, W-122, Q-142, D-159, W-161,

Table 1. Comparison (Percent Identity, Percent Similarity) of Amino Acid Sequences for Mn-SOD of Sweet Potato Callus and Other Organisms

file name	EMBL no.	genus species	% identity	% similarity	English name	strain	tissue
SW-MnSOD	L77078	*Impomea batatas*			sweet potato	Tainong 57	callus
pssodr	X60170	*Pisum sativum*	72.807	83.333	pea		
npmnsod	X14482	*Nicotiana plumbaginifolia*	75.000	83.333	tobacco		curled-leaved
hevsodmn	L11707	*Hevea brasiliensis*	74.783	81.739	rubber tree		
ricsodaoa	L19436	*Oryza sativa*	71.053	79.825	rice	Nipponbare	
mzemtmsoda	L19461	*Zea mays*	67.401	79.295	maize	W64A	
mmnsodr	X04972	*Mus musculus*	52.752	65.138	mouse		
hsmnsod	X14322	*Homo sapiens*	52.572	63.063	human		
scsodmng	X02156	*Saccharomyceaes cerevisiae*	47.273	65.455			
ecsod	X03951	*Escherichia coli*	43.590	60.000			
bacsoda	M81188	*Bacillus stearothermphilus*	44.898	61.224			
tth[a]		*T. thermophilus*					

[a] Stallings et al. (1985).

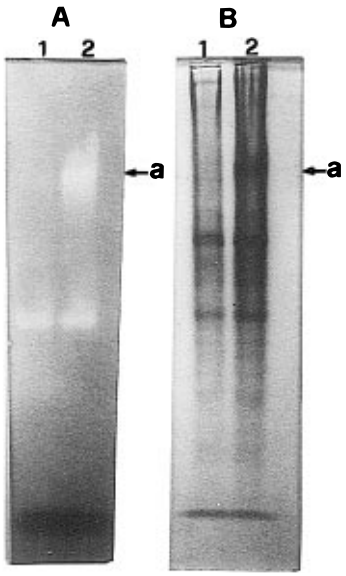

Figure 3. Activity staining and Coomassie blue staining of the crude extract of recombinant sweet potato Mn-SOD cDNA: (A) staining for activity; (B) staining for protein by Coomassie blue; (lane 1) BL21(DE3) carrying pET-28a(+) as control; (lane 2) BL21(DE3) carrying recombinant Mn-SOD cDNA. ←a denotes Mn-SOD activity (panel A) and Mn-SOD protein (panel B), respectively.

and H-163 in this study) is almost strongly conserved in all of the known sequences. Stallings et al. (1985) suggested that the observed helical conformation is required to juxtapose the three residues (H-22, H-26, and Y-30) in such an arrangement that is crucial for Mn-SOD catalysis based on the structure of Mn-SOD from *Thermus thermophilus* at 2.4 Å resolution. Studies are currently underway to elucidate the structure–function relationship of the sweet potato Mn-SOD.

As shown in Figure 3, an achromatic zone (panel A, lane 2, ←a) denoted the activity of recombinant Mn-SOD from crude enzyme extract matching the predominant protein by Coomassie blue staining (panel B, lane 2, ←a). This indicates that this recombinant Mn-SOD cDNA can express active Mn-SOD enzyme in *E. coli* BL21(DE3).

CONCLUSION

A full-length cDNA encoding a putative Mn-SOD from the callus of sweet potato was amplified by PCR technique. This clone comprises a complete open reading frame coding for 233 amino acid residues. The coding region was introduced into an expression vector, pET-28a(+), and transformed into *E. coli* BL21(DE3). The expression of the recombinant Mn-SOD cDNA was confirmed by enzyme activity staining on native acrylamide gel.

ACKNOWLEDGMENT

We thank Mr. Hsiang-Ting Liu and Keng-Yen Fu for technical assistance during the work. We sincerely thank Mr. Heng-Long Hwang for a generous gift of callus.

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Received for review July 8, 1996. Revised manuscript received November 5, 1996. Accepted November 14, 1996.[⊗] This work was supported by the National Science Council of the Republic of China through Grant NSC 86-2313-B-019-040 to C.-T. L.

JF960484B

[⊗] Abstract published in *Advance ACS Abstracts*, January 1, 1997.