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CLONING AND CHARACTERIZATION OF A COPPER/ZINC-SUPEROXIDE DISMUTASE GENE FROM *ASPERGILLUS JAPONICUS*.[1]

Chi-Tsai Lin[2,3], Ming-Tse Lin[2], Dey-Chyi Sheu[2], and Kow-Jen Duan[2]

ABSTRACT: In order to clone a Cu/Zn-SOD gene from *Aspergillus japonicus*, the highly conserved regions of the SOD sequence of maize and other species were compared and two oligonucleotides were synthesized. The *Aspergillus japonicus* cDNA was then used as the template to get a 0.3 kb fragment by PCR technique. The 0.3 kb DNA fragment has high homology compared with maize SOD-4 cDNA. Then we used the 0.3 kb fragment as probe to screen *Aspergillus japonicus* genomic libraries. A 0.4 kb DNA fragment from purified positive plaque was subcloned into pGEM-7zf(+) vector and sequenced. Nucleotide sequence analysis of this clone revealed that it comprised an open reading frame coding for 120 amino acid residues. The encoded polypeptide is highly homologous with other plant SODs. The residues required for coordinating copper and zinc are also conserved as they are among all reported Cu/Zn-SOD sequences.

KEYWORDS: *Aspergillus japonicus*, copper/zinc-superoxide dismutase (Cu/Zn-SOD).

INTRODUCTION

From 1939 to 1941, a copper-containing blue-green protein was isolated later proved to be important in the body's defense against free radicals. This protein, now known as superoxide dismutase (SOD), isolated from bovine erythrocyte, and later found to be in almost all living cells, was proved by McCard and Fridovich in 1969 to be an enzyme catalyzing the dismutation reaction of the superoxide anion radical (McCard and Fridovich, 1969).

O_2^-, H_2O_2, OH · , singlet oxygen, and lipid hydroperoxide are the five most important free radicals related to health and medicine. Normally the body possesses adequate defense systems against attack by oxygen free radicals. There are occasions when the defense systems become inadequate: 1. When there is excessive production (air, food, smoking, etc.). 2. When there is inadequate intake of antioxidant nutrients. 3. When receive medical treatment such as drugs, chemotherapy, and radiation therapy, etc. 4. The aging process causes reduced production of antioxidant enzymes (Su, 1993)

(1) The sequence is available from the Genome Sequence DataBase under the accession number L32834.

(2) Department of Biogenineering, Tatung Institute of Technology. 40 Chungshan North Road, 3rd sec. Taipei, Taiwan. R.O.China.

(3) Corrsepondende author.

The study of antioxidants and their applications in health and medicine has become an important aspect in medical science. Especially, superoxide dismutase (SOD) is one of the most important antioxidants in human body. SODs are metalloproteins and are classified into three types depending on the metal found in the active site. They are Mn-, Fe-, and Cu/Zn-SOD. Its application in medicine in an injective form has been explored with positive results. However, it is expensive and its half-life in the circulatory system is short. The other SOD-like small molecules from plants are being used as oral antioxidant supplement.

Many SOD DNAs from various sources other than *Aspergillus japonicus* have been sequenced and compared. We report here a 0.3 kb SOD DNA from *Aspergillus japonicus* cDNA was amplified by PCR. The SOD DNA was used as a probe to select a 0.4 kb SOD DNA from an *Aspergillus japonicus* genomic library. Here we report the 0.4 kb SOD DNA sequence and its deduced amino acid sequence compared with other plant SOD sequences.

MATERIALS AND METHODS

***Aspergillus japonicus* mycelium** *Aspergillus japonicus* was cultured at 30℃ in a 5 liter jar fermentor (3 liter of working volume containing 1% malt extract, 1% yeast extract, 25% sucrose) under aerated conditions (3 l/min) at 400 rpm for 24 hours. The mycelia were harvested for the following mRNA preparation.

mRNA preparation and cDNA synthesis Mycelia were frozen in liquid nitrogen and ground to powder in a ceramic mortar. Total RNA was prepared by the guanidium procedure, the poly(A)$^+$ RNA was isolated according to the oligo-(dT) affinity method (Lin *et al*, 1991), and double strand cDNA was synthesized using RiboClone cDNA synthesis System (C1660) kit from Promega.

Primer Two converging primers (5'-TTCATGGGTTCCATGTGC-3', 5'-GTTTCCGGT GCTCTTGCT-3') were synthesized according to the sequence of maize SOD-4 (Cannon and Scandalios, 1989).

Polymerase chain reaction Conditions for PCR were as follows. A 100-μl reaction mixture containing 100 ng *Aspergillus japonicus* cDNA as template, 45 pm of each 18-mer primer, 10 μl reaction buffer, 10 μl 1.25 mM dNTPs, and 2.5 unit Taq polymerase was reacted for 24 cycles of amplification (each cycle: 92℃ for 2 min, 46℃ for 2 min, 72 ℃ for 2 min).

Subcloning and DNA sequence analysis Twenty μl of PCR product was applied to 0.6% agarose, and NA-45 DEAE membrane (Schleicher & Schuell) was used to absorb the 0.3 kb DNA band. The 0.3 kb DNA was recovered, blunted the ends , phosphorylated at its 5'-ends, and ligated with pGEM-7Zf(+) vector at *Sma*I site (Promega). The

constructed plasmid was transformed to *E. coli* JM109 host cell. The positive clones were screened with γ -P^{32}-ATP labeled primers and were sequenced bidirectionally by the dideoxy chain termination method (Sanger *et al.*, 1977).

Construction of genomic libraries and screening Sixty gram mycelium was frozen in liquid nitrogen and ground to powder in a ceramic mortar. The chromosomal DNA was prepared by the ultracentrifugation procedure. The pure chromosomal DNA was partially digested by *Eco*RI and ligated with λ gt10 as vector. The recombinant DNAs were packaged with Stratagene's Gigapack and then a genomic library was constructed by using C600 as the host. A positive clone containing 0.4 kb SOD insert selected by plaque hybridization with P^{32}-labeled 0.3 kb SOD DNA fragment, and the 0.4 kb SOD DNA was then subcloned into pGEM-7Zf(+) using JM109 as host. Nucleotide sequence was determined in both directions by the dideoxy chain termination method.

Data Analysis The data analysis was done on the DNASIS software purchased from Hitachi.

RESULTS AND DISCUSSIONS

By using PCR technique, we have amplified and selected a positive clone containing 0.3 kb DNA fragment from *Aspergillus japonicus* cDNA template. After sequenced and compared it with maized SOD cDNA, the homology was as high as 73%. Then we used the 0.3 kb fragment as probe to screen genomic libraries. A positive clone containing 0.4 kb insert was screened and sequenced.

Fig. 1 shows its nucleotide and deduced amino acid sequences. There is no intron in the determined sequence, which is the same as yeast (Bermingham-McDonogh, *et al.*, 1988) but different from other eukaryotes: seven introns in rice (Sakamoto, *et al.*, 1992), four introns in human (Levanon, *et al.*, 1985), one intron in *Drosophila* (Seto, *et al.*, 1987), and three introns in *Neurospora* (Chary, 1990).

The deduced sequence of 120 amino acid showed high homology with the sequences of the cytosolic Cu/Zn-SOD from several other plant species(Fig. 2). In these comparision, we found that it lacks 34 amino acid residues at N-terminal and 2 extra amino acid residues at C-terminal. To investigate whether the lacking 34 amino acid residues are essential or not in Cu/Zn-SOD molecule, we will subclone the DNA sequence to an expression vector in the near future, and check the enzyme activity of the gene product.

The residues that are conseusus in Cu/Zn-SOD are those essential for maintaining the structure of the active site. These residues include the six histidines and the one aspartate that constitute the ligands of the Cu^{2+}(His 45, 47, 62 and 119) and Zn^{2+}(His 62, 70, 79, and Asp 82). The Arg 141 positioned closely to the Cu^{2+} is important for catalysis and highly conservative, too. The bridge (imidazole of histidine 62) is released from and ligated to the Cu^{2+} by O_2^- during its successive reduction and reoxidation. The Arg 141

```
  1 GAATTCGAGC TCGGTACCCC TCCATGGATT CCATGTGCAC GCGCTCGGTG ACACCACTAA
  1   N  S  S   S  V  P   L  H  G  F   H  V  H   A  L  G   D  T  T  N

 61 TGGCTGCATG TCAACTGGAC CACACTTCAA TCCTACTGGG AAGGAACATG GGGCACCACA
 21   G  C  M   S  T  G   P  H  F  N   P  T  G   K  E  H   G  A  P  Q

121 AGATGAGAAC CGCCATGCCG GTGATCTTGG AAATATAACA GCTGGAGCAG ATGGTGTTGC
 41   D  E  N   R  H  A   G  D  L  G   N  I  T   A  G  A   D  G  V  A

181 TAATGTCAAT GTCTCTGACA GCCAGATCCC CCTTACTGGA GCACACTCCA TCATTGGCCG
 61   N  V  N   V  S  D   S  Q  I  P   L  T  G   A  H  S   I  I  G  R

241 AGCTGTTGTT GTCCATGCTG ATCCTGATGA TCTTGGCAAG GGTGGACATG AGCTTAGCAA
 81   A  V  V   V  H  A   D  P  D  D   L  G  K   G  G  H   E  L  S  K

301 GACCACTGGA AATTCGAATT CCTCCATGGA TTCATGTGCA CATGGAATCC AGGGGATCCT
101   T  T  G   N  S  N   S  S  M  D   S  C  A   H  G  I   Q  G  I  L

361 CTAGAGTCGA CCTGCAGGAT GAAGCTTGAA TTC
      *
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Fig. 1. Nucleotide sequence of *Aspergillus japonicus* SOD(AS-SOD) genomic DNA and the deduced amino acid sequence. Numbers to the left refer to nuclotide or amino acid residuces. The asterisk denotes the stop signal.

ligated to the Cu^{2+} by O_2^- during its successive reduction and reoxidation. The Arg 141 is positioned in the solvent access channel close to the Cu^{2+} such that an O_2^-, while hydrogen bonded to this arginine could also ligate to the copper(Fridovich, 1986). In the present sequence, the seven residues coordinating copper and zinc are conserved, but Arg 141 is naturally replaced by Ser. We propose that the enzyme owns only 20% activity because we have succeeded to replace Arg 141 by Ser in sweet potato Cu/Zn-SOD, the mutant enzyme only maintain about 20% activity compared to normal ones (unpublished).

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                                                                  * *
AS-SOD                                                      NSSSVP LHGFHVHALG
SW-SOD     MVKAVAVLSS SEGVSGTIFF SQEGDGPTTV TGNVSGLKPG ..........
50
TSOD       ........N. .......YL. T.V.VA.... N..I...... ..........
MSOD-4     ........G. ....K..... T......... ..S...... ..........
MSOD-2     ........AG TD-.K..... .......... ..SI...... ..........
RSODA      .....V..G. ..I.K...H. V......... ..S....... .....I....

                      *        *          *    *
AS-SOD     DTTNGCMSTG PHFNPTGKEH GAPQDENRHA GDLGNITAGA DGVANVNVSD
SW-SOD     .......... .....A.... ...G.D.... .......V.E ..T.SFTIT.
100
TSOD       .......... ..Y....... ...E..V... .......V.E ..T.SFTIT.
MSOD-4     .......... ..Y...S... ...E...... .....V.... .....I..T.
MSOD-2     .......... .....V.L.. ...EF.D... .....V...E ...V....T.
RSODA      .......... ..Y....... ...E..T... .....V...E .....IH.V.

                             *
AS-SOD     SQIPLTGAHS IIGRAVVVHA DPDDLGKGGH ELSKTTGNSN SSMDSCAHGI
SW-SOD     K......AN. V........G .......... ....S...AG GRVACGII.L
150
TSOD       K......PQ. .......... .......... ....S...AG GRIACGII.L
MSOD-4     .......PN. .......... .......... ....S...AG GRVACGII.L
MSOD-2     .....A.P.. .......... .......... ....S...AG GRAVCGII.L
RSODA      .......PN. .......... .......... ........AG GRAVCGII.L


AS-SOD     QGIL  154
SW-SOD     ..
TSOD       ..
MSOD-4     ..
MSOD-2     ..
RSODA      ..
```

Fig. 2. Comparison of amino acid sequences of SODs: AS-SOD, *Aspergillus japonicus* (this study) ; SW-SOD, sweet potato SOD; TSOD, tomato SOD; MSOD-4, maize SOD-4; MSOD-2, maize SOD-2; RSODA, rice SODA. Numbers refer to SW-SOD. Six sequences are shown only where they differ from AS-SOD. '.' refers to identities with AS-SOD. '-'denotes deletion. Residues coordinating copper and zinc are indicated with asterisks. The two cysteines that form disulfide bridge are boxed (Lin *et al*, 1993).

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麴菌銅鋅型超氧歧化酶基因選殖

林棋財、林銘澤、許坯棻、段國仁

摘　　要

依據玉米Cu/Zn-SOD之核酸序列，合成兩股引子，並以麴菌cDNA當模板，以PCR方法增生出一段0.3 kb DNA，經次選殖及定序，其序列與玉米Cu/Zn-SOD之序列同殖性高達73%，進一步以此0.3 kb DNA當探針，進行麴菌基因庫之篩選，結果篩得一株含有0.4 kb之正反應株，經定序分析，發現可推導出含120個胺基酸序列，其序列和其它生物Cu/Zn-SOD之序列同質性很高，並含有與銅和鋅進行相對應(coordinating)之胺基酸序列。