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A Cu,Zn superoxide dismutase (SOD1) from *Cordyceps militaris*: cDNA cloning, expression and characterization

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Objectives

The first line of antioxidant defense against reactive oxygen species includes the enzymatic activity of the superoxide dismutase (SOD) that catalyzes the disproportionation of superoxide to hydrogen peroxide and water. The SOD mainly removes highly toxic O_2^- and also prevents O_2^- mediated reduction of iron and subsequent OH^- generation.

Along with an interest in SOD as a first line of defense against damage mediated by the superoxide anion, the SOD1 enzyme has been subjected to investigation in the molecular and cellular level. Until now fungal Cu,Zn SOD (SOD1) genes had been studied extensively from various species. Previously, in *C. sinensis*, the antioxidant activities of the SOD1 in fruiting bodies have been reported without isolating the corresponding gene. However, SOD gene in entomopathogenic fungi as well as in *Cordyceps* species is not available currently. Thus, our objective in initiating this study was to illustrate the structure of the SOD1 gene from the *C. militaris*, which is one of the most valued *Cordyceps* species.

This paper describes the molecular cloning of SOD1 cDNA from *C. militaris*. The *C. militaris* SOD1 cDNA is expressed functionally in baculovirus-infected insect cells and the purified recombinant *C. militaris* SOD1 is assayed. Furthermore, SOD1 expression in *C. militaris* is analyzed.

Materials and Methods

Materials - The entomopathogenic fungus *Cordyceps militaris*

Methods - cDNA library screening, nucleotide sequencing and data analysis, Genomic DNA isolation and Southern blot analysis, RNA isolation and Northern blot analysis, Construction of baculovirus transfer vector, Cell culture and construction of recombinant virus, SDS-polyacrylamide gel electrophoresis, Purification of recombinant *C. militaris* SOD1, Preparation of polyclonal antiserum and Western blot analysis, Determination of enzyme activity

Results and Discussion

A cDNA encoding the Cu,Zn superoxide dismutase (SOD1) of *Cordyceps militaris*, which is one of the entomopathogenic fungi called a vegetable wasp and plant worm, was cloned and characterized. The SOD1 cDNA contains an open reading frame of 462 bp encoding 154 amino acid residues (Fig. 1 and 2). The deduced amino acid sequence of the *C. militaris* SOD1 cDNA showed 88% identity to *Claviceps*

purpurea SOD1, 82% to *Neurospora crassa* SOD1, and 75% - 64% to other fungi SOD1 (Fig. 3). The *C. militaris* SOD1 possesses the typical metal binding ligands of six histidines and one aspartic acid common to fungi SOD1 (Fig. 2). Phylogenetic analysis confirmed a closer relationship of the deduced amino acid sequences of the *C. militaris* SOD1 gene to the *C. purpurea* and *N. crassa* within the ascomycetes group (Fig. 3). The cDNA encoding *C. militaris* SOD1 was expressed as a 17-kDa polypeptide in the baculovirus-infected insect Sf9 cells (Fig. 4) and the enzyme activity of the purified recombinant *C. militaris* SOD1 was approximately 568 U per mg of recombinant SOD1 (Fig. 5). Southern blot analysis of the genomic DNA suggested the presence of the *C. militaris* SOD1 gene as a single copy (Fig. 6) and Northern blot analysis showed increment of SOD1 transcript signal with growth stage (Fig. 7). Similarly, the *C. militaris* SOD1 enzyme assay also exhibited increment of activity with growth stage (Fig. 8). This result of *C. militaris* SOD1 is the first molecular characterization of SOD1 gene from any entomopathogenic fungus.

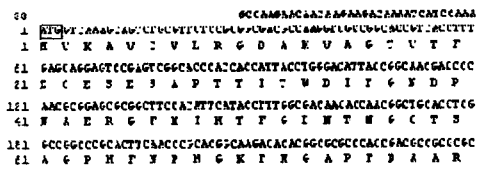

Fig. 1. The nucleotide and deduced protein sequence of the *C. militaris* SOD1 cDNA. The start codon of ATG is boxed and the termination codon is shown by asterisk. In the cDNA sequence, the polyadenylation sequence is underlined. This cDNA sequence has been deposited in GenBank under accession number AY176061.

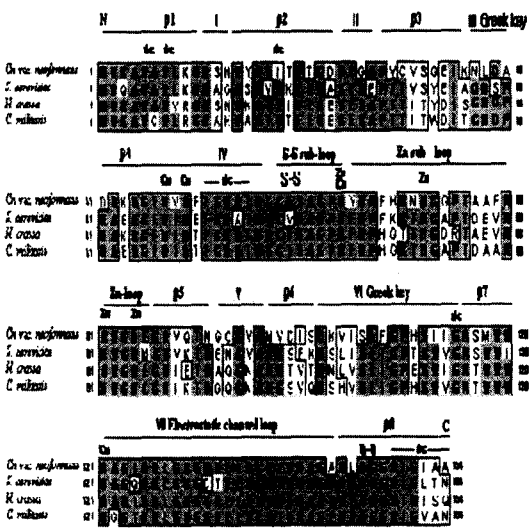

Fig. 2. Comparison of the deduced amino acid sequences of *C. militaris* SOD1 with the *Cn* var. *neoformans*, *S. cerevisiae*, and *N. crassa* SOD1. Residues are numbered according to the aligned three fungal SOD1 sequences, and invariant residues are shaded black. The eight β-strands of the β-barrel, the seven connecting loops or turns (Roman numerals), and the N-terminal (N) and C-terminal (C) sequences not involved in β–strands, are shown above the alignment. Structural alignments are taken from the bovine SOD1 crystal structure (Tainer et al., 1982). Residues that form disulfide bridge (S-S), ligate the metals (Cu or Zn), or are involved in dimmer contact (dc) are also shown above the alignment. The SOD1 sequences were taken from the following sources: *Cn* var. *neoformans*

(Chaturvedi et al., 2001), *S. cerevisiae* (Steinman, 1980), and *N. crassa* (Chary et al., 1990).

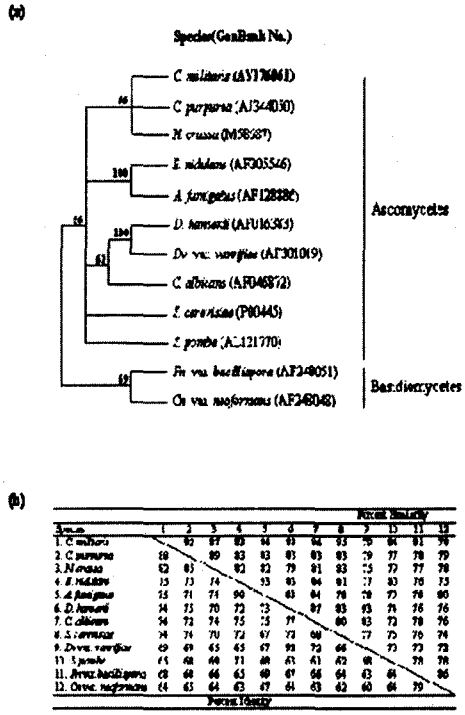

Fig. 3. Phylogenetic relationship among fungal SOD1 sequences. (a) A maximum parsimony analysis for the *C. militaris* SOD1 and the other known fungal SOD1 sequences. The accession numbers of the sequences in the GenBank are as follows: *C. militaris* (AY176061; this study), *C. purpurea* (AJ344050), *N. crassa* (M58687), *E. nidulans* (AF305546), *A. fumigatus* (AF128886), *D. hansenii* (AF016383), *C. albicans* (AF046872), *S. cerevisiae* (P00445), *D. vanrijiae* var. *vanrijiae* (AF301019), *F. neoformans* var. *bacillispora* (AF248051), *S. pombe* (AL121770), and *C. neoformans* var. *neoformans* (AF248048). The tree was obtained by bootstrap analysis with the option of heuristic search and the numbers on the branches represent bootstrap values for 1,000 replicates. (b) Pairwise identities and similarities of the deduced amino acid sequence of *C. militaris* SOD1 among fungal SOD1 sequences.

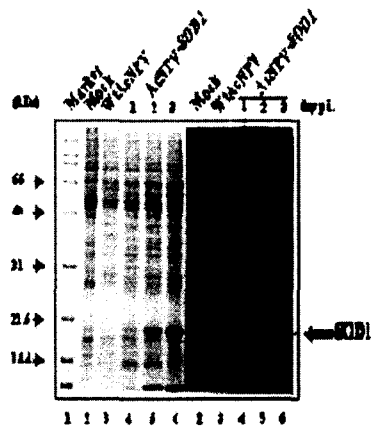

Fig. 4. Expression of the *C. militaris* SOD1 in recombinant baculovirus-infected insect Sf9 cells. Sf9 cells were mock-infected (lane 2) or infected with the wild-type AcNPV (lane 3) and the recombinant AcNPV (lanes 4, 5 and 6) at an MOI of 5 PFU per cell. Cells were collected at 1 (lane 4), 2 (lanes 3 and 5) and 3 (lane 6) days p.i. Total cellular lysates were subjected to 12% SDS-PAGE (left panel), electroblotted and incubated with antiserum to recombinant *C. militaris* SOD1 (right panel). The arrow on the right of the panel indicates the 17 kDa recombinant *C. militaris* SOD1 polypeptide. Molecular weight standards were used as size marker (lane 1).

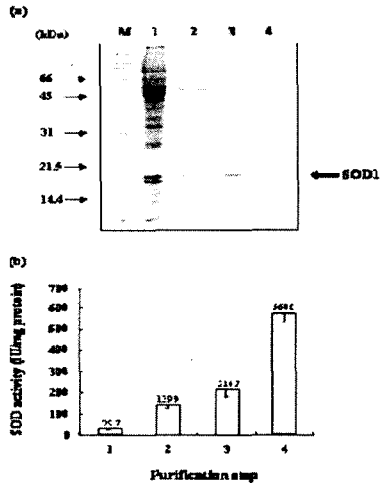

Fig. 5. Purification of the recombinant *C. militaris* SOD1 expressed in baculovirus-infected insect cells. (a) SDS-PAGE of *C. militaris* SOD1 purification step using FPLC techniques. The sample of each purification step using FPLC techniques was analyzed by 12% SDS-PAGE. Lanes: M, molecular weight standards; 1, cellular lysates of recombinant baculovirus-infected cells harvested at 3 days p.i.; 2, ammonium sulfate fraction; 3, desalting eluent; 4, gel-filteration eluent. The arrow on the right of the panel indicates the purified recombinant *C. militaris* SOD1 polypeptide of 17 kDa. (b) Specific activity of *C. militaris* SOD1 purification step using FPLC techniques. Steps: 1, cellular lysates of recombinant baculovirus-infected cells harvested at 3 days p.i.; 2, ammonium sulfate fraction; 3, desalting eluent; 4, gel-filteration eluent.

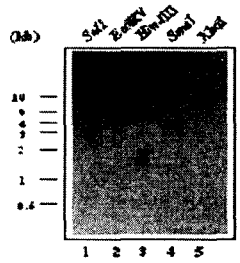

Fig. 6. Southern blot analysis of *C. militaris* genomic DNA for SOD1. Genomic DNAs were digested with five restriction enzymes, *Sal*I (lane 1), *Eco*RV (lane 2), *Hind*III (lane 3), *Sma*I (lane 4) or *Xba*I (lane 5), and hybridized with radiolabelled 652 bp *C. militaris* SOD1 cDNA. Size markers are shown on the left.

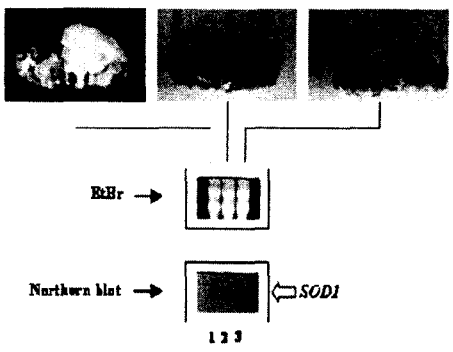

Fig. 7. Northern blot analysis of *C. militaris* SOD1. Total RNA was isolated from mycelium of 1 week p.i. (lane 1) and fruiting body of 2 (lane 2) or 3 (lane 3) weeks p.i., respectively (upper panel). The RNA was separated by 1.0% formaldehyde agarose gel electrophoresis (middle panel), transferred on to a nylon membrane, and hybridized with radiolabelled 652 bp *C. militaris* cDNA (lower panel). Transcripts are indicated on the right side of the panel by arrow.

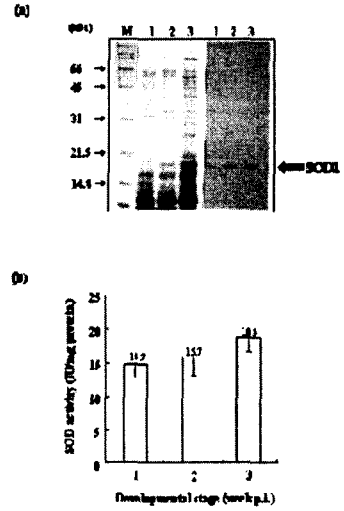

Fig. 8. Expression of SOD1 in *C. militaris*. (a) SDS-PAGE and Western blot analysis of total cellular lysates according to *C. militaris* growth stage. Total cellular lysates were prepared from mycelium of 1 week p.i. (lane 1) and fruiting body of 2 (lane 2) or 3 (lane 3) weeks p.i., respectively. The cellular lysates were subjected to 12% SDS-PAGE (left panel), electroblotted and incubated with antiserum to recombinant C. militaris SOD1 (right panel). The arrow on the right of the panel indicates the 17 kDa C. militaris SOD1 polypeptide. Molecular weight standards were used as size marker (M). (b) Enzyme activity assay of C. militaris SOD1 with growth stage. The SOD1 activity was assayed from mycelium (1 week p.i.) and fruiting body (2 or 3 weeks p.i.) of C. militaris.

References

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