Isolation of a Germin-like Protein with Manganese Superoxide Dismutase Activity from Cells of a Moss, Barbula unguiculata*

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A novel extracellular Mn-superoxide dismutase (SOD) was isolated from a moss, Barbula unguiculata. The SOD was a glycoprotein; the apparent molecular mass of its native form was 120 kDa, as estimated by gel filtration chromatography, and that of its monomer was 22,072 Da, as estimated by time of flight mass spectrometry. The protein had manganese with a stoichiometry of 0.80 Mn/monomer. The cDNA clone for a gene encoding the extracellular Mn-SOD was isolated. Sequence analysis showed that it has a strong similarity to germin (oxalate oxidase) and germin-like proteins (GLPs) of several plant species and possesses all the characteristic features of members of the germin family. The clone encoding this extracellular Mn-SOD was therefore designated B. unguiculata GLP (BuGLP). BuGLP had no oxalate oxidase activity. In addition, the cDNA for a gene encoding the moss mitochondrial Mn-SOD was isolated. Its amino acid sequence had little similarity to that of BuGLP, even though a close similarity was observed among the mitochondrial Mn-SODs of various organisms. BuGLP was the first germin-like protein that was really demonstrated to be a metalloprotein with Mn-SOD activity but no oxalate oxidase activity.

Superoxide dismutase (SOD)† (EC 1.15.1.1) is of major importance in protecting living cells against superoxide anion toxicity produced under oxidatively stressed circumstances. Phylogenetic distribution of the three types of SOD, CuZn-SOD, Mn-SOD, and Fe-SOD, in plants and microorganisms has been of general interest (1). Fe-SOD is found in prokaryotes, eukaryotic algae (2, 3), and bryophytes (4), but in higher plants it is detected only in a number of unrelated plant families (1, 5). CuZn-SOD is not generally found in prokaryotes (6) except for the periplasm of a few Gram-negative bacteria (7). Most eukaryotic algae lack CuZn-SOD (8), except for some green algae (9). All of the land plants examined have both cytosolic and chloroplast CuZn-SODs. As for Mn-SOD, it is found in the cytosol of all prokaryotes and in the mitochondria of all eu-karyotes examined.

Extracellular SODs (EC-SODs) are found in animals (10, 11), prokaryotes (12, 13), and plants (14, 15). They are all CuZn-SODs. Some Gram-positive bacteria produce extracellular SODs: Nocardia asteroides secretes into the medium a unique form of SOD containing Fe, Mn, and Zn (16), and Streptococcus pyogenes secretes a SOD that contains the typical amino acid sequence for Mn-SODs (17).

Germin is a water-soluble glycoprotein with a monomer molecular mass of about 25 kDa and forms oligomers that are highly resistant to proteolysis (18, 19). Germins are expressed during seed germination of wheat and barley (18, 19) and in mature leaves in response to pathogen attack (20–22), and they are identified as oxalate oxidase from the amino acid sequence homology (18). Thus, germins are suggested to catalyze cell-wall reinforcement by oxidative cross-linking (23) or to protect leaves against infectious microorganisms by generating H$_2$O$_2$ (22, 24). In plants other than wheat and barley, proteins closely related to germins are identified and termed germin-like proteins (GLPs) (25). Some of these GLPs have no oxalate oxidase activity, and the biological function of some GLPs is not comparable to germins.

Our interest in this study has been focused on determining how land plants evolved SOD molecules to adapt cells to the oxygen-accumulated atmosphere. We have taken notice of SODs of the bryophytes, which are considered to be the first land plants and to occupy a critical position in the evolution of land plants. We first studied SODs of liverwort, Marchantia paleacea var. diptera, which is evolutionarily the most basal lineage among bryophytes, and found that it has a cytosolic CuZn-SOD with properties similar to CuZn-SODs found in chloroplasts in higher plants (26, 27). Then, we expanded the study to the SODs of a moss, Barbula unguiculata, that is considered morphologically to be more closely related to vascular plants. During the study, we found that cells of the moss excreted Mn-SOD in the medium. The extracellular Mn-SOD was found, quite interestingly, a germin-like protein with Mn-SOD activity but no oxalate oxidase activity. The protein is the first germin-like protein with SOD activity carrying manganese as a prosthetic group.

EXPERIMENTAL PROCEDURES

**Plant Material and Culture Conditions**—Cells of B. unguiculata (AS cell line) were propagated by shaking on a gyratory shaker at 110 rpm at 25 ± 1 °C in the light as described previously (28).

**Preparation of Intracellular Crude Extracts and Extracellular Supernatant**—Intracellular crude extracts were prepared as described previously (29) except that the concentration of EDTA was 0.1 mM instead of 1 mM in the grinding medium and that 15 cycles of sonication were carried out for disruption of cells. Extracellular supernatant was ob-
tained from the cells (150 g of fresh cells) suspended in 1 M NaCl (500 ml), followed by gentle stirring for 30 min at 4 °C, and the cells were glass-filtered with suction.

**Identification of Three SOD Isozymes**—Identification of SOD isozymes was carried out as described previously (4) by nondenaturing polyacrylamide gel electrophoresis with SOD activity staining.

**Purification of Extracellular Mn-SOD—Ammonium sulfate was added to the extracellular supernatant up to 90% saturation. The precipitate was dissolved in a minimum amount of 20 mM Tris-HCl (pH 8.0), dialyzed against the same buffer, concentrated by ultrafiltration (Centriflo CF25, Amicon), and applied to a column (2.5 × 100 cm) of Sephadex G-100 (Amersham Pharmacia Biotech). The fractions containing SOD activity were pooled and applied to a column (1.6 × 5 cm) of concanavalin A-agarose (Honen Co.) equilibrated with the same buffer. The column was washed with 500 ml of the buffer, and then the adsorbed proteins were eluted with 100 ml of the buffer containing 0.5 M α-methyl-D-glucoside.

**Characterization of Extracellular Mn-SOD—Superoxide dismutase assay was carried out essentially as described by McVord and Friedrich (31) using cytochrome c as the detector and xanthine-xanthine oxidase as the superoxide generator.

Oxalate oxidase activity was assayed by two methods. One was essentially as described by Dumas et al. (32) in which the activity was detected with gel electrophoresis followed by transfer onto a nitrocellulose sheet and activity staining. The other was the spectrophotometric assay, as described by Zhang et al. (33).

Molecular mass of the native form of the Mn-SOD was estimated by gel filtration with Sephadex G-100. The molecular mass of the monomer form was estimated by a REFLEX™ matrix-assisted laser desorption ionization time of flight mass spectrometer (Bruker). Data were acquired in the positive linear mode at 30 kV. A saturated solution of sinapinic acid in a mixture of 0.1% trifluoroacetic acid and 90% acetonitrile was used as a matrix. Singly charged (M+H)+ and doubly charged (M+2H)2+ ions of bovine serum albumin were used as standards to calibrate the mass spectrum (m/z 66,431 and 33,216, respectively).

Manganese in the purified enzyme was determined by a Z-9000 Zeeman atomic absorption spectrometer (Hitachi). The amino-terminal sequence and an inner amino acid sequence were analyzed using a gas-phase protein-peptide sequencer (model 473A, Applied Biosystems).

Detection of glycoprotein was carried out as described by Zacharius et al. (34) using the periodic acid-Shiff technique on the electrophoretic gel.

**Isolation of cDNA for the Moss Extracellular Mn-SOD—Total RNA was isolated from 3-day-cultured cells (2 g) by the standard guanidine isothiocyanate extraction and cesium chloride ultracentrifugation method (35). First strand cDNA was synthesized from total RNA with a system for rapid amplification of cDNA 3'-ends (Life Technologies, Inc.). The sense primer used was 5'-GA/CT/GA/AG/GA/TG/GG/TT/ CT/GG/CA/AG/GA/TG/TT/GT/TA/GG/GA/GG/GA/AG/GC/ C/G/AG/GA/TG/GG/CT/CC/GA/AG/GA/TG/GG/CT/CC/CA/AG/GA/TG/GG/CT-3', which was synthesized to the amino-terminal amino acid sequence of the moss extracellular Mn-SOD and in reference to the conserved amino acid sequences of plant germin. Reaction conditions of PCR amplification were as follows: 30 cycles of 30 s of denaturing at 94 °C, 30 s of annealing at 50 °C, and 1 min of elongation at 72 °C. The PCR products (about 800 base pairs) were amplified, cloned into pCRII vector with the TA-cloning kit (Invitrogen, San Diego, CA), and sequenced on both strands.

**Isolation of cDNA for the Moss Mitochondrial Mn-SOD—The rice mRNA was purified with Oligoex™-dT30 Super (Takara Shuzo, Kyoto, Japan), and a cDNA library was constructed as described previously (27). To prepare the probes for isolation of cDNAs, reverse transcription-PCR was first performed with Superscript™ II RNAse H reverse transcriptase (Life Technologies, Inc.). The pair of primers used were as the sense primer, 5'-AA/GC/TA/GC/CT/GG/GT/A/G/C/G/ C/G/AG/GA/TG/GG/CT/CC/GA/AG/GA/TG/GG/CT/CC/CA/AG/GA/TG/GG/CT-3', and the antisense primer, 5'-TA/GC/AG/TG/ C/TG/CC/AG/AG/GA/GG/CT-3', which were synthesized in reference to the most conserved region of the known sequences of plant Mn-SOD. Reaction conditions were as follows: 40 cycles of 30 s of denaturing at 94 °C, 30 s of annealing at 40 °C, and 1 min of elongation at 70 °C. A PCR product (about 400 base pairs) was amplified, subcloned into pCRII vector with the TA-cloning kit (Invitrogen, San Diego, CA), and sequenced to confirm that it was part of the cDNA for the moss mitochondrial Mn-SOD. The cDNA library was screened with the PCR product, which was labeled with digoxigenin using a digoxigenin DNA labeling and detection kit (Roche Molecular Biochemicals). Inserts from the moss cDNA clones that hybridized to digoxigenin-labeled DNA probes were extracted from recombinant plasmid, subcloned into the pUC

**RESULTS**

**Isolation of an Extracellular Mn-SOD—Intracellular crude extracts and extracellular supernatant were subjected to non-denaturing gel electrophoresis, and SOD activity was stained (Fig. 1). There existed three kinds of SOD isozyme in the intracellular extracts (Fig. 1A): the upper band, which was insensitive to either cyanide or H2O2, indicates Mn-SOD; the two bands sensitive to either cyanide or H2O2 indicates CuZn-SOD; and the band insensitive to cyanide but sensitive to H2O2 indicates Fe-SOD. In the extracellular supernatant (Fig. 1B), there was a broad SOD activity band with very slow migration on native PAGE that is insensitive to either cyanide nor H2O2, suggesting the presence of an EC Mn-SOD.

**EC Mn-SOD was purified by the procedure summarized in Table I. After ammonium sulfate precipitation, Sephadex G-100 chromatography was adopted. The apparent molecular mass of EC Mn-SOD estimated from the chromatography was about 120 kDa. The fractions containing SOD activity were pooled and applied to the concanavalin A-agarose affinity column. EC Mn-SOD was eluted in the buffer containing 0.5 M α-methyl-D-glucoside with a 12-fold purification and a yield of 17%.

**Characterization of EC Mn-SOD—Although the SOD activity staining of purified EC Mn-SOD showed a broad band with a slow migration on native PAGE, it migrated on SDS-PAGE as a single sharp band with an apparent molecular mass of 28 kDa (Fig. 2A). When the SDS gel was stained with periodic acid-Schiff reagent, the protein band turned visible (Fig. 2B), indicating clearly that EC Mn-SOD is a glycoprotein, as shown in all examples of germin and GLPs (25). Presence of the carbohydrate chain in the protein was also confirmed by effective purification of EC Mn-SOD by binding it to concanavalin A-agarose followed by displacing it with α-methyl-D-glucoside. The affinity binding to concanavalin A shows that the carbohydrate chain is a 1,1 linked mannose core structure.

The molecular mass of monomer of EC Mn-SOD, estimated by time of flight mass spectroscopy, was 22,072 Da, and that of

![Fig. 1. SOD isozymes in intracellular extracts and extracellular supernatant from suspension-cultured cells of B. unguiculata. Intracellular crude extracts (50 µg of protein) (A) and extracellular supernatant (10 µg of protein) (B) were subjected to non-denaturing gel electrophoresis. Three SOD isozymes were identified by active staining of gels that were left untreated (lane 1) or preincubated with 1 mM KCN (lane 2) or 5 mM H2O2 (lane 3) for 1 h. MT, mitochondrial.](image-url)
Germin-like Protein with Mn-SOD Activity

Summary of extracellular Mn-SOD purification

| Treatment                                    | SOD activity (units) | Protein (mg) | Specific activity (units/mg) | Recovery (%) |
|----------------------------------------------|----------------------|--------------|-------------------------------|-------------|
| Extracellular supernatant                    | 37,700               | 61.4         | 613                           |             |
| Ammonium sulfate precipitation               | 26,100               | 32.5         | 803                           | 69.2        |
| Sephadex G-100 chromatography                | 11,700               | 2.03         | 5750                          | 31.0        |
| Con A-agarose affinity chromatography        | 5500                 | 0.77         | 7090                          | 17.2        |

FIG. 2. SDS-PAGE of the purified extracellular Mn-SOD. The purified enzyme (4 μg of protein) was subjected to SDS-PAGE with Coomassie Brilliant Blue R-250 staining for protein (A) and periodic acid-Schiff staining for carbohydrate (B). The mobilities and molecular masses (in kDa) of marker proteins are indicated at the left.

The doubly charged ion (M+2H)2+ was 10,980 Da. Involvement of manganese ion in the mass number was not clear because it could be removed during measurement. There exists the discrepancy of the molecular mass of monomer obtained from SDS-PAGE, mass spectrometry and amino acid sequence. SDS-PAGE is known not always to show the exact molecular masses, and the most reliable value are from mass spectrometry. However, it is difficult in this situation to determine the extent of oligomerization of the molecular mass of 120 kDa of the active form of EC Mn-SOD, estimated from gel filtration. Although native forms of germins of GLPs were reported to be dimeric to function. Each subunit contains around 250 amino acid residues and has a molecular mass of about 23 kDa. The residues that ligate the metal ion are the same in all

FIG. 3. Amino acid sequence of mature BuGLP and alignment with those of germins/oxalate oxidases and a GLP from Arabidopsis thaliana. The sequences are aligned visually for maximum similarity. The numbering of residues is based on the sequence of BuGLP. The residues that are identical to those of BuGLP are shown as white letters on black. Underlining indicates consensus sequences corresponding to germin box 1 and box 2, in which asterisks and circles, respectively, indicate the amino acid residues critical for the function of germins/oxalate oxidases (see text). Sources of sequences deduced from the cDNAs and their GenBank™ accession numbers are as follows: BuGLP, this work; oxox, oxalate oxidase from barley Hordeum vulgare, GenBank™ accession number Y14203; ger2.8, germin gf-2.8 from wheat, GenBank™ accession number M63223; ger3.8, germin gf-3.8 from wheat, GenBank™ accession number M63224; glp6, germin-like protein from A. thaliana, GenBank™ accession number U75194.

The deduced BuGLP consists of 194 amino acids and has a molecular mass of 20,766 Da. Time of flight mass spectrometry gave a larger value, 22,072 Da. This difference, 1306 Da, could be mainly ascribed to attachment of a saccharide chain composed of around seven monosaccharides. However, the exact content of saccharide chain could not be deduced because the composition of the saccharide was not identified yet, and the contribution of manganese ion to the monomer mass number in mass spectrometry was ambiguous. The fact that the array of 54 amino acids from the amino-terminal was sequenced, but Asn-55 was not, and the fact that the consensus sequence for the asparagine-type sugar chain, NX(T/S), appears once in residues 55–57 in the BuGLP sequence clearly indicated that Asn-55 could be only the site for the saccharide chain.

BuGLP Has No Oxalate Oxidase Activity—Wheat and barley germins are identified as oxalate oxidase (18). As for GLPs in plants, some have no oxalate oxidase activity, and their biological functions are not comparable to those of germins. Whether BuGLP has oxalate oxidase activity or not was examined by two methods, the direct staining of the nitrocellulose sheet blotted from the electrophoretic gel and the spectrophotometric method. No activity was detected by these methods.

Primary Structure of the Moss Mitochondrial Mn-SOD—Most of the mitochondrial Mn-SODs from various organisms have to be dimeric to function. Each subunit contains around 200 amino acid residues and has a molecular mass of about 23 kDa. The residues that ligate the metal ion are the same in all

Primary Structure of EC Mn-SOD—The amino-terminal amino acid sequence of EC Mn-SOD indicated that it was a germin-like protein. To isolate cDNA for the SOD, degenerate oligonucleotide primers were designed and used for reverse transcription PCR. A PCR-amplified DNA fragment of approximately 800 base pairs was found to encode a peptide very similar to the original peptide sequence. The PCR fragment could be removed during measurement. There exists the discrepancy of the molecular mass of monomer obtained from SDS-PAGE, mass spectrometry and amino acid sequence. SDS-PAGE is known not always to show the exact molecular masses, and the most reliable value are from mass spectrometry. However, it is difficult in this situation to determine the extent of oligomerization of the molecular mass of 120 kDa of the active form of EC Mn-SOD, estimated from gel filtration. Although native forms of germins of GLPs were reported to be dimeric to function. Each subunit contains around 250 amino acid residues and has a molecular mass of about 23 kDa. The residues that ligate the metal ion are the same in all
forms of the enzyme, namely three histidines and an aspartate. (36). The moss *B. unguiculata* also has a Mn-SOD in mitochondria (Fig. 1). The isolation of BuGLP that has Mn-SOD activity has raised a further interest in the sequence similarity between BuGLP and the mitochondrial Mn-SOD of the moss, especially the similarity in the manganese ligating residues. Therefore, we isolated the single, full-length cDNA encoding the mitochondrial Mn-SOD. Fig. 4 shows the sequence alignment of the mitochondrial Mn-SODs from the moss and other organisms. A close sequence identity (42–64%) was observed between the moss Mn-SOD and others. The amino acid residues conserved in all forms of mitochondrial Mn-SOD also exist in the moss mitochondrial Mn-SOD, especially all of the metal binding ligands; the first, His-28, in the motif27LHHXKHLHTYV; the second, His-74, in the motif running from residue 66 to the end of the helix base, FNGGHHXNHSIFWK; and the third and fourth, Asp-161 and His-165, in the long motif running from residue 156 in the last β-strands through to the fifth main helix, 156PLLIGDVWEHAYLYQKNVRDPYKNIW. Thus, the moss mitochondrial Mn-SOD is a typical mitochondrial Mn-SOD.

There is little sequence similarity between the moss mitochondrial Mn-SOD and BuGLP. The percentage of residue identity for matched residues, if any, was calculated to be only 15%. From a standpoint of the sequence similarity, it is therefore clear that the present EC Mn-SOD belongs to a family of germin-like protein rather than the Mn-SOD commonly found in mitochondria.

**DISCUSSION**

The present report deals with distinctive nature of an extracellular protein newly isolated from a moss, *B. unguiculata*. The sequence of the protein showed clearly the similarity to germin or GLPs of plant origins but not to the mitochondrial Mn-SOD of the same moss. As for activity, on the contrary, it had SOD activity with manganese as a prosthetic group but none of the oxalate oxidase activity commonly shown in germins. Although there are no reports that germins or GLPs possess SOD activity, the present results on the sequence similarity, together with the in vivo oligomeric form (120 kDa) with glycosylated monomers (22,072 Da), indicates that the EC Mn-SOD is a member of GLPs. Although there is little sequence similarity between BuGLP with Mn-SOD activity and the moss mitochondrial Mn-SOD, one interesting question is whether the amino acid residues, which are highly indicative of a role in manganese binding and enzyme activity, are similar between them. Gane et al. (37) showed the germin/oxalate oxidase three-dimensional model and described that the three histidine residues (His-88, His-90, and His-134, numbered as for BuGLP in Fig. 3) lie on neighboring antiparallel β-strands and form a cluster of adjacent side-chain imidazole groups. They predicted that the histidine cluster is the metal-binding site, the oxalate oxidase active site, although the metal cofactor of germin/oxalate oxidase has never been demonstrated. Kotsira and Clonis (30) employed chemical techniques to modify barley root germin to identify amino acid residues essential for enzyme activity and suggested that germin is a metalloenzyme, the metal(s) of which is involved in the oxidative mechanism, although the metal has not yet been identified. BuGLP has Mn-SOD activity, and manganese was actually identified with a stoichiometry of 0.80 atom/monomer. This is the first identification of metal in germins and GLPs. The primary sequence of BuGLP indicates that the protein has the germin box, including three histidine residues (His-88, His-90, and His-134). This, together with the fact that three histidines and an aspartate in mitochondrial Mn-SOD have been shown to ligate manganese (36), indicates that the histidine cluster in BuGLP could be assigned as the manganese-binding site. More detailed experiments are being conducted to prove the hypothesis.

The specific role of germins is considered to be generation of Ca**²⁺** and H₂O₂ by degradation of oxalate, both of which are required for peroxidase-mediated reactions, such as cross-linking reaction of cell wall polysaccharides and lignification during germination (23). GLPs are isolated in gymnosperms, such as pines, and in dicot species (25), but some of GLPs have no oxalate oxidase activity. Thus, it is still unclear whether their biological function is comparable to germins despite numerous studies on GLPs and their expression. In plants, extracellular CuZn-SODs are identified (14, 15), the physiological function of which is considered to be the production of H₂O₂ from superoxide, which also facilitates the biosynthesis of lignin. The fact that BuGLP is extracted readily by 1 M NaCl from the moss cells suggests that BuGLP is localized outside of the cell and probably associated with the cell wall with no covalent cross-link. The evidence, together with the lack of oxalate oxidase activity in BuGLP, makes it possible to speculate that BuGLP is also involved in supplying H₂O₂ by disproportionation of superoxide for the cross-linking of cell wall.

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