Identification of Manganese Superoxide Dismutase from Sphingobacterium sp. T2 as a Novel Bacterial Enzyme for Lignin Oxidation

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Supporting Information

ABSTRACT: The valorization of aromatic heteropolymer lignin is an important unsolved problem in the development of a biomass-based biorefinery, for which novel high-activity biocatalysts are needed. Sequencing of the genomic DNA of lignin-degrading bacterial strain Sphingobacterium sp. T2 revealed no matches to known lignin-degrading genes. Proteomic matches for two manganese superoxide dismutase proteins were found in partially purified extracellular fractions. Recombinant MnSOD1 and MnSOD2 were both found to show high activity for oxidation of Organosolv and Kraft lignin, and lignin model compounds, generating multiple oxidation products. Structure determination revealed that the products result from aryl-Cα and Cα-Cβ bond oxidative cleavage and O-demethylation. The crystal structure of MnSOD1 was determined to 1.35 Å resolution, revealing a typical MnSOD homodimer harboring a five-coordinate trigonal bipyramidal Mn(II) center ligated by three His, one Asp, and a water/hydroxide in each active site. We propose that the lignin oxidation reactivity of these enzymes is due to the production of a hydroxyl radical, a highly reactive oxidant. This is the first demonstration that MnSOD is a microbial lignin-oxidizing enzyme.

The aromatic heteropolymer lignin is an important component of the lignocellulose matrix of plant cell walls, comprising 15−30% dry weight of lignocellulose.1 Lignin is liberated during physicochemical pretreatment of biomass for cellulosic bioethanol production and is also produced industrially from pulp/paper manufacture via the Kraft process but is currently a low value byproduct that is burned for energy or used in the production of concrete, asphalt, and polymeric materials.1,2 The aromatic content of lignin is a potentially valuable source of renewable aromatic chemicals, and the valorization of lignin via either chemical or biocatalytic routes is of considerable current interest but has proved very challenging.2

Microbial degradation of lignin has been mainly studied in basidiomycete fungi: white-rot fungi such as Phanerochaete chrysosporium produce extracellular lignin peroxidase and manganese peroxidase enzymes that can oxidize lignin, and some fungi produce extracellular laccases that can also attack lignin.1 Despite reports of bacterial oxidation of lignin,3,4 until recently the enzymology of bacterial lignin breakdown (summarized in Figure 1) was poorly understood. In 2011, we reported a dye-decolorizing peroxidase DypB from Rhodococcus jostii RHA1 that played a significant role in the lignin-degrading ability of this strain, and the recombinant protein was shown to oxidize both Mn(II) and lignin model compounds, catalyzing oxidative Cα-Cβ bond cleavage of a β-aryl ether lignin dimer.5 A further Dyp2 peroxidase has been identified in Amycolatopsis sp. 75iv2 that shows higher activity for Mn(II) oxidation.6 Bacterial laccase enzymes have also been identified in Streptomyces coelicolor A3(2), S. viridosporus T7A,

Figure 1. Bacterial enzymes for oxidation of lignin.

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and *Amycolatopsis* sp. 7Siv2, which catalyze C₅ oxidation of a lignin model compound, and whose genetic knockout significantly reduces the ability of the host to metabolize lignin. Given the complexity of the lignin polymer, it seems likely that there is a group of lignin-oxidizing enzymes, and that further lignin-oxidizing enzymes are still to be discovered.

Using a colorimetric assay method, we have previously reported a screening method for isolation of lignin-oxidizing bacterial strains from environmental soil samples. Using this method, we reported the isolation of 12 novel bacterial lignin-oxidizing strains, including a thermotolerant *Sphingobacterium* sp. T2 isolate which grows on minimal media containing wheat straw lignocellulose as the sole carbon source, or minimal media containing Kraft lignin (see Supporting Information S1), and which showed >10-fold higher activity for lignin oxidation than other isolates in our assay. Another *Sphingobacterium* isolate with high activity for lignosulfonate degradation has also subsequently been reported. In this paper, we report the identification from proteomic analysis and genome sequencing of two extracellular manganese superoxide dismutase enzymes in *Sphingobacterium* sp. T2 that show novel reactivity for lignin oxidation.

## RESULTS

Proteomic Analysis of *Sphingobacterium* sp. T2. Extracellular fractions of *Sphingobacterium* sp. T2 grown on Luria–Bertani broth were partially purified via Q sepharose anion exchange and phenyl sepharose hydrophobic interaction chromatography. Several active fractions were observed (see Supporting Information S2), which showed activity using both the nitrated lignin colorimetric assay and ABTS oxidation. Proteomic analysis via tryptic digestion and MALDI-MS peptide identification, followed by matching to libraries of genomic data, gave a number of matching sequences from *Sphingobacterium spiritorum* strains ATCC 33300 and ATCC 33861, *Leadbetterella byssophilia* DSM 17132, *Pedobacter* sp. BAL39 and *Chryseobacterium gleum*. The list of proteins (see Supporting Information Figure S3) includes proteins OmpA and TonB, both known to be major cell surface proteins in *Bacteroides fragilis*, and several enzyme activities. Of particular interest are two sequences for manganese superoxide dismutase. Although there are no reports of superoxide dismutase involvement in lignin breakdown, this enzyme catalyzes the interconversion of dioxygen with reactive oxygen species superoxide and peroxide, consistent with an observation that *Sphingobacterium* T2 showed higher lignin oxidation activity in the presence of dioxygen, rather than hydrogen peroxide.

The genomic DNA sequence of *Sphingobacterium* sp. T2 (deposited as DDBJ/EMBL/GenBank accession JXAC00000000) contains two genes encoding manganese superoxide dismutases, named *sod1* and *sod2*, matching the proteomic amino acid sequences obtained above. Both encoded proteins showed sequence similarity to the SodA family of superoxide dismutases, with Sod1 and Sod2 showing 49% and 47% sequence identity to *E. coli* SodA, respectively, and 73% sequence identity to each other. The Sod1 amino acid sequence contains a 25 amino acid N-terminal protein targeting sequence MMKMNIFKTALVATLAFQTFFA identified by software SignalP 4.0. In the *Sphingobacterium* sp. T2 genome sequence, *sod1* is located adjacent to an ECF sigma factor and antisigma factor regulatory genes, while *sod2* is located within a cluster of 14 genes containing two araC regulatory genes and three ABC transporter component genes (see Supporting Information Figure S24). The genome contains no dyp-type peroxidase genes and no putative laccase genes, and no recognizable aromatic degradation gene clusters, quite different from the genome sequence of *Rhododendron japonicum* RHA1, which contains large numbers of gene clusters for aromatic degradation. The only annotated peroxidase genes are a catalase/peroxidase (KatG) of the same class as an enzyme reported to show activity for oxidation of a lignin model compound, glutathione peroxidase, cytochrome c peroxidase, a thiol peroxidase, and an alkyl hydroperoxidase. We therefore decided to express the recombinant enzyme for the two MnSOD enzymes and the catalase/peroxidase KatG enzyme.

Expression and Assay of KatG, MnSOD1, and MnSOD2. The genes encoding *Sphingobacterium* sp. T2 KatG, MnSOD1, and MnSOD2 were amplified by PCR and expressed as His₆ fusion proteins in *E. coli*. In the case of MnSOD1, removal of the N-terminal signal sequence was found to aid protein expression in *E. coli*. Each recombinant protein expressed as a soluble protein and was purified by metal affinity chromatography, giving yields of 3.8, 19.5, and 23 mg protein/L culture, respectively (see Supporting Information Figures S4 and S7).

Recombinant *Sphingobacterium* KatG was purified as an 86 kDa protein, was found to be active for oxidation of guaiacol (2-methoxyphenol, *v*ₘₐₓ 0.005 μmol min⁻¹ mg protein⁻¹, *Kₘ 2.1 mM) and ABTS (νₘₐₓ 0.006 μmol min⁻¹ mg protein⁻¹, *Kₘ 5.3 μM) in the presence of 4 mM hydrogen peroxide (see Supporting Information Figure S5), and showed weak activity for oxidation of bromophenol blue, bromothymol blue, and reactive black 5 dyes. However, recombinant KatG showed no detectable reaction with wheat straw lignocellulose or alkali Kraft lignin, by HPLC analysis; hence this enzyme does not appear to be responsible for the lignin oxidation activity of *Sphingobacterium* sp. T2.

Recombinant *Sphingobacterium* MnSOD1 and MnSOD2 were purified as 22.6 and 20.3 kDa proteins, respectively. The recombinant enzymes were found to be active for superoxide dismutation, as measured by inhibition of pyrogallol auto-oxidation (1,2,3-trihydroxybenzene, specific activities: SOD1 400 units/mg; SOD2 124 units/mg), and inhibition of cytochrome c oxidation (specific activities: SOD1 3090 units/mg; SOD2 860 units/mg). Both purified recombinant proteins showed some UV–vis absorbance peaks in the range 360–600 nm: recombinant SOD1 is a pale pink color, showing a weak absorbance peak at 465 nm (ε = 470 M⁻¹ cm⁻¹), comparable to reported *λ*ₘₐₓ values of 480 nm for the human and *Anabaena* MnSOD enzymes; recombinant SOD2 is colorless but shows absorbance peaks at 411 and 503 nm (see Supporting Information Figures S8, S9).

Analysis of metal content using ICP-MS revealed that recombinant SOD1 contained 91% total metal content as Mn, with 7.4% Fe. In contrast, recombinant SOD2 was found to contain 54.7% Zn, 22.6% Mn, 18.4% Fe, and 4.9% Cu. In order to investigate the metal dependence of SOD2 for activity, 20 μM SOD2 apo-enzyme was incubated with FeSO₄, CuSO₄, or MnSO₄ in molar ratios of 1:4, 1:2, 1:1, and 2:1 metal ion/enzyme ratio. Activity using the pyrogallol assay was only observed in the presence of Mn³⁺, with optimum activity requiring 1–2 mol equiv of Mn²⁺ (see Supporting Information S10).

Upon incubation of 720 μg of SOD1 or SOD2 with a suspension of Organosolv lignin in 50 mM potassium...
phosphate buffer 7.8, to which was added 1.25 mM KO$_2$, a visible change in color from yellow to orange was observed over 60 min, and a change in the solubility of the lignin was observed, becoming noticeably more water-soluble (see Figure 2A). Analysis of reaction components by reverse phase HPLC revealed the presence of a number of new peaks, together with an envelope of products in the range 6−20 min with increasing intensity vs time, as shown in Figure 2B. Reaction was also observed with alkali Kraft lignin, generating multiple reaction products in the range 6−20 min with increasing intensity vs time, as shown in Figure 2B. Reaction was also observed with alkali Kraft lignin, generating multiple reaction products in the range 6−20 min with increasing intensity vs time, as shown in Figure 2B.

Analysis of the reaction products from processing of Organosolv lignin by GC-MS gave 10 new product peaks, which were identified by comparison with authentic standards, as shown in Figure 3 (see Supporting Information Figures S12−S21). Products vanillic acid (1), dihydroxybenzoic acid (2), 5-hydroxyvanillic acid (3), 4-hydroxybenzoic acid (4), and vanillin (5) result from cleavage of the C$_{4}$−C$_{3}$ bond in lignin aryl-C$_{4}$ units, while 2-methoxyhydroquinone (7), guaiacol (8), and catechol (9) result from cleavage of the aryl-C$_{6}$ bond in lignin aryl-C$_{3}$ units. 4-(2-Hydroxyethyl)guaiacol (6) contains an aryl-C$_{2}$ skeleton but is deoxygenated at C$_{β}$. Oxalic acid (10) has been observed previously as a metabolite of microbial lignin oxidation.8,20 For the background reaction in the presence of KO$_2$ only, the only product peaks that could be detected by GC-MS were small amounts of vanillic acid (1) and oxalic acid (10), as shown in Figure 3.

In order to study which of the observed products may be derived from the predominant β-aryl ether (β-O-4) linkage present in polymeric lignin, incubations of SOD1 and SOD2 with a β-aryl ether model compound guaiacylglycerol-β-guaiacylglycerol ether were carried out. Products 2-methoxyhydroquinone (7) and guaiacol (8) were observed, consistent with aryl−C$_{6}$ bond cleavage. Incubation of SOD1 or SOD2 with guaiacol (8) gave catechol (9) as a product, indicating that the oxidative demethylation of guaiacol is catalyzed by SOD1 and SOD2.

In order to examine which of the observed enzymes may be unique to the predominant β-aryl ether (β-O-4) linkage present in polymeric lignin, incubations of SOD1 and SOD2 with a β-aryl ether model compound guaiacylglycerol-β-guaiacylglycerol ether were carried out. Products 2-methoxyhydroquinone (7) and guaiacol (8) were observed, consistent with aryl−C$_{6}$ bond cleavage. Incubation of SOD1 or SOD2 with guaiacol (8) gave catechol (9) as a product, indicating that the oxidative demethylation of guaiacol is catalyzed by SOD1 and SOD2.

In order to examine which of the observed enzymes may be unique to the Sphingobacterium MnSOD enzymes, manganese superoxide dismutase genes from Escherichia coli and Thermus thermophilus were cloned and overexpressed in E. coli as His$_{6}$ fusion proteins, and the purified recombinant enzymes were assayed (see Supporting Information S22). When incubated with Organosolv lignin and KO$_2$, only very small amounts of product metabolites were observed by HPLC analysis,
corresponding to 5–6% of the products formed by SpMnSOD1, similar to the background KO2 reaction.

The generation of multiple reaction products implies the generation of a reactive oxidant by the Sphingobacterium MnSOD enzymes. In order to investigate turnover-dependent enzyme inactivation, SOD activity was measured under the lignin oxidation conditions, using the pyrogallol assay. It was found that 75% activity was lost after 30 min under these conditions, but thereafter 10–20% residual activity was retained over 3–4 h (see Supporting Information S28). Analysis of recombinant SpMnSOD1 by electrospray mass spectrometry verified the expected mass at 26,394 Da, which was still the major species present after 1 h under the assay conditions (see Supporting Information S24), indicating that there is no rapid chemical modification of the enzyme.

Crystal Structure of MnSOD1. Diffracting crystals of Sphingobacterium sp. (pdb: 5A9G) T2MnSOD (SpMnSOD1) were obtained and X-ray diffraction data collected to 1.35 Å. The structure of SpMnSOD1 was determined using molecular replacement using the MnSOD structure from Bacillus subtilis (pdb: 2RCV).23 The crystal structure of SpMnSOD1 revealed a typical MnSOD homodimer comprising two characteristic Fe/MnSOD subunits (Figure 4). The SpMnSOD1 monomers superpose with an RMSD of 0.108 Å (165 CA atoms) and are therefore essentially structurally identical. The SpMnSOD1 monomer and dimer share high structural homology with other MnSOD enzymes for which crystal structures have been determined. Structural superposition of SpMnSOD1 with homologues from E. coli (pdb code 1DSN; salmon), T. thermophilus (3MDS; yellow), B. subtilis (2RCV; light green), D. radiodurans (1Y67; magenta), and Anabena (1GV3; cyan) are shown in ribbon representation. Figure drawn with Pymol.45

Each SpMnSOD1 subunit contains a five-coordinate trigonal bipyramidal Mn ion ligated by His26 and water/hydroxide as apical ligands and His76, Asp163, and His167 as equatorial ligands (Figure 5A). The Mn ligands and inner sphere residues are surrounded by outer sphere residues including the highly...
Anabena PCC 7120 (pdb code 1GV3; cyan; monomer superposition = 0.445 for 137 CA; dimer superposition = 0.841 for 314 CA). Numbering refers to SpMnSOD1. C) The SIGMAA weighted 2mFo–DFc electron density using phases from the final model of the SpMnSOD1Mn3− binding site is contoured at the 1.0 Å level, where s represents the RMS electron density for the unit cell. Contours more than 1.4 Å from any of the displayed atoms have been removed for clarity. Figure drawn with PyMOL.45

The presence of an extracellular superoxide dismutase enzyme is unusual, since bacterial superoxide dismutases are usually intracellular;13 however an extracellular MnSOD enzyme has been reported in Streptococcus pyogenes,29 which shares 42% and 48% sequence identity with SpMnSOD1 and SpMnSOD2, respectively. Database searches using the BLAST algorithm (see Supporting Information Figures S25, S26) reveal that homologues for SpMnSOD1 and SpMnSOD2 are found in Sphingobacteriales and Flavobacteriales, and also in strains of Acinetobacter (γ-proteobacteria). Interestingly, an extracellular manganese superoxide dismutase has been reported in a gene cluster for metabolism of poly(cis-1,4-isoprene) in Gordonia polysoprenivorans VH2 and is required for efficient growth on poly(cis-1,4-isoprene) as carbon source.30 Schulte et al. proposed that the SodA enzyme is probably involved in protection against reactive oxygen species during rubber breakdown,30 but this enzyme also shares 32% and 37% sequence identity with SpMnSOD1 and SpMnSOD2, respectively, so it might also possess oxidative C–C bond cleavage activity. Given the close similarity in structure between the Sphingobacterium MnSOD enzymes and E. coli and T. thermophilus MnSOD enzymes, it is interesting that the latter enzymes are much less active for lignin oxidation, implying that there is some special reactivity possessed by the Sphingobacterium MnSOD enzymes.

Following lignin oxidation by superoxide dismutase enzymes, the subsequent metabolism of the lignin oxidation products is unclear, since the genome of Sphingobacterium sp. T2 does not appear to contain any conventional aromatic degradation gene clusters. We have previously found that this strain is able to utilize vanillic acid as the sole carbon source for growth,3 which suggests that it is able to metabolize such intermediates. The reaction products that we have observed from oxidation of Organosolv lignin are highly oxidized, so we suspect there may be a pathway similar to the phloroglucinol pathway observed in Pelobacter acidigallici (Bacteroidaceae),31 Eubacterium oxidoreducens,32 and Coprococcus sp.,33 in which a 1,3,5-trihydroxybenzene tautomerizes to a nonaromatic cyclohexane-1,3,5-trione, which is then a substrate for hydrolytic cleavage, rather than oxidative ring cleavage.

Treatment of Organosolv lignin with Sod1 or Sod2 generates multiple reaction products, of which we have characterized 12 structures shown in Figure 3. The formation of the reaction products shown in Figure 2 from lignin indicates that aryl–Cα oxidative bond cleavage has taken place (products 7–10), as well as Cα–Cβ oxidative cleavage (products 1–5), decarboxylation (e.g., conversion of 1 to 9) and O-demethylation (products 2, 10). The generation of such a range of reaction products and reaction type from Organosolv or Kraft lignin implies the generation of a highly reactive oxidant. The reaction cycle of manganese superoxide dismutase, shown in Figure S, involves two half-reactions, namely oxidation of superoxide to dioxygen by Mn(III), and reduction of superoxide to hydrogen peroxide by Mn(II).13,15,16,19 Two possible hypotheses for interaction with lignin are illustrated in Figure 7. The first possibility (Figure 7A) is that the Mn(III) form of the metal cofactor might oxidize lignin, analogous to the generation of Mn(III) by fungal manganese peroxidase, which then acts as a diffusible oxidant to attack lignin.14 However, the manganese cofactor in superoxide dismutases is generally tightly bound.
and we have seen no evidence that it is released by the *Sphingobacterium* SOD enzymes.

The second hypothesis (Figure 7B) is that the SpMnSOD enzymes are able to further reduce peroxide to hydroxyl radical, which is the reactive oxidant to attack lignin. It is reported that bovine erythrocyte Cu/Zn superoxide dismutase can generate hydroxyl radicals, but that *E. coli* MnSOD, which in our hands shows very little lignin oxidation activity, does not generate hydroxyl radical.\(^{35}\) The observation of oxidation products containing additional phenolic hydroxyl groups (e.g., products 3, 6, 7) is consistent with the known ability of hydroxyl radical to carry out phenolic hydroxylation;\(^{36}\) hence this appears to be a possible mechanism for lignin oxidation via these enzymes. We note that nature uses the hydroxyl radical to attack lignin in a different context: brown rot fungi utilize Fenton chemistry to generate a hydroxyl radical to attack lignin.\(^{37,38}\) There are also literature reports of the production of hydroxyl radicals in white rot fungus *Phanerochaete chrysosporium*,\(^{39-41}\) though subsequent data implied that this is not a major contributing mechanism in white-rot fungal lignin degradation.\(^{42}\)

We therefore propose a possible mechanism shown in Figure 8 for the generation of the observed products from Organosolv lignin. Hydroxyl radical is reported to cause demethoxylation of methoxylated aromatic compounds, via addition of hydroxyl radical to the aromatic ring.\(^{37}\) If hydroxyl radical attacked at aryl C-1 of an aryl-C<sub>3</sub> unit, then C→C fragmentation as shown in Figure 8 would generate compound 7, observed in incubations of SpMnSOD enzymes with both Organosolv lignin and a β-aryl ether model compound. The hydroxyl radical is also known to cause oxidative fragmentation of ketones such as α-oxo-γ-methylthiobutyric acid;\(^{41,43}\) therefore we propose that oxidative cleavage of a benzyl ketone intermediate could lead to vanillic acid 1 via cleavage A, and guaiacol 8 via cleavage B. Demethoxylation of guaiacol 8, a known reaction for hydroxyl radical,\(^{37}\) would generate catechol 9. Further hydroxylation of vanillic acid 1, a known reaction for hydroxyl radical,\(^{36}\) would then generate 5-hydroxyvanillic acid 3.

The crystal structure of *Sphingobacterium* sp. T2MnSOD1 determined here shows high structural similarity with MnSOD structures from *E. coli* and *T. thermophilus* that we have shown experimentally to have low activity for lignin oxidation. Hence, we note that nature uses the hydroxyl radical to attack lignin in a different context: brown rot fungi utilize Fenton chemistry to generate a hydroxyl radical to attack lignin.\(^{37,38}\) There are also literature reports of the production of hydroxyl radicals in white rot fungus *Phanerochaete chrysosporium*,\(^{39-41}\) though subsequent data implied that this is not a major contributing mechanism in white-rot fungal lignin degradation.\(^{42}\)

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we suggest that amino acid replacements near to the active site may affect the environment of the active site and perhaps alter the redox potential of these enzymes, leading to a change in reactivity of these enzymes. There are several amino acid replacements close to Mn ligands His-26 and His-76 (see Figure 6) that would potentially change the charge balance near the active site, whose precise function will be studied in future work. Another unsolved question is how the enzyme protects itself from chemical modification by the hydroxyl radical oxidant that is generated. We have shown that turnover-dependent inactivation of the enzyme does occur to some extent, but not completely (see Supporting Information Figure S23). Furthermore, there is no chemical modification of the polypeptide observed by mass spectrometry (see Supporting Information Figure S24), nor in the crystal structure of SpMnSOD1.

The identification of the Sphingobacterium sp. T2 manganese superoxide dismutases as lignin-oxidizing enzymes expands the range of bacterial enzymes capable of lignin oxidation.5–7 These enzymes could be valuable biocatalysts for the valorization of lignin produced as a byproduct of biofuel production and pulp/paper manufacture, including Kraft lignin for which the SpMnSOD enzymes show activity.4 The identification of reaction products from oxidation of polymeric lignin helps to define further the metabolic pathways for microbial lignin breakdown.20

■ METHODS

Protoxic Identification. Cultures of Sphingobacterium sp. T2 were grown in Luria–Bertani broth (1.5 L) at 45 °C for 24 h. After centrifugation (6000g, 20 min), protein was precipitated by the addition of 70% ammonium sulfate, followed by centrifugation (15 000g, 20 min). Pellets were resuspended in 20 mM potassium phosphate buffer (pH 7.4), applied to a Q sepharose anion exchange column, and eluted in a gradient of 0−2 M NaCl in 20 mM potassium phosphate buffer (pH 7.4), at a flow rate of 1.6 mL min−1. The flow-through fraction from the Q sepharose column was most active and was further purified by Phenyl Sepharose column chromatography, eluting with 20 mM sodium phosphate (pH 7.4) containing a gradient of 2 to 0 M (NH₄)₂SO₄ at a flow rate of 1.6 mL min−1. Fractions were assayed for activity using the nitratated lignin assay in the presence and absence of H₂O₂. Active fractions were analyzed by SD-SD PAGE (see Supporting Information S2), and Coomassie-stained gel bands were processed and proteomic analysis carried out by the WPH Proteomics Facility (School of Life Sciences, University of Warwick). After trypsic digestion, the extracted peptides from each sample were analyzed by nano LC-ESI-MS/MS using Nano Acuity/Ultima Global Instrumentation (Waters), using a 30 min LC gradient. The data were processed to generate peak list files using Protein Lynx Global Surveyor v 2.5.1. The observed molecular ion data for trypptic digests were compared with protein databases, and a number of matches were found (see Supporting Information Figure S3).

Expression of Recombinant KatG and MsSOD1&2. Cloning primers were designed for the Sphingobacterium sp. T2 katG, sod1, and sod2 genes, and E. coli and T. thermophilus MsSOD containing a S′-CACC overhang (see Supporting Information S4, S7, S23 for primer sequences). The katG, sod1, and sod2 genes were amplified from Sphingobacterium sp. T2 genomic DNA by polymerase chain reaction, using Platinum Pfx-DNA polymerase from Invitrogen, following the manufacturer’s instructions. The amplified genes were cloned using the Champion pET Directional TOPO Expression Kit (Invitrogen) into expression vector pET200 (katG) or pET151 (sod1, sod2, EcSOD, TisSOD), and transformed into Escherichia coli DH5α (katG) or TOP10 competent cells (Invitrogen). The recombinant plasmids were then transformed into BL21 E. coli BL21(DE3)pLysS (Invitrogen), for protein expression.

Cultures of each recombinant strain were grown at 37 °C in 500 mL of Luria–Bertani media containing 40 μg/mL kanamycin (for KatG) or 100 mg mL−1 ampicillin, induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at OD₆₀₀ = 0.6, then incubated overnight at 15 °C with shaking at 180 rpm. In the case of SOD1 and SOD2, 1 mM MnSO₄ was also added at induction. The cell pellet was harvested by centrifugation (6000g, 15 min). The cells were resuspended in 20 mM Tris pH 8.0 containing 10 mM imidazole, 0.5 M NaCl, and 1 mM PMSF, passed through a cell disruptor, centrifuged (10 000g, 35 min), and the supernatant was filtered with a Whatman 0.2 μm syringe filter. The soluble protein fraction was loaded on to a 5 mL pre-equilibrated Ni-NTA column (Qiagen) with 20 mM Tris pH 8.0 buffer containing 20 mM imidazole, 0.5 M NaCl, and eluted with 20 mM Tris pH 7.8 containing 300 mM imidazole, 0.5 M NaCl. The purified enzyme was concentrated with an Amicon centrifugal unit (10 kDa cut off) and desalted with a PD-10 column, eluting with 50 mM sodium phosphate buffer pH 7.8.

Enzyme Assays. KatG assay was carried out in 1 mL of 50 mM sodium acetate (pH 5.0) containing 0.5 mM ABTS, 4.0 mM H₂O₂, to which was added recombinant KatG. Reactions were monitored at 414 nm (ε₄₁₄ = 36 600 M⁻¹ cm⁻¹). Guaiacol oxidation was carried out in 1 mL of 50 mM sodium acetate (pH 5.0) containing 100 μM guaiacol, 4.0 mM H₂O₂, to which was added recombinant KatG, monitoring at 465 nm (ε₄₆₅ = 26 600 M⁻¹ cm⁻¹). SOD activity was assayed via inhibition of xanthine auto-oxidation in 50 mM Tris buffer pH 8.2 containing 0.1 mM EDTA, or via ferricytochrome c assay in 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 50 μM xanthine, 20 μM cytochrome c, and 0.01 units xanthine oxidase.

Incubation with Lignin or Lignin Model Compounds. Solutions of 5 mg of wheat straw Organosolv lignin or alkali Kraft lignin or powdered micianus lignocellulose (5 mg mL⁻¹ in DMSO) or lignin model compound (10 mg mL⁻¹ in methanol) were prepared. Reactions were carried out in a 3 mL volume using 50 mM NH₄HCO₃ buffer pH 7.8 containing 0.1 mM EDTA, containing either 200 μL of lignin model compound or 500 μL of lignin sample, and 0.8 mg of SOD enzyme. A total of 1 mL of a saturated solution of KO₂ in dry methanol was added and the reaction was monitored at 414 nm, with monitoring at 465 nm, which was added recombinant KatG. Reactions were assayed via reaction as described above, samples were centrifuged (13 000 rpm, microcentrifuge) for 10 min prior to analysis by HPLC. HPLC method A: analysis was conducted using a Phenomenex Luna 5 μm C18 reverse phase column (4.6 mm) on a Hewlett-Packard Series 1100 analyzer, at a flow rate of 0.5 mL/min, with monitoring at 310 nm. The gradient was as follows: 20 to 30% MeOH/H₂O over 5 min, 30 to 50% MeOH/H₂O from 5 to 12 min, and 50 to 80% MeOH/H₂O from 12 to 25 min. Method B: column and flow rate as above, monitoring at 270 and 310 nm. Buffer A: H₂O with 0.1% v/v formic acid; buffer B, MeOH. Gradient: start with 5% buffer B, 5−30% B over 0−20 min; 30% B for 10 min; 30−100% B for 15 min; 100% B for 8 min; 100−50% B over 8 min. Reaction products were extracted into ethyl acetate, and the organic layer was evaporated. The products were dissolved in chloroform, dried (Na₂SO₄), and filtered. The samples were analyzed by LC-MS or by GC-MS, either directly or after derivatization with N,O-bis(trimethylsilyl)acetamide (see Supporting Information S12).

Crystalization and Structure Determination of SpMnSOD1. Methods for protein crystallization, X-ray data collection, structure determination, and refinement are described in Supporting Information S29, with crystallographic data and refinement statistics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00298.
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extracellular superoxide dismutase (SodA) as a radical scavenger in poly(cis-1,4-isoprene) degradation. 


