pH-Dependent Relationship between Catalytic Activity and Hydrogen Peroxide Production Shown via Characterization of a Lytic Polysaccharide Monooxygenase from *Gloeophyllum trabeum*

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**ABSTRACT** Lytic polysaccharide monooxygenases (LPMOs) are copper-dependent enzymes that perform oxidative cleavage of recalcitrant polysaccharides. We have purified and characterized a recombinant family AA9 LPMO, LPMO9B, from *Gloeophyllum trabeum* (GtLPMO9B) which is active on both cellulose and xyloglucan. Activity of the enzyme was tested in the presence of three different reductants: ascorbic acid, gallic acid, and 2,3-dihydroxybenzoic acid (2,3-DHBA). Under standard aerobic conditions typically used in LPMO experiments, the first two reductants could drive LPMO catalysis whereas 2,3-DHBA could not. In agreement with the recent discovery that H$_2$O$_2$ can drive LPMO catalysis, we show that gradual addition of H$_2$O$_2$ allowed LPMO activity at very low, substoichiometric (relative to products formed) reductant concentrations. Most importantly, we found that while 2,3-DHBA is not capable of driving the LPMO reaction under standard aerobic conditions, it can do so in the presence of externally added H$_2$O$_2$. At alkaline pH, 2,3-DHBA is able to drive the LPMO reaction without externally added H$_2$O$_2$, and this ability overlaps entirely the endogenous generation of H$_2$O$_2$ by GtLPMO9B-catalyzed oxidation of 2,3-DHBA. These findings support the notion that H$_2$O$_2$ is a cosubstrate of LPMOs and provide insight into how LPMO reactions depend on, and may be controlled by, the choice of pH and reductant.

**IMPORTANCE** Lytic polysaccharide monooxygenases promote enzymatic depolymerization of lignocellulosic materials by microorganisms due to their ability to oxidatively cleave recalcitrant polysaccharides. The properties of these copper-dependent enzymes are currently of high scientific and industrial interest. We describe a previously uncharacterized fungal LPMO and show how reductants, which are needed to prime the LPMO by reducing Cu(II) to Cu(I) and to supply electrons during catalysis, affect enzyme efficiency and stability. The results support claims that H$_2$O$_2$ is a natural cosubstrate for LPMOs by demonstrating that when certain reductants are used, catalysis can be driven only by H$_2$O$_2$ and not by O$_2$. Furthermore, we show how auto-inactivation resulting from endogenous generation of H$_2$O$_2$ in the LPMO-reductant system may be prevented. Finally, we identified a reductant that leads to enzyme activation without any endogenous H$_2$O$_2$ generation, allowing for improved control of LPMO reactivity and providing a valuable tool for future LPMO research.

**KEYWORDS** LPMO, lignocellulose, brown rot fungi, hydrogen peroxide, lytic polysaccharide monooxygenase, wood decay

Lignocellulosic biomass is the most abundant biogenic material on Earth. In nature, microorganisms that degrade lignocellulose utilize a plethora of different enzymes that attack the various components of the plant cell wall (lignin, cellulose, and hemi-
celluloses). Among these are traditional hydrolytic cellulases and hemicellulases (1), as well as oxidoreductases with a wide range of functions (2). Crucial among these oxidoreductases are enzymes called lytic polysaccharide monooxygenases (LPMOs), whose beneficial effect on biomass degradation was first described in 2005 for chitin (3) and subsequently for cellulose (4). The genomes of lignocellulose-degrading organisms often encode multiple LPMOs. These enzymes are found in all three domains of life and are classified in the carbohydrate-active enzymes (CAZy) database (5) in the auxiliary activity (AA) families 9, 10, 11, 13, 14, and 15. LPMOs are mono-copper oxidases that cause chain breaks in crystalline and amorphous polysaccharides, such as cellulose, hemicelluloses, chitin, and starch (6–9). Cellulose-active fungal LPMOs form family AA9. Depending on the organism, the number of LPMO genes in fungi can range from a few to dozens (10, 11).

_Gloeophyllum trabeum_ is a model wood-degrading basidiomycete that causes brown rot by the selective removal of the cell wall polysaccharides without the mineralization of lignin (12). Brown rot fungi form a polyphyletic group, characterized not by their shared ancestry but by the type of decay they cause (13). While brown rot fungi are limited in their number of lignocellulose active enzymes compared to the number in other decay organisms such as white rot fungi, they are hypothesized to use a Fenton-like mechanism involving iron, oxalic acid, and phenolic secondary metabolites to generate reactive oxygen species (ROS) that depolymerize wood cell wall polysaccharides (12, 14–16). These mechanisms are well known from _G. trabeum_, which secretes oxalic acid to chelate transition metals and to maintain a low pH in its surroundings (12, 14, 15). While the genome of _G. trabeum_ encodes a limited number of traditional hydrolytic cellulases and hemicellulases (nine glycoside hydrolase 3 [GH3], five GH5, and three GH10 enzymes and two GH12s, one GH74, and five GH43s, respectively) compared to the numbers in white rot fungi, it encodes a considerable number of AA family enzymes, including six LPMOs (4 AA9s and 2 AA14s), 24 glucose-methanol-choline (GMC) oxidoreductases (AA3), and 2 copper radical oxidases (AA5) (10). GMC oxidoreductases are flavoenzymes that oxidize various sugars and alcohols with the concomitant reduction of either O2 (to H2O2) or quinones/phenoxyl radicals (to phenols) or other compounds (17).

The role of H2O2 in wood-degrading fungi has traditionally been attributed to oxidative depolymerization of plant cell wall lignin by lignolytic peroxidases and polysaccharides via nonenzymatic generation of reactive oxygen species (ROS). Recent developments regarding the oxidative mechanisms of LPMOs, however, have shed new light on the potential role of H2O2 during enzymatic depolymerization of cell wall polysaccharides (2, 18). Since their identification as oxidative enzymes, LPMOs were thought to use molecular oxygen as cosubstrate (8, 19), hence the name monooxygenase (Fig. 1). The suggested mechanism for the monooxygenase reaction entails a one-electron reduction of Cu(II) to Cu(I), followed by the binding of O2 and formation of a superoxide intermediate [Cu(II)-O-O·] (20). A second electron and two protons are then required to complete the catalytic cycle, via different possible routes (6, 21), leading to incorporation of a hydroxyl group at the C-1 or C-4 in the scissile glycosidic bond, which is followed by spontaneous bond cleavage (19). This mechanism has recently been challenged by Bissaro et al. (18), who suggested that H2O2 is the natural cosubstrate of LPMOs (Fig. 1). In this scenario a one-electron “priming” reduction of the active site Cu(II) to Cu(I) is followed by a reaction with H2O2 that leads to hydrogen abstraction and subsequent hydroxylation of the substrate via different possible routes (18). Subsequent experimental (22–24) and computational (25, 26) studies have supported H2O2-driven LPMO catalysis, but the nature of the natural cosubstrate, O2 or H2O2, remains under debate.

No matter the true mechanism, it is clear that LPMOs need to be reduced to become active. The source of the necessary reducing power in _vivo_ is not known, but it is well established that LPMOs are promiscuous when it comes to interacting with reductants in _vitro_ (27, 28). Several AA3 enzymes (27, 29), including well-known CDHs (30, 31) and an AA12 pyranose dehydrogenase (32), are able to drive LPMO action. In addition to
enzymatic electron donors, phenolics (28, 33) and lignin-derived compounds (34) can also drive LPMO reactions. While cellobiose dehydrogenase (CDH) sometimes is considered a natural reductant for LPMOs, several brown rot fungi, including G. trabeum, lack genes that encode CDH but have several genes encoding other AA3 family enzymes (e.g., aryl alcohol oxidases) that may reduce LPMOs (27, 29) and that are expressed during growth on lignocellulosic biomass (35). Of note, several AA3 enzymes produce H2O2 and may fuel H2O2-driven LPMO catalysis (2).

The observed promiscuity of LPMOs when it comes to reductants can likely be attributed in part to the open structure of the LPMO active site, where the copper is directly exposed on the flat catalytic surface of the enzyme. Previous studies have reported the redox potential of LPMO-Cu(II)/LPMO-Cu(I) to be 155 to 326 mV (most are >240 mV), while that of soluble copper is approximately 160 mV [Cu(II)/Cu(I)] (29).

Reactions in the presence of reductant and absence of substrate have shown that LPMOs can act as oxidases, reducing O2 to produce H2O2 (36). There are two potential pathways for the observed generation of H2O2. In one scenario, single-electron reduction of molecular oxygen in the LPMO active site is followed by the release of superoxide that will then undergo spontaneous disproportionation or react with a reductant. The other possible mechanism involves H2O2 production in the active site of the enzyme, meaning that the first single-electron reduction of molecular oxygen is followed by delivery of an additional electron and two protons to reduce copper-bound superoxide to H2O2. There is some disagreement in the literature as to why H2O2
production is not observed in reaction mixtures containing an LPMO substrate. Some investigators argue that the oxidase reaction is suppressed by the formation of a productive enzyme-substrate complex and that substrate cleavage is a monoxygenase reaction (24), whereas others argue that, even in the presence of substrate, non-substrate-bound LPMOs generate H₂O₂, which is not observed because substrate-bound LPMOs use the H₂O₂ in carrying out a peroxygenase reaction (2, 18). Of note, the role of the interaction of the LPMO with the reductant varies quite considerably between these two scenarios.

We have studied the properties of a not previously characterized LPMO, LPMO9B, from the brown rot fungus *G. trabeum* (*GtLPMO9B*), focusing on the effects of reductant, pH, and H₂O₂ on catalytic activity. We demonstrate how the activity of *GtLPMO9B* is modulated by different reductants, reductant concentrations, and supply of H₂O₂. Importantly, these studies revealed that a reductant not previously used in LPMO activity studies, 2,3-dihydroxybenzoic acid (2,3-DHBA), is capable of activating the LPMO for H₂O₂-driven catalysis but, in contrast to, e.g., ascorbic acid (AscA), cannot drive the LPMO reaction on its own. This interesting observation provides a useful tool for further LPMO studies and lends support to the proposal that H₂O₂ is a natural cosubstrate of LPMOs.

**RESULTS AND DISCUSSION**

**Enzyme production.** Recombinant *GtLPMO9B* was successfully produced in *Pichia pastoris*, and the purified enzyme revealed an approximate mass of 50 kDa when analyzed by SDS-PAGE. Sequence analysis of secreted *GtLPMO9B* shows a predicted mass of 24.5 kDa, indicating significant posttranslational modification (glycosylation) of the recombinant protein. *GtLPMO9B* carries two putative N-glycosylation sites (Asn138 and Asn217) and five putative O-glycosylation sites (Thr26, Ser99, Ser100, Ser139, and Ser192), as determined by the NetNGlyc, version 1.0 (37), and NetOGlyc, version 4.0 (38), servers of the Technical University of Denmark. *GtLPMO9B* is a single-domain AA9 LPMO, and the closest characterized relative is *GtLPMO9A-2* from *G. trabeum* (76.6% sequence identity), and the second closest relative with a known structure (PDB accession number 4EIS) is *Neurospora crassa* LPMO9M (*NcLPMO9M*) (54.8% identity).

A structural model of *GtLPMO9B* built with PHYRE2 (39) showed the presence of a typical catalytic copper site comprising two conserved histidines (His1 and His86) and a tyrosine (Tyr175) in the proximal axial coordination position (see Fig. S1 in the supplemental material). The potential glycosylation sites are not located on the catalytic surface in the enzyme model; the closest residues, Asn138 and Ser139, are more than 10 Å away from the His brace. Still, since glycosylation was considerable, we cannot exclude the possibility that the attached glycans interact with the catalytic surface.

**Substrate and reductant specificity.** The activity of *GtLPMO9B* was tested on a wide range of substrates, including phosphoric acid-swollen cellulose (PASC), soluble cello-oligosaccharides (Glc₅ and Glc₆), konjac glucomannan, lichenan from Icelandic moss, birchwood xylan, galactomannan, wheat arabinoxylan, barley beta-glucan, ivory nut mannan, and xyloglucan from tamarind seed (tamarin xyloglucan [TXG]) using ascorbic acid (AscA) as a reducing agent. Product analysis by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) revealed reductant-dependent product formation for cellulose and xyloglucan only (Fig. 2). Figure 2A shows that *GtLPMO9B* produces both C-1- or C-4-oxidized cello-oligosaccharides from PASC, which implies that also native and double-oxidized oligomers are prominent (40). Of the *G. trabeum* LPMO9s, *GtLPMO9B* shares 76.6% identity with the AA9 domain of *GtLPMO9A-2* (characterized by Kojima et al. [41]). Both enzymes are C-1/C-4-oxidizing LPMOs and have activity on cellulose and xyloglucan (Fig. 2) (41). *GtLPMO9A-2* has broad specificity and is able to cleave the xyloglucan backbone regardless of the substitution pattern of glucosyl residues. Of note, in a previous study, *GtLPMO9B* was shown to increase the activity of the *G. trabeum*
endoglucanase GtCel5B and xylanase GtXyl10G on pretreated oak and kenaf (a hemp type) (42), but in this study the activity of the LPMO alone was not analyzed.

Analysis of product formation after incubating GtLPMO9B with PASC for 24 h in the presence of 1 mM concentrations of various reducing agents at pH 6.5 showed C-1- or C-4-oxidized product levels similar to those obtained with AscA for gallic acid (GA), pyrogallol, caffeic acid, catechol, and hydroquinone, all of which are di-hydroxy or tri-hydroxy aromatic compounds (results not shown; note that these were endpoint measurements; kinetic data for selected reductants are discussed below). Monohydroxy coniferyl alcohol, a natural lignin precursor, gave low product yields, with peak intensities >15 times lower than those with AscA, whereas no product formation was observed in reactions with 2,3-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, vanillic acid, guaiacol, veratryl alcohol, 2,4-hexadiene-1-ol, and 4-hydroxybenzoic acid under these standard conditions.

**Activity with AscA.** The activity of GtLPMO9B with ascorbic acid (AscA) as the reducing agent was tested under aerobic conditions using either millimolar concentrations of the reductant only or multiple additions of reductant and H₂O₂ at micromolar concentrations. The multiple-addition strategy was used to keep the maximum concentrations of H₂O₂ low since high concentrations may lead to autocatalytic

**FIG 2** Products generated by GtLPMO9B. (A) HPAEC-PAD chromatogram showing soluble native and oxidized cello-oligosaccharides released from PASC. The peaks were annotated based on Isaksen et al. (46); double-oxidized oligomers elute at 40 to 43 min. (B) HPAEC-PAD chromatogram showing soluble native and oxidized xyloglucan oligomers released from tamarind xyloglucan (TXG). Reaction mixtures contained 0.2% (wt/vol) PASC or TXG, 1 μM GtLPMO9B, and 1 mM AscA (solid line) or no AscA (dashed line) and were incubated in 50 mM bis-Tris-HCl buffer (pH 6.5) for 24 h at 45°C.
inactivation of the LPMO (18). Solubilized oligomeric products were treated with *Trichoderma reesei* Cel7A (TrCel7A) to generate a mixture containing only C-1-oxidized cellobiose (cellobionic acid or GlcGlcA) and C-4-oxidized cellobiose (Glc4gemGlc) as oxidized products. The amounts of these two oxidized disaccharides were quantified by HPAEC-PAD using in-house-prepared standards and summed to determine total product formation by the LPMO. The ratio of C-1- to C-4-oxidized products was constant in all experiments carried out at standard pH.

Figure 3A shows that in the initial linear phase of reactions with 1 mM AscA at pH 6.5, i.e., conditions that are generally used in LPMO research, 1 μM GtLPMO9B produced approximately 0.88 μM oxidized products per minute, and the reaction stopped after
about 120 min. Increasing the concentration of AscA to 5 mM gave an increased initial rate (1.18 μM·min⁻¹), but product formation leveled off already after 60 min, meaning that the product final yield was reduced.

Notably, the final yield of oxidized products in reactions with millimolar concentrations of AscA (1 mM and 5 mM) remained far below the theoretical yield, which would be at least 250 μM product if molecular oxygen was limiting or 1 mM and 5 mM, respectively, if AscA was limiting. This is commonly observed for in vitro LPMO reactions. Some oxidations will be overlooked because the oxidized sites remain on the insoluble substrate (43). While this fraction may be as high as 50% in some cases (43, 44), it cannot explain why the total yield in the reaction with 5 mM AscA is only 60 μM. A more likely explanation is that the LPMOs become inactivated during the reaction due to oxidative damage in their active sites (18, 24, 45).

Figure 3C shows that GtLPMO9B incubated under aerobic conditions with AscA indeed produces H₂O₂ in the absence of substrate.

In the presence of O₂ and AscA and in the absence of substrate, LPMOs produce H₂O₂ (36, 46), and it has been claimed that H₂O₂ production by non-substrate-bound reduced enzyme contributes to driving LPMO reactions under commonly used conditions (18). It has further been shown that accumulation of H₂O₂ and/or poor substrate binding correlates with LPMO inactivation (18, 23, 45). Figure 3C shows that GtLPMO9B incubated under aerobic conditions with AscA indeed produces H₂O₂ in the absence of substrate.

Activity with GA. Of the other tested reductants, gallic acid (GA) was considered the most interesting one because it is known to react more slowly with O₂ (27) and could thus perhaps lead to more controlled reactions, with less inactivation. Indeed, in contrast to results in reactions with AscA, in reactions with GA, product formation continued after 4 h (Fig. 4A). In accordance with the presumed slower generation of reactive species by GA and in contrast to what was observed with AscA (Fig. 3A), increasing the concentration of GA increased both the reaction rate and the final yield after 24 h (Fig. 4A). Figure 4C shows that H₂O₂ accumulation in the absence of substrate was lower for GA than for AscA (both at 30 μM concentration; the H₂O₂ production rates were 0.29 μM·h⁻¹ and 4.8 μM·h⁻¹, respectively).

To investigate whether this difference could be attributed to H₂O₂ scavenging by GA, we incubated 30 μM reductant (AscA, GA, or 2,3-DHBA) with 30 μM H₂O₂ for 1 h (pH 6.5 at room temperature) (Fig. S2). This revealed that while AscA and 2,3-DHBA removed 54% and 31% of H₂O₂, respectively, GA removed 94%. We interpret these observations to indicate that GA is protecting the LPMO from excessive H₂O₂ generated in the reaction with PASC (Fig. 4A), explaining the prolonged longevity of the enzyme in the reaction. Notably, this finding highlights the fact that the Amplex Red assay should be used with caution to determine absolute H₂O₂ production by LPMOs (36) in the presence of reductants. Reductants may react with H₂O₂, which will mask H₂O₂ production by LPMOs, and, thus, the values obtained in the Amplex Red assay reflect the total accumulation and not production. These observations underpin the impact of the choice of reductant on the activity and longevity of LPMOs.

Interestingly, replacing 15 μM AscA with 15 μM GA in reactions with multiple additions of reductant and H₂O₂ (Fig. 4B) revealed much less difference between the reductants. Compared to results with reaction mixtures containing millimolar amounts of GA, this H₂O₂-based setup gave fast kinetics and progress curves with GA that were quite similar to those obtained with AscA under the same conditions. The observation that GA and AscA gave similar results when applied at low concentrations in reactions with added H₂O₂ indicated that a difference in reductant performance in standard
O₂-driven reactions is not due to differences in the ability to carry out the first priming reduction of the LPMO but, rather, to a difference in the ability to promote generation and accumulation of H₂O₂ in the reaction mixture.

**Activity with 2,3-DHBA and hydrogen peroxide.** Considering the observation that GA was equally as effective as AscA in reactions with added H₂O₂, we then tested whether something similar might apply to the reductants that were not able to drive LPMO reactions under standard (aerobic, no added H₂O₂) conditions. Of three reductants tested, 2,3-dihydroxybenzoic acid (2,3-DHBA), 3,5-DHBA, and vanillic acid, one, 2,3-DHBA, indeed led to the formation of oxidized products in reaction mixtures with added H₂O₂ at pH 6.5 (Fig. 5). This experiment shows that 2,3-DHBA was able to prime

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**FIG 4** Activity of GtLPMO9B with gallic acid (GA) as a reductant. The reaction mixtures used in the experiments shown in panels A and B were incubated at 45°C and 1,000 rpm, and mixtures contained 1 μM enzyme, 50 mM bis-Tris-HCl buffer at pH 6.5, 0.2% (A) or 0.5% (B) PASC (wt/vol), and 1 mM (solid line) GA or 5 mM (dashed line) GA added only at time zero (A) or with repetitive addition of 15 μg GA and 50 μM H₂O₂ every 15 min, starting at time zero (B). Solubilized oxidized products were quantified as C-1-oxidized (cellobionic acid) and C-4-oxidized (Glc4gemGlc) dimers using HPAEC-PAD, and the sum of the two was calculated. Note the difference in time scale between the graphs shown in panels A (24 h) and B (240 min). (C) H₂O₂ accumulation in a reaction mixture containing 1 μM GtLPMO9B and 30 μM GA without PASC (dashed line) and in a control reaction mixture containing 1 μM CuSO₄ instead of the LPMO (solid line); reaction mixtures without GA did not show H₂O₂ accumulation. Error bars show standard deviations (n = 3 independent experiments).
GtLPMO9B but was unable to drive LPMO reaction without the added H2O2. Accordingly, under the conditions used, H2O2 production in a reaction without substrate at pH 6.5 was not detected (see Fig. 6, discussed in detail below). This finding supports the notion that LPMO activity requires H2O2, which either can be generated in the reaction, as in standard reactions with AscA, or needs to be added, as in reactions with 2,3-DHBA.

Experiments with various amounts of added H2O2 showed that larger amounts led to faster initial enzyme rates and faster inactivation (Fig. 5A). Estimated initial reaction rates were 0.52 μM·min⁻¹, 0.88 μM·min⁻¹, and 1.30 μM·min⁻¹, in reaction mixtures supplied with 20, 35, and 50 μM H2O2 every 15 min, respectively. Enzyme inactivation, which is likely due to accumulation of H2O2 in solution, became visible after 60 min in the reaction with 50 μM H2O2, after 120 min in the reaction with 35 μM H2O2, and not at all (within the 240 min sampling period) in the reaction with 20 μM H2O2. In agreement with recent studies describing how LPMO activity may be controlled and maintained during cellulose saccharification by controlled addition of H2O2 (47), the present results show that keeping the H2O2 concentration at an appropriate, low concentration is essential for LPMO performance.

Figure 5B shows that, within the tested range of 2.5 to 15 μM, in reactions with 20 μM H2O2, the reductant (2,3-DHBA) concentration had a relatively modest effect on LPMO performance, including the initial rate of the reaction. When 5 or 2.5 μM 2,3-DHBA combined with 20 μM H2O2 feeds was used, 147 and 122 μM soluble

**FIG 5** Activity of GtLPMO9B with gradual addition of 2,3-dihydroxybenzoic acid (2,3-DHBA) and H2O2. The reaction mixtures were incubated at 45°C and 1,000 rpm and contained 1 μM enzyme, 50 mM bis-Tris-HCl buffer at pH 6.5, and 0.5% (wt/vol) PASC and were subjected to repetitive additions, every 15 min, starting at time zero, of 15 μM 2,3-DHBA and either 0, 20, 35 or 50 μM H2O2 (indicated in the figure) (A) or 20 μM H2O2 and either 2.5, 5 or 15 μM 2,3-DHBA (B). Solubilized oxidized products were quantified as C-1-oxidized (cellobionic acid) and C-4-oxidized (Glc4gemGlc) dimers using HPAEC-PAD, and the sum of the two was calculated. Error bars show standard deviations (n = 3 independent experiments).
oxidized products were detected after 240 min, i.e., 1.8- and 3.1-fold more than the total amount of added reductant (80 and 40 \( \mu M \), resulting from 16 additions of 5 \( \mu M \) or 2.5 \( \mu M \) each, respectively). This confirms the notion that only nonstoichiometric amounts of reductant (with respect to amounts of oxidized products) are required when the LPMO is fueled with \( \text{H}_2\text{O}_2 \). After 240 min, the cumulative \( \text{H}_2\text{O}_2 \) concentration would be 320 \( \mu M \), which would imply that in the most stable reaction depicted in Fig. 5A (15 \( \mu M \) 2,3-DHBA, 20 \( \mu M \) \( \text{H}_2\text{O}_2 \) curve), giving a product yield after 240 min of 165 \( \mu M \), 51.5% of the \( \text{H}_2\text{O}_2 \) has been converted to soluble oxidized cello-oligomers. Of note, only soluble oxidized sites were measured, whereas existing data (43, 48) indicate that in the early phases of LPMO reactions, a considerable fraction of oxidized sites may remain in the insoluble fraction. Thus, the actual level of incorporation of \( \text{H}_2\text{O}_2 \) into oxidized sites will be higher than 51.5%, and the present data are compatible with an expected 1:1 stoichiometry between the amount of added \( \text{H}_2\text{O}_2 \) and the amount of generated oxidized sites.

**The effect of pH on the LPMO–AscA/2,3-DHBA systems.** As the redox potential of 2,3-DHBA is strongly affected by pH (49), we tested the activity of GtLPMO9B (1 \( \mu M \)) with either 1 mM AscA or 2,3-DHBA at pH 6.0, 6.5 (the pH used in the experiments described above), 7.0, 8.0, and 9.0 on 0.5% PASC, in the presence of \( \text{O}_2 \) only for 24 h. The reactions were evaluated based on C-1-oxidized products only as C-4-oxidized products are unstable at alkaline pH (40). AscA was able to drive GtLPMO9B action in the full pH range of 6.0 to 9.0. On the other hand, while 2,3-DHBA was not able to drive the LPMO reaction at pH 6.5 in the absence of \( \text{H}_2\text{O}_2 \) (Fig. 5A and 6A), it was able to do so at pH 8.0 and 9.0 (Fig. 6A). Importantly, Fig. 6B shows that the pH dependency of LPMO activity (Fig. 6A) correlates with the pH dependency of the ability of the GtLPMO9B/2,3-DHBA system to generate \( \text{H}_2\text{O}_2 \); production of \( \text{H}_2\text{O}_2 \) was detected at pH 8.0 and 9.0 but not at pH 6.0 and 7.0. Of note, the same type of pH dependency applies to control reactions with \( \text{CuSO}_4 \) (Fig. 6B), albeit with lower \( \text{H}_2\text{O}_2 \) production rates than with reaction mixtures containing the LPMO.

Since the redox potential of 2,3-DHBA becomes less positive with increasing pH (49), possibly as a result of deprotonation of a phenolic hydroxyl group (49), it is conceivable that increased pH leads to an increased ability to generate \( \text{H}_2\text{O}_2 \) by LPMO-dependent or LPMO-independent reduction of \( \text{O}_2 \) (Fig. 1). Figure 6B shows that accumulation of \( \text{H}_2\text{O}_2 \) is much higher in reaction mixtures containing the LPMO and 2,3-DHBA than in control reaction mixtures lacking the enzyme. Thus, by far most of the \( \text{H}_2\text{O}_2 \) generated in the LPMO-containing reaction mixtures is generated by the LPMO and not by enzyme-independent processes, i.e., direct oxidation of 2,3-DHBA, possibly catalyzed by free copper in solution. Apparently, at pH 6 to 7, neither LPMO-dependent nor LPMO-independent \( \text{H}_2\text{O}_2 \) generation is sufficient to drive the reaction. However, at this pH, 2,3-DHBA does reduce the LPMO, allowing \( \text{H}_2\text{O}_2 \)-driven catalysis.

**Concluding remarks.** In this study, we analyzed the properties of the second of a total of four predicted family AA9 LPMOs encoded by the genome of *Gloeophyllum trabeum*, GtLPMO9B. The substrate specificity of this single-domain LPMO is similar to that of the previously characterized GtLPMO9A-2 (41).

The interplay of LPMOs with reducing compounds, reactive oxygen species, and other redox enzymes is of great current interest (2, 27, 28). We therefore used GtLPMO9B to study some of these issues, which led to the discovery of a reductant, 2,3-DHBA, which had remarkable effects on LPMO catalytic activity.

The present data indicate that, at pH 6.5, 2,3-DHBA reduces Cu(II) to Cu(I) via a single-electron reduction and that this reduced copper center, in what is now a primed LPMO, reacts with externally supplied \( \text{H}_2\text{O}_2 \) to perform catalytic cleavage of the cellulose substrate. The absence of LPMO activity in the absence of externally supplied \( \text{H}_2\text{O}_2 \) correlates with the lack of \( \text{H}_2\text{O}_2 \) generation by the 2,3-DHBA–LPMO redox system. It is remarkable that \( \text{H}_2\text{O}_2 \) production was not detected at pH 6 or 7 since a reduced LPMO [Cu(I)] will react with molecular oxygen to generate superoxide (20) and since redox potentials suggest that a reductant that is capable of reducing the LPMO (E_p of...
would also be capable of reducing superoxide to H$_2$O$_2$ ($E_0$ of +890 mV) (60). One explanation could be that the superoxide remains bound to the now-oxidized copper ion [Cu(II)], which would strongly affect its redox potential.

We would argue that the present results support the notion that H$_2$O$_2$ is the natural substrate for LPMOs (18), but it must be noted that the option that LPMOs can use molecular oxygen directly cannot be dismissed (24, 50). Interestingly, regardless of the true nature of the oxygen cosubstrate, the present data show that GtLPMO9B at pH 6 to 7, with 2,3-DHBA acting as electron donor, is not able to reduce this cosubstrate to a species powerful enough to carry out hydrogen abstraction from the polysaccharide substrate. Use of 2,3-DHBA enables good control of LPMO activity, and we expect that this reductant will be useful in future LPMO studies. Preliminary studies with 2,3-DHBA and another LPMO, C-4-oxidizing NcLPMO9C from Neurospora crassa, gave results similar to those described above (Fig. S3). It is worth noting that, in contrast to use of AscA, e.g., use of 2,3-DHBA may allow reducing the LPMO without creating conditions that lead to enzyme inactivation, and this can be done under aerobic conditions.

**MATERIALS AND METHODS**

**Cloning and expression of GtLPMO9B.** The gene encoding GtLPMO9B (UniProt accession number S7RK00) including its native signal sequence was codon optimized for Pichia pastoris (GenScript, NJ, USA). The synthetic gene was inserted into the pPink-GAP vector, which was then transformed into *P. pastoris*.
PichiaPink strain 4 cells (Invitrogen, CA, USA), as described earlier (51). Transformants were screened for protein production in BMGY medium (containing 1% [vol/vol] glycerol).

The best-producing transformant was grown in 25 mL of buffered minimal glycerol complex (BMGY) medium (containing 1% [vol/vol] glycerol) overnight in a 100-mL shake flask at 29°C and 200 rpm. The culture was then used to inoculate 500 mL of BMGY medium (containing 1% [vol/vol] glycerol) in a 2-L baffled shake flask. The culture was inoculated at 29°C and 200 rpm for 48 h. After 24 h of incubation the medium was resupplemented with 1% (vol/vol) glycerol. After 48 h the culture was centrifuged at 4°C and 10,000 × g for 10 min to remove the cells. The supernatant was filtered through a 0.2-μm-pore-size polyethersulfone membrane (Millipore, MA, USA), dialyzed against 50 mM bis-Tris-HCl buffer (pH 6.5), and concentrated to 100 mL with a VivaFlow 50 tangential crossflow concentrator (molecular weight cutoff (MWCO) of 10 kDa; Sartorius Stedim Biotech, Germany).

**Purification of recombinant protein.** The recombinant GtLPMO9B protein was purified in a two-step protocol using hydrophobic interaction chromatography (HIC) followed by size exclusion chromatography (SEC), as described previously (52). Purified GtLPMO9B was copper saturated by incubating the enzyme with an excess of Cu(II)SO4 (at −3:1 molar ratio of copper/enzyme) for 30 min at room temperature as described previously (53). The Cu(II)-loaded sample of GtLPMO9B was buffer exchanged to 50 mM bis-Tris-HCl buffer (pH 6.5), using Amicon Ultra centrifugal filters (MWCO of 3 kDa; Merck Millipore, Burlington, MA, USA). The resulting protein solution was filtered through a sterile 0.22-μm-pore-size Millipore GV filter (Merck Millipore, Burlington, MA, USA) and stored at 4°C. Protein concentrations were determined by measuring absorbance at 280 nm in a spectrophotometer, calculating the concentration based on the extinction coefficient (ε = 55,475 M⁻¹cm⁻¹; calculated using ProtParam (54)).

**Screening of substrates and reductants.** Activity of GtLPMO9B was tested on the following substrates: phosphoric acid-swollen cellulose (PASC) prepared from Avicel as described previously (55), konjac glucomannan, galactomannan, ivory nut mannan, xyloglucan from tamarind seed, lichenan from Icelandic moss, wheat arabinoxylan, and barley beta-glucan, all purchased from Megazyme (Bray, Ireland), and birchwood xylan, obtained from Sigma-Aldrich (St. Louis, MO, USA). Substrate specificity was tested by setting up reaction mixtures (100 μL) containing 0.2% (wt/vol) substrate, 1 μM GtLPMO9B, and 1 mM ascorbic acid (AscA) in 50 mM bis-Tris-HCl buffer, pH 6.5. The samples were incubated in an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany) at 45°C and 1,000 rpm for 24 h. Control reactions were performed in the absence of ascorbic acid. Reactions were stopped by separating the soluble fractions from the insoluble substrates by filtration using a 96-well filter plate (Millipore) operated with a vacuum manifold.

Activity assays with PASC, under the conditions described above, were also done with the following alternative reducing agents: gallic acid, pyrogallol, caffeic acid, catechol, hydroquinone, coniferyl alcohol, 2,3-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, vanillic acid, guaiacol, veratryl alcohol, 2,4-hexadiene-1-ol, and 4-hydroxybenzoic acid. All reducing agents were purchased from Sigma-Aldrich.

**GtLPMO9B activity on PASC: time series and quantification of oxidized products.** Reaction mixtures (600 μL) contained 1 μM GtLPMO9B, 0.2% (wt/vol) PASC, and 1 mM reductant in 50 mM bis-Tris-HCl buffer, pH 6.5. The reaction mixtures were incubated in an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany) at 45°C and 1,000 rpm. At various time points (20, 40, 60, 120, and 240 min), 100-μL samples were taken and boiled for 5 min. For gallic acid, a final sample was taken after 24 h. Separation of soluble and insoluble material was achieved by centrifugation at 11,000 × g for 10 min. The soluble fractions (25 μL) were mixed with 24 μL of 150 mM sodium-acetate buffer (pH 4.5) and 1 μL of TrCel7A from Trichoderma reesei (≈1 μM) and incubated for 24 h at 37°C. The treatment with TrCel7A converts soluble oxidized cello-oligomers to cellobiotic acid (C-1 oxidized) and Glc4gemGlc (C-4 oxidized). Soluble fractions treated in this way were subsequently analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The amount of released oxidized products was quantified using the following standards: 4-hydroxy-β-D-xyl-Hexp-(1→4)-β-D-Glcp (Glc4gemGlc), prepared as described previously (56), and cellobionic acid, prepared by treating cellobiotic acid with cellobiose dehydrogenase from Myriococcum thermophilum (MCDH) (57). All experiments were performed in triplicate.

**The effect of pH on GtLPMO9B activity.** GtLPMO9B (1 μM) was incubated with 0.5% (wt/vol) PASC in 50 mM buffer solution with 1 mM reductant in a final volume of 100 μL. For the reaction mixtures at pH 6.0, 6.5, or 7.0, 50 mM bis-Tris-HCl was used as the buffering agent, while 50 mM Tris-HCl was used for the reaction mixtures at pH 8.0 or 9.0. The samples were incubated in an Eppendorf ThermoMixer at 45°C and 1,000 rpm for 24 h. The reactions were stopped by boiling for 5 min. Samples were hydrolyzed with TrCel7A as previously described, and oxidized product was quantified as cellobionic acid. All reactions were performed in triplicate.

H2O2 as cosubstrate. In reaction mixtures with added H2O2, 1 μM GtLPMO9B was mixed with 0.5% (wt/vol) PASC in 50 mM bis-Tris-HCl buffer (pH 6.5), and various initial concentrations of reductant and H2O2 (2.5 and 20, 5 and 35, or 15 and 50 μM, respectively) were added. GtLPMO9B was premixed with PASC in the buffer (588 μL), after which we added first 6 μL of freshly prepared reductant solution with a concentration of 0.25, 0.5, or 1.5 mM reductant and then 6 μL of a freshly prepared 2,3,5, or 5 mM H2O2 stock solution. Reductant and H2O2 in that order, were subsequently added to the reaction mixtures every 15 min for a total period of 240 min. Added volumes were adapted to the changes in total reaction volume that were due to previous additions and sampling so that the added amounts corresponded to final concentrations identical to the concentrations at time (t) 0. Reactions were stopped by boiling and freezing (−20°C) prior to further analysis. The amount of released oxidized product was quantified as...
H$_2$O$_2$ production in the absence of substrate at different pHs. The production of H$_2$O$_2$ by GLtPMO98 at different pHs in the absence of substrate was monitored using an assay with Amplex Red and horseradish peroxidase (HRP), both purchased from Sigma-Aldrich (St. Louis, USA), according to the method of Kitt et al. (36). In brief, 100-μl reaction mixtures were prepared in 96-well microtiter plates containing 1 μM GLtPMO98, 5 U of horseradish peroxidase, 50 μM Amplex Red, and 30 μM reductant in the appropriate buffer. The reactions were initiated by addition of the reductant, followed by real-time measurement of absorbance at 540 nm for 120 min in a Thermo Scientific Multiscan FC microplate reader at room temperature. For pH 6.0, 6.5, and 7.0, 50 mM bis-Tris-HCl buffer was used, while at pH 8.0 and 9.0, 50 mM Tris-HCl buffer was used. In control reaction mixtures, 1 μM GLtPMO98 was replaced by either 1 μM CuSO$_4$ or water. Standard curves for quantification of H$_2$O$_2$ were made in the concentration range of 0.5 to 20 μM, at the pHs used in the experiment. All reactions were performed in triplicate.

H$_2$O$_2$ scavenging by reductants. Reductants (AscA, GA, and 2,3-DHBA) were incubated with H$_2$O$_2$ at a 1:1 molar ratio (30 μM) in 50 mM bis-Tris-HCl buffer, pH 6.5, for 60 min at room temperature. In the control reaction, reductant was replaced with water. H$_2$O$_2$ concentrations were determined by real-time measurement of absorbance at 540 nm for 120 min in a Thermo Scientific Multiscan FC microplate reader at room temperature. For pH 6.0, 6.5, and 7.0, 50 mM bis-Tris-HCl buffer was used, while at pH 8.0 and 9.0, 50 mM Tris-HCl buffer was used. In control reaction mixtures, 1 μM GLtPMO98 was replaced by either 1 μM CuSO$_4$ or water. Standard curves for quantification of H$_2$O$_2$ were made in the concentration range of 0.5 to 20 μM, at the pHs used in the experiment. All reactions were performed in triplicate.

Detection of oxidized products. Oxidized products were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). HPAEC was performed on a Dionex ICS5000 system, equipped with a CarboPac PA1 analytical column (2 by 250 mm) and a CarboPac PA1 guard column (2 by 50 mm), using a 50-min gradient (58) for hemiacetalic and hemi-etheric substrates. Chromatograms were recorded and analyzed using Chromeleon, version 7.0, softwar (Thermo Fisher Scientific, Waltham, MA, USA).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02612-18.

**SUPPLEMENTAL FILE 1** PDF file, 1.6 MB.

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