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3 **Fenton-type chemistry by a copper enzyme:**
4 **molecular mechanism of polysaccharide oxidative cleavage**
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17 **Abstract:** The discovery of Lytic Polysaccharide Monooxygenases (LPMOs) has been
18 instrumental for the development of economically sustainable lignocellulose biorefineries.
19 Despite the obvious importance of these exceptionally powerful redox enzymes, their mode of
20 action remains enigmatic and their activity and stability under process conditions are hard to
21 control. By using enzyme assays, mass spectrometry and experiments with labeled oxygen
22 atoms, we show that H₂O₂, and not O₂ as previously thought, is the co-substrate of LPMOs. By
23 controlling H₂O₂ supply, stable reaction kinetics and high enzymatic rates are achieved, the
24 LPMOs work under anaerobic conditions, and the need for adding stoichiometric amounts of
25 reductants is alleviated. These results offer completely new perspectives regarding the mode of
26 action of these unique mono-copper enzymes, the enzymatic conversion of biomass in Nature,
27 and industrial biorefining.
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30 **Keywords:** biomass, monooxygenase, copper enzyme, hydrogen peroxide, cellulose, chitin
31

32 **Abbreviations:** ascorbic acid (AscA); cellobiose dehydrogenase (CDH); chlorophyllin (Chl); glucose-
33 methanol-choline (GMC) oxidoreductase; glycoside hydrolase (GH); hydrogen atom abstraction (HAA);
34 lytic polysaccharide monooxygenases (LPMO); particulate methane monooxygenases (pMMO);
35 superoxide (O₂^{•-}); superoxide dismutase (SOD); xanthine (XTH); xanthine oxidase (XOD).
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38 The depolymerization of complex plant biomass, primarily composed of cellulose,
39 various hemicelluloses and lignin, relies on a network of enzymatic and chemical reactions that
40 is still full of mysteries. Until recently, the degradation of the recalcitrant polysaccharides in
41 plant biomass was thought to be achieved by an arsenal of hydrolytic enzymes called glycoside
42 hydrolases (GHs) (1). In some ecosystems, the enzymatic deconstruction process is supported by
43 Fenton chemistry, i.e. transition metal-driven *in situ* generation of H₂O₂-derived hydroxyl
44 radicals, one of the most powerful oxidizing species found on Earth (2), which can oxidize both
45 polysaccharides and lignin in plant biomass (3). In 2010, a new class of enzymes was discovered,
46 which carry out oxidative cleavage of polysaccharides (4). These enzymes, today known as lytic
47 polysaccharide monooxygenases (LPMOs) (5), are single-copper redox enzymes (6, 7), that can
48 hydroxylate the C1 or C4 positions of scissile glycosidic bonds (4, 8–10).

49 Despite their abundance in Nature (5, 11) and their obvious industrial importance, for
50 example in the production of cellulosic ethanol (12), the mode of action of LPMOs remains
51 enigmatic, although some catalytic mechanisms have been proposed (7, 8, 10, 13, 14). It is well-
52 established that one LPMO reaction cycle requires the recruitment of two electrons (4, 14–16).
53 The first electron is often thought to be acquired via reduction of the LPMO's Cu(II) center to
54 Cu(I) (11). When and how oxygen and the second electron are recruited remains an enigma. It
55 appears impossible that an electron provider such as cellobiose dehydrogenase (CDH) (11)
56 carries out direct reduction of the active site copper while the LPMO is bound to the substrate,
57 whereas it is unlikely that the protein unbinds during catalysis to allow such a direct second
58 reduction step. The existence of an internal electron channel that would allow electron delivery
59 to a substrate-bound enzyme has therefore been postulated (10, 17, 18).

60 Interestingly, a recent study has shown that unprecedented high levels of LPMO activity
61 may be obtained when the enzyme is exposed to visible light in the presence of chlorophyllin
62 (Chl) and ascorbic acid (AscA) (19). Although this study fell short of mechanistic explanations,
63 the effect was attributed to the generation of high-energy electrons provided by photoexcited
64 Chl, with AscA regenerating the Chl. From the increasing amount of publicly available data it
65 appears clear that LPMO catalytic rates are indeed dependent on the nature of the redox partner
66 (11, 20), which is intriguing, since it has been shown that the rate of LPMO reduction in solution
67 is much higher (11, 16) than reported overall rates for LPMO action (4, 14, 21). These
68 observations made us postulate that a chemical species, common to all known reaction systems

69 but accumulating at different rates, plays an unsuspected key role in the LPMO mechanism.
70 Looking for a potential culprit for LPMO activity, we studied the Chl/light, Chl/light-AscA and
71 AscA systems for LPMO activation. A bacterial C1-specific cellulose-active LPMO10 from
72 *Streptomyces coelicolor* (ScLPMO10C) was used as primary model enzyme.

73 When using the Chl/light-AscA system, with relatively high light intensities, a strong
74 increase in LPMO activity was indeed observed, notably accompanied by an almost immediate
75 inactivation of the enzyme (**Fig. 1A**). Since light-exposed chlorophyll may produce superoxide
76 ($O_2^{\bullet-}$) (22), we investigated whether addition of superoxide dismutase (SOD) or superoxide-
77 consuming chemicals to the Chl/light-AscA system would allow better control of the reaction,
78 which turned out not to be the case (**Fig. S1**). On the other hand, we found that both the catalytic
79 rate and apparent inactivation of the enzyme could be modulated by varying the amount of AscA
80 (**Fig. 1A; Fig. S2**) or the light intensity (**Fig. S3**). Interestingly, in the absence of AscA, the
81 Chl/light system yielded good LPMO activity and apparent inactivation of the enzyme was much
82 reduced, as illustrated by a more linear progress curve for LPMO activity (**Fig. 1A**). Under these
83 latter conditions, low concentrations of SOD were beneficial for LPMO activity, whereas high
84 concentrations of SOD were detrimental due to rapid inactivation of the enzyme (**Fig. 1A; Fig.**
85 **S4**). These results show that the levels of superoxide and/or the products of SOD, O_2 and H_2O_2 ,
86 affect LPMO activity.

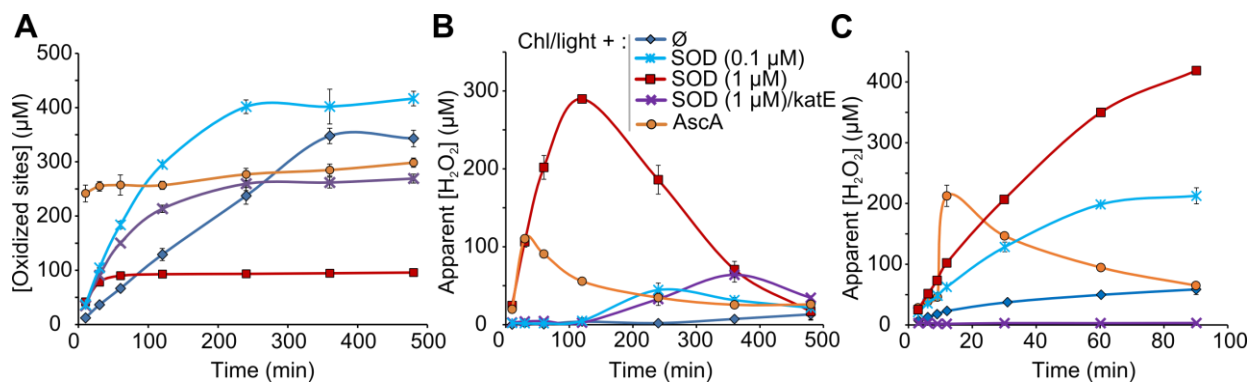
87 **Figure 1C** shows that, in the absence of an LPMO, the Chl/light system produces H_2O_2
88 and that production is strongly increased by adding SOD, which enzymatically converts
89 superoxide to H_2O_2 , or AscA, which chemically reduces superoxide to H_2O_2 (**Fig. S5**). These
90 H_2O_2 production levels in the absence of the LPMO (**Fig. 1C**) correlate well with the initial rates
91 observed in the LPMO reactions (**Fig. 1A**). Moreover, rapid enzyme inactivation in the LPMO
92 reactions (**Fig. 1A**) correlates with the H_2O_2 production potential (**Fig. 1C**) of the system used
93 and is associated with accumulation of H_2O_2 in the reaction mixture (**Fig. 1B**). Notably, in the
94 control reaction with only Chl/light, yielding relatively stable reaction kinetics (**Fig. 1A**),
95 accumulation of H_2O_2 was not observed (**Fig. 1B**), whereas the Chl/light system does produce
96 H_2O_2 (**Fig. 1C**). Addition of catalase reduced the detrimental effect of adding high amounts of
97 SOD, reflected in slower inactivation of the LPMO (**Fig. 1A**) and reduced accumulation of H_2O_2
98 (**Fig. 1B**). All together, these results suggest that H_2O_2 is an unsuspected co-substrate for LPMOs
99 and that too high levels of H_2O_2 are detrimental. The high initial LPMO rate observed when

100 using the Chl/light+AscA system (**Fig. 1A**) is likely related to fast H₂O₂ production (up to 200
101 μ M within the 12 first minutes of the reaction; **Fig. 1C**), which leads to rapid inactivation of the
102 enzyme.

103 Control reactions with only AscA, well known for its ability to drive LPMO activity,
104 yielded more modest H₂O₂ levels (< 40 μ M within the first 60 min, **Fig. S6C**), which is likely
105 related to AscA being less capable of engaging in the thermodynamically challenging reduction
106 of O₂, compared to Chl/light. Reactions similar to those in **Fig. 1** but only using AscA generally
107 yielded less clear results (**Fig. S6A&B**), which is likely due to the many possible redox reactions
108 involving AscA, superoxide and H₂O₂ (**Fig S5**). However, the same overall trend stood out: both
109 higher LPMO activity and faster apparent enzyme inactivation were correlated with higher H₂O₂
110 levels.

111 Reactions with the Chl/light system (i.e. no AscA) seemingly lack a reductant needed to
112 reduce the LPMO copper, which led us to speculate that O₂^{•-} could be involved in LPMO
113 reduction (**pathway (iv) Fig. S5**). Indeed, chemical (KO₂) or enzymatic (xanthine/xanthine
114 oxidase) O₂^{•-} generating systems could drive LPMO activity, albeit at low levels (**Fig. S7**).
115 Control experiments without any reductant but with exogenous H₂O₂ did not lead to cellulose
116 oxidation (**Fig. S8**). This latter observation (**Fig. S8**) indicates that only the reduced LPMO can
117 react with H₂O₂ and is crucial for the discussions below.

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120 **Fig. 1. LPMO activity and apparent hydrogen peroxide production when using the**
121 **Chl/light system for driving the reaction.** Panels A and B show time-courses for the release of
122 aldonic acid products (A) and H₂O₂ levels (B) upon incubating Avicel (10 g.L⁻¹) with
123 ScLPMO10C (0.5 μ M). Reactions were carried out in sodium phosphate buffer (50 mM, pH 7.0)
124 at 40 °C, under magnetic stirring, with Chl (500 μ M) exposed to visible light ($I = 25\% I_{\max}$,
125 approx. 42 W.cm⁻²). Note that, compared to the previous study by Cannella et al. (19), we used
126 higher light intensities, which likely explains why in our hands, the Chl/light system also works
127 in the absence of a reductant (blue diamonds). Reaction conditions varied in terms of the
128 presence of SOD (0.1 or 1 μ M), a catalase, katE (10 μ g.mL⁻¹), or AscA (1 mM). **Panel C** shows

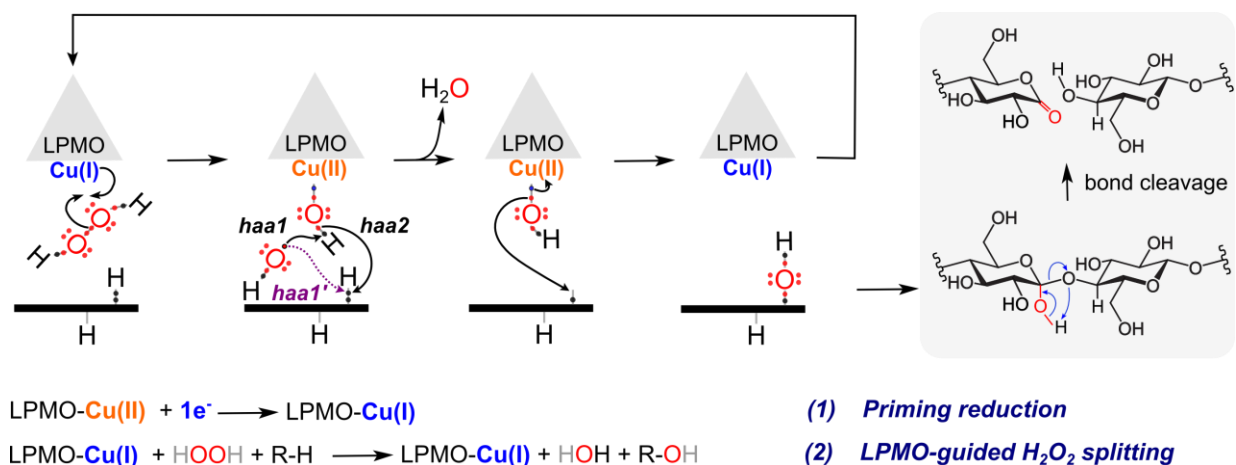
129 the apparent production of H₂O₂ in the absence of ScLPMO10C, with all other conditions being
130 the same as for Panels A & B. Control reactions in the dark did not yield detectable levels of
131 oxidized products (**Fig. S3A**), with the exception of reactions with AscA (**Fig. S6**). The legend
132 code displayed in panel B applies also to panels A and C. Error bars show \pm s.d. (n = 3).
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134 To determine the role of H₂O₂, we then analyzed initial LPMO rates in the presence of a
135 reductant and varying concentrations of exogenous H₂O₂ (**Fig. S9**). A spectacular increase in
136 initial LPMO rates was observed at the lower H₂O₂ concentrations, with up to 26-fold more
137 soluble oxidized products being released from Avicel by ScLPMO10C after 2 minutes when
138 incubated in the presence of 200 μ M H₂O₂ (**Fig. S9C**). This increase in activity is in the same
139 order of magnitude as the increases reported for the Chl/light+AscA system (**Fig. S3E** and (19)).
140 At higher H₂O₂ concentrations, the LPMO reactions stopped very rapidly. Exogenous H₂O₂
141 affected the activity of a fungal LPMO9 from *Phanerochaete chrysosporium* K-3 (PcLPMO9D)
142 (**Fig. S9D-F**), another type of cellulose-active bacterial LPMO10, ScLPMO10B (**Fig. S9G-I**),
143 and a chitin-active LPMO10, CBP21 (**Fig. S9K-L**), in a similar manner, but significant
144 differences were observed in terms of the degree of activity enhancement and the sensitivity to
145 H₂O₂ (note that the rate enhancement for CBP21 is >100-fold; **Fig. S9L**) Control reactions in
146 which the enzyme was replaced by Cu(II)SO₄ did not show any oxidized products (**Fig. S10**).

147 The results described above suggest a catalytic mechanism in which an H₂O₂-derived
148 oxygen atom, rather than an O₂-derived oxygen atom, would be introduced into the
149 polysaccharide chain. In the proposed mechanism (**Fig. 2**), a priming reduction of the LPMO-
150 Cu(II) to LPMO-Cu(I) occurs first. H₂O₂ would then bind to the Cu(I) center and homolytic bond
151 cleavage, similar to what happens during Fenton chemistry, would produce a hydroxyl radical.
152 This likely leads to formation of a Cu(II)-hydroxide intermediate and a substrate radical by one
153 of several possible pathways (**Fig. S11**). In each of these mechanisms, the reaction between a
154 copper-hydroxyl intermediate and the substrate radical leads to hydroxylation of the substrate
155 and to regeneration of the Cu(I) center, which can enter a new catalytic cycle.

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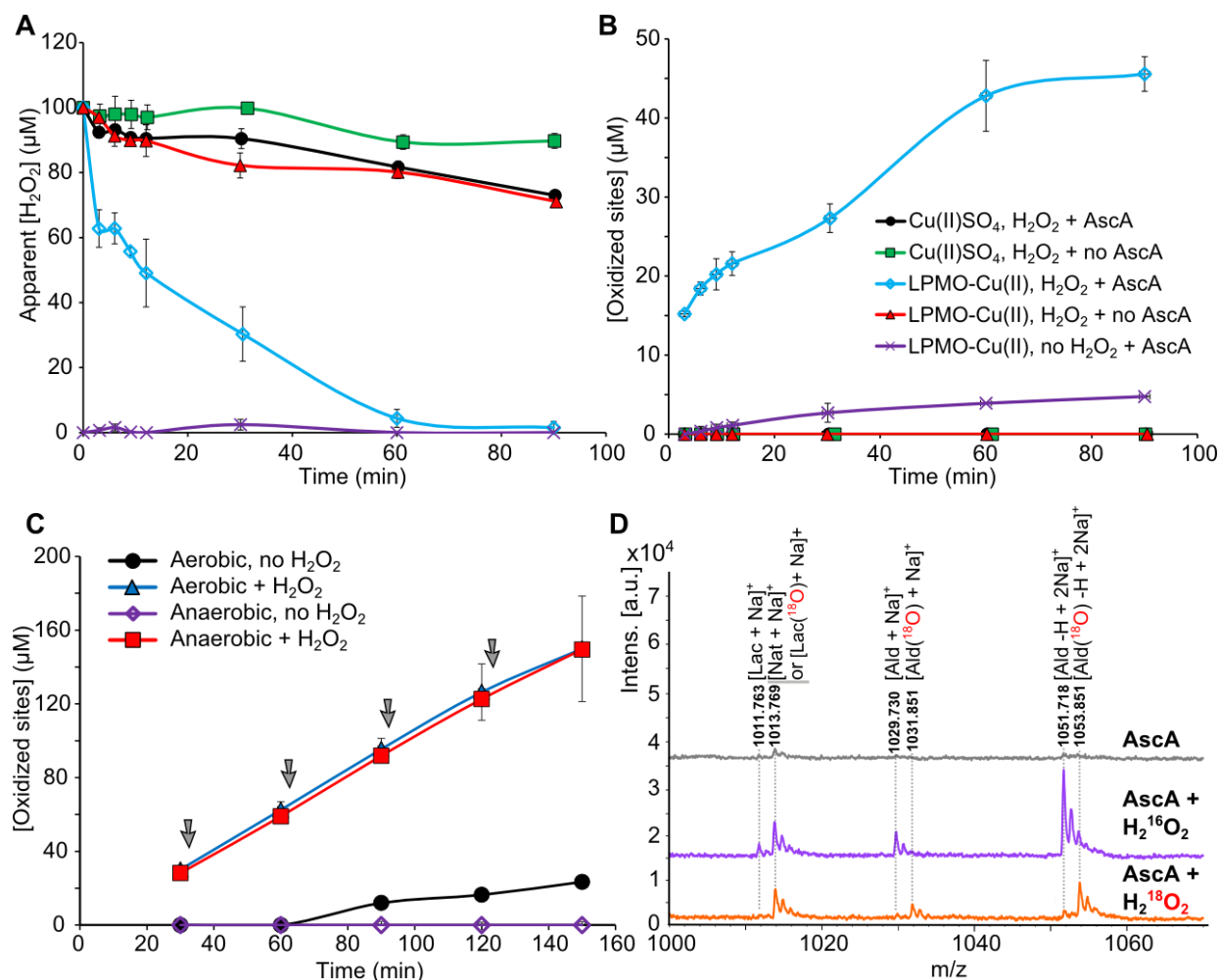
160 **Fig. 2. Proposed LPMO-guided H₂O₂ splitting mechanism for enzymatic oxidative cleavage**
 161 **of polysaccharides.** In the proposed mechanism, LPMO-Cu(II) is first reduced to LPMO-Cu(I)
 162 (“priming reduction”), followed by H₂O₂ binding and homolytic bond cleavage. This cleavage
 163 leads to the Fenton-like generation of a hydroxyl radical, catalyzing HAA either from the Cu(II)-
 164 hydroxide (haa1) or from the substrate (haa1’). The former scenario would generate a copper-
 165 oxyl intermediate that can abstract a hydrogen atom from the substrate (haa2). In both scenarios
 166 a water molecule is eliminated and attack of the Cu(II)-hydroxide on the substrate radical leads
 167 to hydroxylation of the substrate and to regeneration of the Cu(I) center, which can enter a new
 168 catalytic cycle. The resulting hydroxylated polysaccharide undergoes molecular rearrangement
 169 leading to lactone formation and bond cleavage (15). The previously proposed reaction scheme
 170 for an O₂-dependent reaction is: $\text{LPMO-Cu(II)} + \text{O}_2 + \text{R-H} + 2e^- + 2\text{H}^+ \rightarrow \text{LPMO-Cu(II)} + \text{H}_2\text{O}$
 171 $+ \text{R-OH}$. See **Fig. S11** for further details.

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173 To test this pathway and obtain final proof of H₂O₂ being the catalytically relevant co-
 174 substrate of LPMOs, additional experiments were carried out (**Fig. 3**). **Figures 3A&B** show that
 175 LPMO-dependent consumption of H₂O₂ (**Fig. 3A**) correlates with the release of oxidized
 176 products (**Fig. 3B**). Importantly, these experiments were done using catalytic (rather than
 177 putatively stoichiometric) amounts of reductant (10 μM; i.e. 100 times lower than commonly
 178 used concentrations; **Fig. S12**) to assess the concept of a “priming reduction” and to reduce the
 179 effect of AscA on H₂O₂ stability (**Fig. S13**). **Fig. 3B** shows that product levels are much higher
 180 than the total amount of AscA added. This is in agreement with the proposed mechanism in
 181 which a reduced LPMO can catalyze several reactions provided that the co-substrate, H₂O₂, is
 182 supplied. Analogous results were obtained when using a glucose oxidase from *Aspergillus niger*
 183 (*AnGOX*), for controlled *in situ* generation of H₂O₂. **Fig. S14** shows that the glucose/*AnGOX*
 184 system boosts *ScLPMO10C* activity in a dose-dependent manner, but only if the LPMO is
 185 reduced by a reductant added in small amounts (**Fig. S14**).

186 As a consequence of the above findings, LPMOs should be able to work under anaerobic
187 conditions, which indeed was observed (**Fig. 3C**; **Fig. S15**). **Fig. 3C** shows that stable kinetics
188 are obtained by adding H₂O₂ and reducing equivalents gradually to the reaction mixture and that
189 the reaction rate is independent of the presence of O₂. Finally, experiments with a labeled co-
190 substrate, H₂¹⁸O₂, showed that indeed, the oxygen introduced into the polysaccharide chain
191 comes from H₂O₂ and not from O₂ (**Figs. 3D, S16-S18**). For example, **Fig. 3D** shows that when
192 using H₂¹⁸O₂, the characteristic peaks for sodium adducts of the aldonic acid form of an oxidized
193 cellohexaose (*m/z* 1029.7 & 1051.7) shifted by +2 Da. Similar observations were made for the
194 chitin-active LPMO10 CBP21 (**Fig. S16**) and a fungal cellulose-active LPMO9 (**Fig. S17**).
195 Reactions with lower concentrations of H₂¹⁸O₂ showed that even in the presence of a 10-fold
196 surplus of ¹⁶O₂, the oxidized products carry ¹⁸O (**Fig. S18**). Finally, a competition experiment
197 with a peroxidase and an LPMO showed that the peroxidase completely inhibited LPMO
198 activity, despite the presence of O₂ and reducing power (AscA or lactose/CDH; **Fig. S19**).
199 Altogether, the experiments depicted in **Fig. 3** and **Figs S15-S19** unequivocally show that H₂O₂
200 is the catalytically relevant co-substrate for LPMO-catalyzed oxidation of a polysaccharide.

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Fig. 3. Probing the H₂O₂-dependent pathway of LPMOs. Panels A and B show H₂O₂ consumption (A) and soluble product formation (B) during incubation of *Sc*LPMO10C-Cu(II) and Avicel in the presence or absence of initial exogenous H₂O₂ (100 μM). In control reactions *Sc*LPMO10C-Cu(II) was replaced by Cu(II)SO₄ (0.5 μM). The reaction was initiated by addition of AscA (10 μM) where indicated. Note that this is a very low AscA concentration, meant to test the “priming reduction” hypothesis (see text for details). The legend code for panels A and B is indicated in the latter. Note that not all LPMO products are soluble, explaining why the levels of consumed H₂O₂ and detected products are not identical. (C) The graphs show time-courses for release of aldonic acid products by *Sc*LPMO10C from Avicel under anaerobic or aerobic conditions at 30 °C. All the reactions were initiated by addition of AscA (10 μM) supplemented with H₂O₂ (50 μM) when indicated. AscA and H₂O₂ additions were repeated right after sampling, i.e. every 30 minutes (grey arrows). (D) MALDI-TOF MS spectra of products obtained after 4 min reaction in the presence of 100 μM H₂¹⁶O₂ or H₂¹⁸O₂, as indicated, and 1 mM AscA. The spectrum shows the hexose cluster, showing sodium adducts of the native (Nat) hexose, and the two forms of the oxidized hexose, the lactone (Lac) and the aldonic acid (Ald). All reactions (panel A to D) were carried out with *Sc*LPMO10C-Cu(II) (0.5 μM) and Avicel (10 g.L⁻¹) in sodium phosphate buffer (50 mM, pH 7.0) at 40 °C under magnetic stirring (unless stated otherwise). The error bars show s.d. (n = 3).

223 Several of the reaction progress curves discussed above show that LPMOs are readily
 224 inactivated and under some conditions, such as when using the Chl/light-AscA system (**Fig. 1A**,
 225 **Fig. S3D**), inactivation seems to occur within a few minutes. Enzyme inactivation was confirmed
 226 by a series of experiments where the LPMO was pre-incubated and then tested for remaining
 227 activity (**Fig. S20**). Enzyme inactivation was similar in the presence of EDTA, showing that
 228 inactivation is not due to free metal-catalyzed generation of hydroxyl radicals. Importantly,
 229 inactivation was partly avoided by the presence of substrate (**Fig. S20**). Using proteomics
 230 technologies, we found that the inactivated LPMO had undergone several oxidative
 231 modifications that were confined to the catalytic histidines and, to a lesser extent, neighboring
 232 residues (**Figs. 4, S21, S22**). Other residues prone to oxidative damage, such as surface exposed
 233 residues in the LPMO domain, the linker or the CBM were not modified (**Fig. S23**). This leads to
 234 the important conclusion that oxidative damage is not caused by ROS in solution, as has been
 235 suggested (23), but by ROS generated in the catalytic center, i.e. *in situ*, by enzyme-generated
 236 hydroxyl radicals with diffusion-limited timescale reactivity. The protective effect of the
 237 substrate (**Fig. S20, S24**), was reflected in reduced oxidative damage of the N-terminal catalytic
 238 histidine (**Fig. 4B**). The higher sensitivity of the N-terminal histidine may be related to the
 239 orientation of the reactive oxygen species during catalysis.

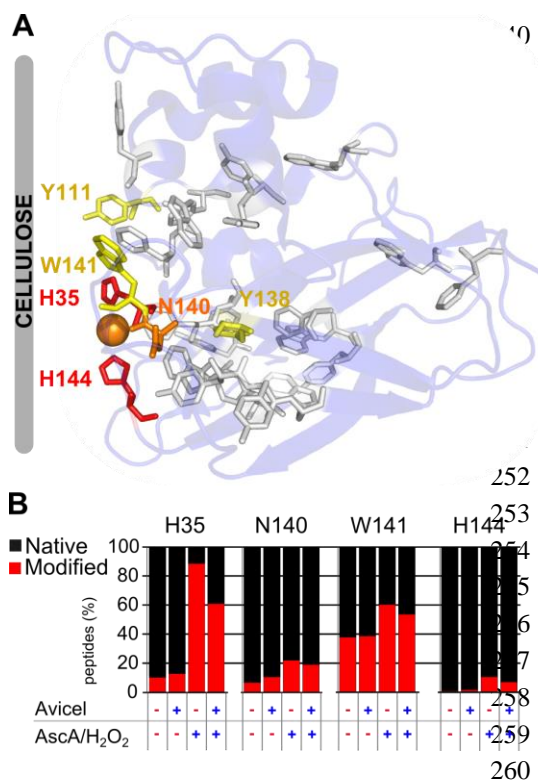


Fig. 4. LPMO self-oxidation and the protective role of the substrate. (A) Mapping of modified residues on the structure of the catalytic domain of ScLPMO10C (PDB 4OY7 (24)) reveals that oxidation occurs in and near the active site, predominantly on the catalytic histidines, H35 and H144. The color code highlights the degree of oxidation: high (red), middle (orange) and low (yellow). For aromatic residues shown as grey sticks no modification was detected (See **Fig. S21-S23**). (B) Impact of substrate on the ratio of modified/native peptides bearing H35, N140, W141 or H144 after a short incubation (i.e. a less drastic treatment compared to panel A). ScLPMO10C (1 μ M) was pre-treated by 20 min incubation in sodium phosphate buffer (50 mM, pH 7.0) at 40 °C under magnetic stirring, in the presence (10 g.L⁻¹) or absence of Avicel and addition of either AscA (1mM)/H₂O₂ (100 μ M) or simply water (control reaction). (See **Fig. S24** for corresponding activity tests).

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262 The present findings unequivocally show that H₂O₂, and not O₂, is the catalytically
263 relevant co-substrate of LPMOs, implying that they should perhaps be called peroxygenases (or
264 LPPO). Basically, LPMOs, after a priming reduction, carry out Fenton-type chemistry in a
265 controlled and substrate-associated manner. From a biological point of view, such a scenario
266 makes more sense than the seemingly somewhat random generation of dangerous hydroxyl
267 radicals in the classical Fenton concept. Although the use of H₂O₂ by redox enzymes (e.g.
268 peroxidases) is well-known, to the best of our knowledge, the biochemistry of the LPMO
269 reaction as unraveled in this study, is unprecedented in Nature (25, 26).

270 LPMOs were initially classified as monooxygenases because experiments with ¹⁸O₂ and
271 using AscA as reductant, showed that one labeled oxygen atom was incorporated in the oxidized
272 product (4). The proposed monooxygenase mechanism is well known (26, 27), seems “logical”,
273 and has not been challenged until now. Strictly spoken, however, the seminal 2010 experiment
274 with labeled oxygen did not show that O₂ is the catalytically relevant co-substrate. In fact, other
275 reactive oxygen species generated from O₂ may have been the co-substrate, including H₂O₂,
276 which indeed is produced under the conditions used (as shown in Fig. S6C). Importantly, the
277 H₂O₂-pathway described here should not be confused with the “peroxide shunt” pathway that has
278 been described for several monooxygenase reactions carried out by binuclear iron/copper
279 enzymes, non-coupled binuclear copper enzymes and mononuclear iron enzymes containing
280 additional co-factors such as porphyrin or biopterin. Such a “peroxide shunt” normally refers to a
281 slow, rather artificial reaction that requires high concentrations of H₂O₂ (10-100 mM) and that is
282 sometimes harnessed to avoid the use of reductants and O₂ (28, 29). These shunt pathways
283 involve the oxidized resting state of the enzyme and tend to lead to unstable reactions with a
284 limited number of turnovers. The situation for LPMOs is very different and truly unique. LPMOs
285 are mono-copper enzymes with no other co-factors, that, after an essential priming reduction,
286 display stable reaction kinetics with multiple turnovers at low (sub mM) H₂O₂ concentrations.

287 Our findings explain several hitherto unexplained phenomena in LPMO biochemistry: (i)
288 The consecutive delivery of two external electrons to the catalytic center is difficult to envisage,
289 but with H₂O₂ being the co-substrate, recruitment of two electrons is not needed. (ii) The widely
290 observed non-linearity of process kinetics is partly due to the self-inactivation of the LPMOs.
291 (iii) The fact that most published catalytic rates for LPMOs are low and similar, and, most
292 remarkably, independent of the LPMO or the substrate used (4, 14, 21), is likely due to the fact

293 that the rate-limiting factor in most experiments was H₂O₂ formation. This point is well
294 illustrated by a recent study demonstrating that the rate of a chitin-active LPMO fueled by the
295 lactose/CDH system and the rate of H₂O₂ production by the latter system (in the absence of an
296 LPMO) are similar (16). (iv) The increase in LPMO rate observed by Cannella et al. in their
297 study on light-activation of LPMOs is due to production of hydrogen peroxide, not to the
298 generation of some sort of “high energy electron” (19). (v) The observation that dehydrogenases
299 can drive LPMO activity while strict oxidases cannot ((30) & Fig. S14) is due to the fact that,
300 while both these enzyme types can produce H₂O₂, only the former can reduce the LPMO (11).

301 As to the level of H₂O₂ under reaction conditions, it is important to note that,
302 notwithstanding the current findings, LPMOs are capable of activating molecular oxygen, albeit
303 at low apparent rate (8, 31). It is well known that LPMOs generate H₂O₂ in the absence of
304 substrate, which leads to the remarkable conclusion that LPMOs can generate their own co-
305 substrate from O₂. This property may have biological implications since H₂O₂ generated by
306 unbound enzymes (several LPMOs display low substrate-binding) may be used by the substrate-
307 bound population to degrade the substrate, explaining why H₂O₂ production by LPMOs is not
308 observed in the presence of substrate (16, 32). It is conceivable that H₂O₂ interacts more strongly
309 with substrate-bound, reduced LPMOs, compared to LPMOs in solution, which would explain
310 why low concentrations of exogenous H₂O₂ are beneficial for activity, whereas higher
311 concentrations lead to self-destructive reactions on unbound enzymes. Notably, the assumption
312 that substrate-affinity has an impact on H₂O₂ management and self-destruction by the LPMOs
313 sheds new light on the role of the CBMs that are appended to some LPMOs, including
314 ScLPMO10C.

315 The link between H₂O₂, Fenton-type systems and enzymatic biomass depolymerization
316 has been a matter of debate, controversy and investigations for several decades. The present
317 findings reveal a novel role for H₂O₂. Glucose-methanol-choline (GMC) oxidoreductases are
318 known H₂O₂ producers and, like LPMOs, abundant in fungal secretomes (11). Some GMC
319 oxidoreductases can reduce LPMOs (11, 30), but their ability to produce H₂O₂, perhaps in a
320 controlled manner, could be another important biological function, as suggested by our
321 experiment showing that a reduced LPMO can be fueled by the H₂O₂ generating glucose/glucose
322 oxidase system. Along the same line, a recent study of the secretome of *Aspergillus nidulans*
323 grown on starches revealed co-secretion of LPMOs, catalase and H₂O₂-producing

324 oxidoreductases (AA3, AA7) in the absence of known H₂O₂-consuming partners such as
325 peroxidases (33). It is noteworthy that the present findings may also be relevant for
326 understanding host-pathogen interactions since for instance LPMO-producing necrotrophic
327 bacteria are known to benefit from H₂O₂ generated by the plant defense system (34).

328 The present findings will have far-reaching implications for the design of biorefining
329 processes, including the production of cellulosic ethanol. LPMOs are important components of
330 current commercial cellulase cocktails (12) but proper aeration and delivery of electrons at
331 industrial scale are considered major challenges as is the instability of LPMOs. We show here
332 that LPMO performance and stability can be controlled by regulating the supply of H₂O₂, a
333 liquid, cheap and easy-to-handle industrial bulk chemical. We further show that LPMOs can act
334 in the presence of only catalytic amounts of reductant, which abolishes reductant-induced
335 undesirable redox side reactions, and in the absence of oxygen, which eliminates the need for
336 aeration. So far, the application of LPMOs has likely been hampered by suboptimal process
337 conditions and it seems evident that further process improvements may be achieved now that the
338 role of H₂O₂ has been uncovered. Notably, overdosing LPMOs can be a problem, since lack of
339 LPMO binding sites on the substrate may lead to LPMO inactivation. It is conceivable that
340 careful balancing of LPMOs and hydrolytic enzymes (e.g. cellulases) is needed, with the
341 cellulases “peeling off” LPMO-disrupted polymer chains from the substrate surface, thus
342 exposing new LPMO binding sites. As to LPMO stability, it is interesting to note that one of the
343 residues most vulnerable to oxidation, the N-terminal catalytic histidine, is methylated in fungal
344 LPMOs. Perhaps this methylation helps protecting the fungal LPMOs from oxidative self-
345 destruction.

346 In the six years after their discovery (4), the role of H₂O₂ in LPMO catalysis has been
347 overlooked, despite intense worldwide research on these enzymes. It is tempting to speculate that
348 a similar situation may exist for other enzymes, in particular for copper monooxygenases that are
349 thought to require two electrons and molecular oxygen. It has not escaped our notice that the still
350 enigmatic particulate methane monooxygenase (pMMO), whose active site bears some
351 resemblance to LPMO active sites, displays LPMO-like H₂O₂-related features: it has been
352 reported that production of H₂O₂ by pMMO is lower in presence of substrate (35) and that H₂O₂
353 binds to and can oxidize the pMMO active site (36). It is conceivable that the present findings

354 have implications beyond understanding and optimizing the enzymatic conversion of recalcitrant
355 polysaccharides by LPMOs.

356

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437

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447

448 **Supplementary Materials:**

449 Materials and Methods

450 Figures S1-S24

451 References (1-60)