The impact of reductants on the catalytic efficiency of a lytic polysaccharide monooxygenase and the special role of dehydroascorbic acid

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Monocopper lytic polysaccharide monooxygenases (LPMOs) catalyse oxidative cleavage of glycosidic bonds in a reductant-dependent reaction. Recent studies indicate that LPMOs, rather than being O2-dependent monooxygenases, are H2O2-dependent peroxygenases. Here, we describe SscLPMO10B, a novel LPMO from the phytopathogenic bacterium Streptomyces scabies and address links between this enzyme’s catalytic rate and in situ hydrogen peroxide production in the presence of ascorbic acid, gallic acid and L-cysteine. Studies of Avicel degradation showed a clear correlation between the catalytic rate of SscLPMO10B and the rate of H2O2 generation in the reaction mixture. We also assessed the impact of oxidised ascorbic acid, dehydroascorbic acid (DHA), on LPMO activity, since DHA, which is not considered a reductant, was recently reported to drive LPMO reactions. Kinetic studies, combined with NMR analysis, showed that DHA is unstable and converts into multiple derivatives, some of which are redox active and can fuel the LPMO reaction by reducing the active site copper and promoting H2O2 production. These results show that the apparent monooxygenase activity observed in SscLPMO10B reactions without exogenously added H2O2 reflects a peroxygenase reaction.

Keywords: dehydroascorbic acid; enzyme kinetics; hydrogen peroxide; LPMO; NMR

Lytic polysaccharide monooxygenases (LPMOs) are monocopper enzymes that are involved in depolymerisation of polysaccharides such as cellulose, other glycans, xylan, chitin, pectin and starch [1–8]. LPMO catalysis relies on the controlled generation of a reactive oxygen species that is powerful enough to hydroxylate glycosidic bonds in crystalline and otherwise inaccessible substrates. The hydroxylation occurs at the C1 or C4 position and results in cleavage of the glycosidic bond, yielding a 1,5-ß-lactone (C1-oxidizing LPMOs) or a 4-ketoaldose (C4-oxidizing LPMOs) [9–11]. Some enzymes generate mixtures of C1- and C4-oxidised products [12]. In nature and in industrial applications, LPMOs provide an essential support to hydrolases whose activity is promoted upon LPMO-catalysed de-crystallisation of recalcitrant polysaccharide substrates [13–15].

Lytic polysaccharide monooxygenases depend on an electron source for their catalytic activity. Most likely due to their solvent-exposed active sites [16,17], these

Abbreviations
DHA, dehydroascorbic acid; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; HRP, horseradish peroxidase; LPMO, lytic polysaccharide monooxygenase.
enzymes can accept a remarkable variety of reductants, ranging from small organic molecules, such as ascorbic acid [1,18], gallic acid [3,19] or L-cysteine [20], to protein partners such as cellobiose dehydrogenase [11,21] and pyrroloquinoline quinone-dependent pyranose dehydrogenase [22].

Historically, LPMOs were thought to be strict monooxygenases (Figure 1A) and involve molecular oxygen as a co-substrate [1,23]. Subsequent studies showed that hydrogen peroxide is able to drive LPMO reactions (Figure 1B), suggesting that these enzymes can operate as peroxygenases [24]. Importantly, peroxygenase LPMO reactions are fast [25–30] compared to monooxygenase LPMO reactions, which are typically two to three orders of magnitude slower. Furthermore, the enzymes clearly prefer H2O2 over O2 in competitive experiments [24].

It is well established that, provided with electron donors, LPMOs are able to reduce molecular oxygen and generate hydrogen peroxide [31,32]. This activity was initially considered as a ‘futile’ uncoupled reaction. However, in light of recent findings on H2O2 being a preferred LPMO co-substrate, hydrogen peroxide production by these enzymes can no longer be considered an irrelevant side process. The notion that standard set-ups for aerobic LPMO reactions (LPMO, 1 mM reductant, substrate, O2) likely lead to generation of H2O2 has led some to suggest that the apparent O2-dependent monooxygenase reactions in fact are peroxygenase reactions that are limited by in situ production of H2O2 [24,26]. The question whether ‘true’ monooxygenase LPMO reactions occur at all is a subject of current debate [28,33,34].

This debate stems in part from data showing that hydrogen peroxide accumulation is not taking place in LPMO reactions with substrates [28,31,33,35]. Some researchers have interpreted these data to show that H2O2 generation by LPMOs is suppressed by substrate binding, which would imply that the observed LPMO activity is due to a true monooxygenase reaction [33]. Other authors have pointed out that the absence of hydrogen peroxide accumulation in LPMO reactions with substrates shows that in situ produced H2O2 is rapidly consumed in a productive peroxygenase reaction [26]. Substrate binding shields the copper site from the solvent, and it is, indeed, conceivable that this prevents LPMOs from generating hydrogen peroxide. Recent experimental work by Filandr et al. [28] lends support to this idea. However, the amount of non-substrate-bound LPMO will vary between different LPMO-substrate combinations. Based on observations that LPMO catalytic domains bind their substrates rather weakly (e.g. [36]), one would expect that typical LPMO reactions contain a significant fraction of free enzymes capable of generating H2O2. Furthermore, commonly used low-molecular-weight reductants react with dissolved oxygen leading to LPMO-independent formation of hydrogen peroxide [37–40]. Several recent studies suggest that these non-enzymatic reactions (sometimes referred to as reductant ‘auto-oxidation’) indeed may be rate-limiting in typical aerobic experiments with AA9 and AA10 LPMOs [28,29,41].

All in all, there is a growing amount of evidence suggesting that LPMO substrate oxidation rates obtained in the absence of exogenously provided H2O2 correlate with the rates of hydrogen peroxide production in the reaction [19,29,41,42]. Despite this growing insight, the factors that limit LPMO activity in standard aerobic reactions remain partly unresolved. For example, while it is well known that LPMO activity depends on the type of reductant [43,44], it remains to be established why this is so. Interpretation of existing literature data is complicated by the fact that researchers use quite different reaction conditions, for example strongly varying reductant concentrations and reaction time scales.

Recently, it became clear that LPMOs can play a role in host-pathogen interactions, and may promote microbial infection in both mammals [45] and plants [8]. Thus, characterisation of LPMOs from microbial pathogens is of significant interest. In this paper, we describe SscLPMO10B, a cellulose-active LPMO from Streptomyces scabies, a bacterium that is known for causing damage to potato crops [46]. Next to characterising this enzyme, we used it as a model to study the link between in situ hydrogen peroxide production and substrate oxidation in the presence of three commonly used reductants (ascorbic acid, gallic acid and L-cysteine). The results show a strong dependency of the LPMO catalytic rate on the level of H2O2 generation in the system. We have also studied SscLPMO10B activity in the presence of dehydroascorbic acid (DHA), which is an oxidised derivative of the most commonly used LPMO reductant, ascorbic acid. Since ascorbic acid plays a role in many natural redox

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**Fig. 1.** Monooxygenase (A) and peroxygenase (B) LPMO reaction pathways. For both routes, initial reduction of Cu(II) to Cu(I) is required for the enzyme to enter the catalytic cycle. The figure was created with BioRender (www.biorender.com).

(A) R-H + O2 + 2e- + 2H+ \text{LPMO} \rightarrow R-OH + H2O

(B) R-H + H2O2 \text{LPMO} \rightarrow R-OH + H2O
processes, further insight into the fate and functionalities of its oxidised derivatives is of general interest [47]. DHA was recently reported to be able to fuel cellulose oxidation by TaLPMO9A [48], but the mechanism behind this remarkable observation remains unknown. To gain further insight into these matters, we carried out kinetic studies of LPMO reactions and used NMR spectroscopy to analyse the fate of DHA. We show that spontaneous degradation of DHA produces a complex set of redox-active derivatives that promote generation of hydrogen peroxide and fuel enzymatic oxidation of cellulose.

Results and discussion

Identification and cloning of putative LPMO genes

The S. scabies genome (strain 87.22, GenBank accession number NC_013929.1) was submitted to the dbCAN2 server for automated annotation of hypothetical LPMO genes. The in silico mining revealed four open reading frames encoding for putative LPMOs, all belonging to AA10 family (Figure 2) and referred to as SscLPMO10A, SscLPMO10B, SscLPMO10C and SscLPMO10D below (GenBank accession numbers WP_106437244.1, WP_173402964.1, WP_013003332.1 and WP_013006341.1, correspondingly). MMseqs2 searches [49] against the PDB database [50] identified ScLPMO10C (cellulose-active C1-oxidizing LPMO) [2], SliLPMO10E (chitin-active C1-oxidizing LPMO) [51] and KpLPMO10A (cellulose- and chitin-active C1/C4-oxidising LPMO) [52] as the closest structurally characterised homologues of SscLPMO10B, SscLPMO10C and SscLPMO10D (56%, 79% and 58% sequence identity between catalytic domains), respectively. The closest structurally characterised homologue of SscLPMO10A is Tma12, an unusual (i.e. non-bacterial) AA10 chitinolytic LPMO from the fern Tectaria macrodonta (47% sequence identity between catalytic domains) [53]. SscLPMO10A lacks a signal peptide, which, considering the crucial role of Histidine 1 in correctly processed LPMOs, is remarkable. This protein also contains a predicted transmembrane segment, localised within an extensive C-terminal region of unknown function (Figure 2).

On the other hand, SscLPMO10B, SscLPMO10C and SscLPMO10D have domain architectures (Figure 2) that are common for secreted bacterial LPMOs. The three corresponding sequences were codon optimised for expression in Escherichia coli and modified to encode for the SmlLPMO10A signal peptide instead of native periplasmic localisation signals, after which the genes were synthesised and cloned into the pET-26(b)+ expression vector.

Production of SscLPMO10B and characterisation of LPMO activity

SDS-PAGE analysis of periplasmic extracts and whole-cell samples obtained from E. coli strains expressing LPMO candidate genes revealed that SscLPMO10C and SscLPMO10D accumulated in the cytoplasm in an insoluble form, while SscLPMO10B was soluble and exported. The latter enzyme was purified from a periplasmic extract by ion-exchange and size-exclusion chromatography (Figure S1) and subjected to copper saturation and desalting. The removal of free copper by desalting was confirmed using a previously described procedure [41] (Figure S2).

The substrate specificity of SscLPMO10B was assessed by setting up 24-h reactions containing 0.5 µM copper-loaded LPMO, 1 mM ascorbic acid and various carbohydrates, including 1% (w/v) Avicel, 0.5% (w/v) PASC, 1% (w/v) CMC, 1% (w/v) β-chitin, 0.5% (w/v) beechwood xylan, 0.5% (w/v) konjac glucomannan, 0.5% (w/v) tamarind xyloglucan, 0.5% (w/v) wheat arabinoxylan and 500 µM D-(+)-cellohexaose. HPAEC-PAD analysis of reaction mixtures showed product formation in the reactions with cellulosic substrates only (Avicel, PASC and the soluble cellulose derivative CMC; Figure 3). The chromatograms showed multiple C1-oxidised products, whereas signals corresponding to C4-oxidised products were not observed.

MALDI-TOF MS analysis of products generated from Avicel (Figure 4) gave a spectrum that is typical...
for strictly C1-oxidising LPMOs, with a clear signal for the sodium salt of the aldonic acid and absence of signals for double oxidised products, which could have emerged if the enzyme had been C1/C4-oxidising.

**Avicel oxidation by SscLPMO10B in the presence of various reductants**

Next, we compared the capacity of various reducing compounds to fuel Avicel solubilisation by SscLPMO10B. The experiment featured three commonly used LPMO reductants (ascorbic acid, gallic acid and L-cysteine) as well as DHA – an oxidised derivative of ascorbic acid, recently reported to drive cellulose oxidation by a AA9 LPMO [48].

The use of ascorbic acid and gallic acid in reactions with SscLPMO10B and Avicel, resulted in slow, but steady oxidation of cellulose over 24 h (Figure 5A). The rates obtained with 1 mM ascorbic acid and 1 mM gallic acid amounted to approximately 0.12 min⁻¹ and 0.25 min⁻¹, respectively. Such excessively low catalytic rates are common among AA10 bacterial LPMOs operating under ‘monooxygenase conditions’ [26,41] and it has been claimed that they reflect the rate of hydrogen peroxide production in the reaction mixture [41].

The reaction with 1 mM L-cysteine displayed a much faster rate (≈1.3 min⁻¹), compared to the experiments with gallic acid and ascorbic acid, but product formation diminished early on in the reaction (after approximately 1 h; Figure 5B). The observed decrease in reaction rate over time can be explained by low reductant stability or by LPMO inactivation, which may occur if too much hydrogen peroxide is being generated in the reaction mixture [24,54] (see below).

Importantly, the experiment with 1 mM DHA confirmed that this oxidised derivative of ascorbic acid can, indeed, drive LPMO reactions with cellulose, not only for fungal AA9s [48] but also for bacterial AA10s (Figure 5A). The rate of DHA-fuelled substrate oxidation amounted to approximately 0.32 min⁻¹, meaning that the reaction with DHA outperformed the reactions with gallic acid and ascorbic acid. The variation in reductant efficiency is addressed further below.

**Hydrogen peroxide production in the presence of various reductants**

Our next goal was to test whether the reductant-dependent differences in catalytic rates on Avicel could be correlated to variation in the rate of in situ hydrogen peroxide production in the reactions. The results of HRP/Amplex Red experiments (Figure 6) revealed that the H₂O₂ production capacity of LPMO reactions indeed strongly depends on the nature of the reducing compound used to sustain the reactions. The highest hydrogen peroxide generation rate was observed in the presence of L-cysteine, whereas the experiment with DHA resulted in the lowest H₂O₂ accumulation rate.
As alluded to above, hydrogen peroxide production in these reactions is a result of both LPMO-dependent and LPMO-independent oxidation of the reductant. Figure 6 shows that the LPMO contribution to the overall hydrogen peroxide production was considerable in the reactions with ascorbic acid and, particularly, l-cysteine. On the other hand, the LPMO did not promote apparent \( \text{H}_2\text{O}_2 \) production in the reactions with gallic acid and DHA. In the case of gallic acid, the LPMO even, seemingly, inhibited \( \text{H}_2\text{O}_2 \) production, which is likely due to a previously observed side reaction in which the LPMO oxidises gallic acid (or gallic acid derivatives) using \( \text{H}_2\text{O}_2 \) \([41,55]\). Such side reactions are likely to become pronounced in the absence of proper LPMO substrates (i.e. the conditions of HRP/Amplex Red assay).

Taken together, the hydrogen peroxide production data (Figure 6) and the substrate oxidation data (Figure 5) obtained with ascorbic acid, gallic acid and l-cysteine indicate that the \( \text{Ssc} \) LPMO10B catalytic rate correlates with the rate of \( \text{H}_2\text{O}_2 \) production in the reaction mixture. Very fast generation of \( \text{H}_2\text{O}_2 \) in the reaction with l-cysteine may explain why the LPMO reaction was so fast and why enzyme inactivation was observed (Figure 5B). Comparison of the rates estimated from the datasets depicted in Figures 5 and 6 shows that the product release rate (Figure 5) is 2–2.5 times lower than the hydrogen peroxide generation rate (Figure 6). This lack of stoichiometry is not surprising, considering that the presence of substrate will reduce the degree of LPMO-dependent \( \text{H}_2\text{O}_2 \) generation \([28,31]\; see below for more discussion). Furthermore, only solubilised oxidised products were...
bic acid (Figure 7A). The addition of hydrogen peroxide to the LPMO reaction with ascorbic acid resulted in an (expected) drastic increase in the product formation rate ($\approx 5.6 \text{ min}^{-1}$) compared to the rate obtained at standard aerobic conditions ($\approx 0.12 \text{ min}^{-1}$). The progress curve shows that product formation diminished after a fast and linear initial phase. This is indicative of enzyme inactivation, which is to be expected in experiments with high amounts of added H$_2$O$_2$ [24,56,57].

Strikingly, the impact of adding H$_2$O$_2$ to a reaction with DHA was minimal, leading to a substrate oxidation rate ($\approx 0.86 \text{ min}^{-1}$) that was only a little higher than the rate observed in the absence of H$_2$O$_2$ ($\approx 0.32 \text{ min}^{-1}$) and much lower than the rate observed in the reaction with H$_2$O$_2$ and ascorbic acid (Figure 7A). This observation suggests that, in contrast to ascorbic acid, DHA is not an efficient reductant for the LPMO, which is a prerequisite for the peroxigenase reaction to happen. This conclusion, however, is in stark contrast with the observation that DHA outperforms ascorbic acid under ‘monooxygenase conditions’ (Figure 5A). One might even wonder whether the results with DHA indicate that SscLPMO10B is a true monooxygenase (so, not dependent on H$_2$O$_2$ formation) or whether DHA perhaps facilitates another, hitherto unresolved catalytic mechanism. Interestingly, Brander et al. [48] recently showed that DHA is able to promote the oxidation of reduced phenolplthalein by an AA9 LPMO in the absence of both oxygen and hydrogen peroxide, which could be taken to suggest that LPMOs catalyse a hitherto unknown reaction.

Looking for possible explanations, we first considered the possibility that DHA may engage in a rapid side reaction with H$_2$O$_2$, thus scavenging the co-substrate. A control reaction with added H$_2$O$_2$ in the presence of both ascorbic acid and DHA (Figure 7B) yielded a progress curve very similar to that obtained in the experiment with H$_2$O$_2$ and only ascorbic acid, which shows that hydrogen peroxide scavenging by DHA was negligible.

We then considered whether an unknown DHA derivative (e.g. a product resulting from spontaneous hydrolysis), rather than DHA itself, could reduce the LPMO and fuel the LPMO reaction. If this would be the case and if the hypothetical DHA derivative would accumulate in LPMO reactions at a relatively slow rate, then one could expect DHA to not work well in the experiments with H$_2$O$_2$ (Figure 7A) due to a short overall incubation time. On the other hand, the SscLPMO10B reactions carried out at standard aerobic conditions (Figure 5A) were incubated for 24 h, potentially allowing for significant amounts of a hypothetical DHA derivative to be formed.
To test this simple but speculative explanation, the Avicel degradation experiment was conducted again, this time using 1 mM DHA that had been pre-incubated with Avicel in reaction buffer at 30°C for 6 h, prior to addition of the enzyme and hydrogen peroxide. Surprisingly, the rate of the DHA-fuelled peroxygenase reaction dramatically increased when using the pre-incubated reductant (Figure 7C), matching the rate obtained in the reaction with H2O2 and ascorbic acid. This observation suggests that, indeed, an unidentified slowly accumulating DHA derivative (or multiple derivatives) can reduce the LPMO. This may explain why DHA works well in the 24-h standard reactions depicted in Figure 5A, whereas DHA performs poorly in the 30-min reactions depicted in Figure 7A.

Importantly, these observations regarding DHA stability can also explain the discrepancy between the very low apparent rates of H2O2 production observed in the presence of DHA (Figure 6) and the efficiency of DHA under ‘monooxygenase conditions’ (Figure 5A). Next to the difference in time scale, complications of the HRP/Amplex Red assay may play a role. It is well established that reductants may, to varying extents, interfere with the HRP/Amplex Red assay signal, leading to underestimation of hydrogen peroxide levels [29,41,58]. This can to some extent be handled by adding reductants to the standard curves for H2O2, as we did in this study. For unstable DHA, the situation is problematic because signal repression will change over time and the compounds involved are unknown (and possibly transient; see below). It is thus possible that the H2O2 production levels observed in the presence of DHA are underestimated.

**Monitoring H2O2 production by SscLPMO10B in the presence of DHA using selective fluorescent probe**

Given the limitations of the standard HRP/Amplex Red assay discussed above, we evaluated Peroxy Orange 1 (PO1, a boron-based fluorescent H2O2 probe [59]) as a new tool to monitor the hydrogen peroxide production in LPMO reactions. PO1 is irreversibly activated by the nucleophilic addition of hydrogen peroxide to the boron atom, resulting in formation of unstable borate ester, which undergoes spontaneous hydrolysis to yield a highly fluorescent stable product [60]. This approach allowed monitoring of H2O2 production over a time scale that is comparable to that of the reactions with cellulose. First, we carried out a simple reaction with 25 μM PO1 and 25 μM H2O2, confirming that the probe can be used to detect hydrogen peroxide under LPMO reaction conditions (Figure S3). Note that relatively low PO1 concentration was used to avoid a precipitation of the probe, which has limited solubility in water [60]. Next, 25 μM PO1 was introduced to the reaction mixture containing 1 mM DHA (Figure 8), leading to a significant increase of the fluorescence over time, compared to a
control reaction with only PO1. Importantly, when 1 \( \mu \text{M} \) \( \text{SscLPMO10B} \) was added to the system alongside DHA, the fluorescence increased even further (Figure 8).

To confirm that the observed PO1-derived fluorescence was indeed due to the accumulation of hydrogen peroxide, a control experiment was carried out using catalase, which can compete with LPMOs for \( \text{H}_2\text{O}_2 \) when applied at sufficiently high concentrations [28]. As expected, addition of catalase repressed \( \text{H}_2\text{O}_2 \) generation both in reactions with DHA alone and in reactions with LPMO and DHA (Figure 8). Quantitative interpretation of fluorescence data obtained with PO1 is complicated by the fact that the probe activation is exceedingly slow (Figure S3), meaning that the assay signal will be affected by potential side reactions that can occur with \( \text{H}_2\text{O}_2 \) in the presence of DHA and LPMO. Nevertheless, Figure 8 clearly shows that hydrogen peroxide is produced in a DHA-dependent manner.

Finally, to prove that DHA-fuelled reactions of \( \text{SscLPMO10B} \) with cellulose depend on in situ produced \( \text{H}_2\text{O}_2 \) under standard aerobic conditions, an inhibition experiment was performed in the presence of Avicel and catalase. As shown in Figure 9, catalase inhibited \( \text{SscLPMO10B} \) activity, confirming that the apparent monooxygenase reaction in the presence of DHA indeed is an \( \text{H}_2\text{O}_2 \)-dependent peroxygenase reaction.

The accumulation of DHA derivatives in the presence and absence of LPMO

It has previously been demonstrated that DHA spontaneously converts to 2,3-diketo-L-gulonic acid (DKG) [61–63] in aqueous solutions. DKG is known to undergo additional transformations in solution giving rise to various compounds, some of which are known to be redox-active [64, 65]. Importantly, one such DKG derivative (called ‘compound 1’) has been isolated by HPLC and shown to be capable of inducing non-enzymatic hydrogen peroxide production, which is a relevant feature in the context of LPMO catalysis [64]. Based on characterisation of compound 1 by electrospray mass spectrometry, Kärkönen et al. proposed the formula \( \text{C}_6\text{H}_8\text{O}_5 \), suggesting that compound 1 has a higher reduction state than DKG (\( \text{C}_6\text{H}_8\text{O}_7 \)) [64]. While the mechanism by which compound 1 is generated remains unclear, its formation likely results from a redox reaction involving DKG and one of the multiple potential products of spontaneous DHA hydrolysis. Interestingly, a hypothetical chemical structure of compound 1 proposed by Kärkönen et al. [64] shows some resemblance to ascorbic acid (\( \text{C}_6\text{H}_8\text{O}_6 \); Figure 10). The structural similarity between these two molecules is supported by the fact that ascorbate oxidase is able to recognise compound 1 as a substrate [64]. In view of these data, it seems feasible that, like ascorbic acid [66], compound 1 can interact with the LPMO active site and reduce it.

**Fig. 8.** \( \text{H}_2\text{O}_2 \) production by \( \text{SscLPMO10B} \) in the presence of DHA. The figure shows the generation of hydrogen peroxide in reactions containing 1 mM DHA and 1 \( \mu \text{M} \) \( \text{SscLPMO10B} \) or 1 mM DHA alone. Note that the addition of LPMO to DHA solution significantly promoted \( \text{H}_2\text{O}_2 \) formation. The experiments were carried out using 25 \( \mu \text{M} \) PO1 (Ex/Em = 540/560 nm) in 50 mM sodium phosphate buffer, pH 6.0, at 30°C. Reaction mixtures supplied with DHA contained 1.5% (v/v) DMSO, whereas reaction mixtures lacking DHA contained 0.5% (v/v) DMSO. Error bars indicate standard deviation between triplicates.
UV-VIS spectroscopy of DHA under standard aerobic LPMO reaction conditions (1 mM DHA, 1% Avicel, no LPMO, pH 6.0) revealed the formation of a pronounced new peak with an absorbance maximum at 268 nm (Figure 11A). This value is very close to the \( \lambda_{\text{max}} \) (271–272 nm) previously reported for compound 1 at mildly acid conditions (pH 5.6) [64]. The peak area increased over time with a concomitant shift in the \( \lambda_{\text{max}} \) (from 268 nm to 285 nm). This notable shift in \( \lambda_{\text{max}} \) over time suggests the formation of additional DHA derivatives. To rule out the possibility that the observed accumulation of DHA derivatives was driven by Avicel or potential contaminations in the Avicel, a control UV-VIS experiment was carried out in the absence of cellulose, showing that the cellulose did not affect the formation of UV-absorbing species and did not affect the shift in \( \lambda_{\text{max}} \) over time (Figure S4). A similar reaction conducted in the presence of both Avicel and LPMO (Figure 11B) gave remarkably lower UV absorption signals after 24 h, lending support to the notion that DHA-derived compounds (or their hypothetical precursors) are involved in LPMO catalysis.

Somewhat surprisingly, a control experiment with DHA and LPMO in the absence of substrate showed even lower UV signals (Figure 11B). While we cannot explain why this is the case, there are plausible explanations, one being that in the absence of a bona fide substrate the LPMO may catalyse H\(_2\)O\(_2\)-dependent oxidation of one or more of the DHA-derived compounds, analogous to findings by Breslmayr et al. [55]. It is also possible that in the absence of substrate, H\(_2\)O\(_2\) production is elevated [28] and that the produced H\(_2\)O\(_2\) reacts directly with DHA-derived redox active compounds. Overall, the results described above support the idea that DHA decomposition involves the formation of reducing species that can drive LPMO action on cellulosic substrates.

To get more insight in the chemistry of DHA and ascorbic acid conversion, we monitored the transformation of these compounds by NMR spectroscopy. Using...
NMR allowed direct observation of the main compounds generated in situ, with high temporal resolution. This contrasts with previous studies on the fate of ascorbic acid and DHA, which were largely based on electrophoretic separation of compounds prior to analysis [64,67]. Firstly, partial assignment of ascorbic acid, DHA and DKG was performed using 10 mM ascorbic acid and DHA solutions. C-H spin pairs were assigned using $^{13}$C-HSQC spectra (Figure S5), and $^{13}$C-HSQC-[1H-1H]-TOCSY correlations were used to establish connectivity. Attempts to assign the quaternary carbons using HMBC correlations were not successful. Nevertheless, the assignment for ascorbic acid fits previously published assignments (Biological Magnetic Resonance Data Bank entry bmse000182; www.bmrb.io), whereas the assignments for DHA and DKG fit the chemical shifts predicted by an empirical neural-network algorithm provided by the NMRShiftDB server (https://nmrshiftdb.nmr.uni-koeln.de/nmrshiftdb/). DHA is known to exist as a single ring or in a bicyclic form. The high chemical shift for the methylene C6 (78.7 ppm) indicates that the bicyclic form of DHA is the only one visible in our NMR experiments. This is an expected observation, as bicyclic DHA is known to be the dominant DHA form [67]. For the single ring form of DHA, a lower chemical shift (around 62 ppm) is to be expected.

Next, based on the assigned chemical shifts, we recorded and analysed NMR spectra of 1 mM DHA solution in 50 mM sodium phosphate buffer, pH 6.0 supplied with 1% (w/v) Avicel in the presence or absence of 0.5 μM LPMO. The NMR analysis clearly showed that the conversion of DHA to DKG was not affected by the presence of the enzyme (Figure 12A,B), indicating that both DHA and DKG are not directly involved in LPMO reduction. Although the UV-VIS data (Figure 11A) clearly showed that further conversion of DKG takes place under these conditions, we did not detect NMR signals corresponding to compound 1 or other DHA derivatives in any of the reactions. This is likely due to the low sensitivity of NMR, which implies that compounds would only be visible at concentrations above ~0.1 mM.

For comparison, the NMR experiment was repeated using ascorbic acid as an electron donor. In contrast to the observations with DHA, the rate of ascorbate consumption increased upon the addition of LPMO (Figure 12C), which is to be expected, considering that this compound can reduce the LPMO directly [66]. The experiment further showed that, as expected, ascorbic acid oxidation resulted in the generation of DHA and DKG (Figure 12D,E). It is not clear from our data to what extent DHA derivatives contribute to enzyme reduction and H₂O₂ production in typical LPMO reactions containing ascorbic acid at high (≥1 mM) concentration. However, it seems reasonable to assume that DHA-related effects could have noticeable impact in certain experiments, for example experiments that involve low amounts of ascorbic acid that will become depleted during prolonged incubation times.

**Concluding remarks**

Taken together, our experiments with SscLPMO10B and four different reductants under conditions referred
to here as ‘standard aerobic reactions’ or ‘monooxygenase conditions’ show that the LPMO catalytic rate depends on the level of in situ hydrogen peroxide production. This study adds to a growing amount of evidence in support of the notion that slow apparent monooxygenase LPMO reactions in fact are peroxygenase reactions, limited by endogenous H₂O₂ supply. It seems clear by now that the impact of the reductant on LPMO activity, which is as such remarkable since reduction of LPMOs is much faster than the apparent monooxygenase reaction [24,43], relates to H₂O₂ production and not to LPMO reduction.

The results of the hydrogen peroxide assays should be interpreted with caution. It is well established that reductants interfere with the HRP/Amplex Red assay signal, for example by reducing Amplex Red radicals back to Amplex Red, leading to reduced resorufin formation and, thus, underestimation of hydrogen.

**Fig. 12.** Time courses of (A) DHA conversion to (B) DKG, and (C) ascorbic acid conversion to (D) DHA and (E) DKG. All experiments were carried out at 30°C in 50 mM sodium phosphate buffer, pH 6.0 prepared in D₂O (D, 99.9%) and supplied with 1% (w/v) Avicel. The reactions containing 0.5 µM LPMO are marked with red colour, whereas the reactions lacking the enzyme are marked with black colour. The signal intensity % in the plots corresponds to integrals in pseudo-2D NMR spectra recorded over 24 h. To facilitate comparison across experiments, all signals were normalised to the DHA signal at the beginning of the reaction for the sample with 0.5 µM SscLPMO10B and 1% (w/v) Avicel (red curve in panel A).
peroxide levels [29,41,58]. While the reductant-mediated repression of the HRP/Amplex Red assay signal can be accounted for when using a well-defined reductant, this cannot be applied to reactions containing DHA, which, as a result of spontaneous DHA conversion, contain unknown amounts of unknown reductants that change over time. When it comes to quantitatively correlating \( H_2O_2 \) production levels to LPMO activity, another complication comes from the fact that the presence of substrate at LPMO turnover conditions will reduce LPMO-dependent \( H_2O_2 \) production [28,31,35,58]. Indeed, recent data indicate that the substrate oxidation rates of bacterial LPMOs show a surprisingly low dependency on the LPMO concentration [28,29,41], which supports the notion that the reaction is limited by the co-substrate and that LPMO-dependent in situ generation of the co-substrate is hampered by substrate binding. While we are not able to quantitatively link \( H_2O_2 \) production levels by a particular LPMO-reductant combination to the rate of cellulose degradation, the results depicted in Figures 5 and 6 show a clear correlation between the two.

Ascorbic acid is an abundant compound in nature that has been studied in multiple contexts [68,69]. In that sense, our findings with DHA not only shed light on an unusual LPMO-activating compound but may also have implications for other redox processes involving ascorbic acid. Our LPMO studies show that, rather than reducing the enzyme directly, DHA is spontaneously converted into a variety of derivatives including at least one that can act as an electron donor for LPMOs and that can promote \( H_2O_2 \) production. We were not able to quantitatively assess the \( H_2O_2 \) production potential of this mixture due to the limitations of the HRP/Amplex red assay that are discussed above; however, our experiments with the PO1 probe confirm that hydrogen peroxide generation is taking place in the presence of DHA. Furthermore, it is clear that the LPMO catalytic rate in reactions with DHA is limited by \( H_2O_2 \) produced in situ, as demonstrated by the catalase inhibition experiment. There is no reason to assume that a DHA-driven reaction is mechanistically different from, for example an ascorbic acid-driven reaction.

**Methods**

**Materials**

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Amplex Red was obtained from Thermo Fisher Scientific (Waltham, MA, USA). A stock solution containing 10 mM Amplex Red was prepared in DMSO and stored at \(-20^\circ C\) in light-protected tubes. Ascorbic acid was stored at \(-20^\circ C\) as filter-sterilised (0.22-µm syringe filter) 100 mM stock solution in metal-free TraceSELECT water (Honeywell, Charlotte, NC, USA). Gallic acid and DHA were stored at \(-20^\circ C\) as filter-sterilised (0.22-µm syringe filter) 100 mM stock solutions in DMSO. The reductant solutions were aliquoted and used only once. Fresh l-cysteine stock solutions were prepared immediately prior to experiments by dissolving the compound in metal-free TraceSELECT water at 100 mM concentration. These solutions were filtered through a 0.22-µm syringe filter and used only once. Horseradish peroxidase type II (HRP; Sigma-Aldrich, St. Louis, MO, USA) was stored in 50 mM sodium phosphate buffer, pH 6.0 at 4°C (at 100 U/ml concentration). Bovine liver catalase (Sigma-Aldrich, St. Louis, MO, USA) was stored in 50 mM sodium phosphate buffer, pH 6.0 at 4°C (at 9500 U/ml concentration). Peroxy Orange 1 (PO1) was stored at \(-20^\circ C\) as 5 mM stock solution in DMSO. Trypsitone and yeast extract were from Thermo Fisher Scientific (Waltham, MA, USA). The model microcrystalline cellulose used in the study was Avicel PH-101. Phosphoric acid swollen cellulose (PASC) was produced from Avicel as described before [70]. Carboxymethyl cellulose (CMC) was stored as 2% (w/v) solution in 50 mM sodium phosphate buffer, pH 6.0. Beechwood xylan, konjac glucomannan, tamarind xylanoglu and wheat arabinoxylan were obtained from Megazymes (Bray, Ireland). β-Chitin extracted from squid pen was purchased from France Chitin (Orange, France).

**Identification and cloning of LPMO genes**

The publicly available *S. scabies* genome (strain 87.22, GenBank accession number NC_013929.1) was mined for hypothetical LPMO encoding sequences using the dbCAN2 server (http://dbcan2.ncbi.nlm.nih.gov) [71]. Putative signal peptides and transmembrane helixes were predicted using the SignalP-5.0 (http://www.cbs.dtu.dk/services/SignalP/) [72] and TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html) servers, respectively. Four open reading frames were found to encode hypothetical AA10 LPMOs (GenBank accession numbers WP_106437244.1, WP_173402964.1, WP_013003332.1 and WP_013006341.1) that are referred to as *SscLPMO10A*, *SscLPMO10B*, *SscLPMO10C* and *SscLPMO10D*, respectively.

The *SscLPMO10B*, *SscLPMO10C* and *SscLPMO10D* full-length sequences (including stop codons) were codon optimised for expression in *E. coli* and modified to encode for the *SmLPMO10A* (CBP21) [1] periplasmic localisation signal (‘MNKTSRTTLSSLGLLLSAAMFGVSVQANA’) instead of native signal peptides. Due to this set-up the C-terminal His-tag encoded by the vector was not included in the expressed proteins. The corresponding genes were
synthesised and cloned into the pET-26(b)+ expression vector (Merck, Darmstadt, Germany) by GenScript (Piscataway, NJ, USA) using NdeI/XhoI restriction sites. Correct synthesis and cloning of the target inserts were confirmed by Sanger sequencing (GenScript, Piscataway, NJ, USA).

Protein expression and purification

SscLPMO10B, SscLPMO10C and SscLPMO10D expression strains were established by heat-shock transformation of BL21 (DE3) competent cells (Invitrogen, Carlsbad, USA) with the LPMO gene-containing pET-26(b)+ plasmids according to the supplier’s protocol. The transformants were incubated in LB medium at 37°C for 1 h, and then plated on LB agar medium with 50 µg/ml kanamycin, followed by overnight incubation at the same temperature. A single colony was picked from the plate and used to inoculate 500 ml of Terrific Broth (TB) medium supplied with 50 µg/ml kanamycin. The culture was incubated for 24 h at 30°C in a LEX-24 Bioreactor (Harbinger Biotechnology & Engineering, Markham, Canada) using compressed air for mixing and aeration. Considerable levels of basal expression were observed when using these conditions; hence, IPTG induction of the T7 promoter was not required.

The cells were collected by centrifugation (6000 × g for 10 min at 4°C) using a Beckman Coulter centrifuge (Brea, CA, USA). Periplasmic extracts were produced by osmotic shocking as described previously [73] and filter-sterilised through 0.22-µm syringe filters (Sarstedt, Nürnbecht, Germany).

The periplasmic extract containing soluble SscLPMO10B was subjected to ion-exchange chromatography with a HiTrap™ Q FF (Q Sepharose) 5-ml column (GE Healthcare, Chicago, USA). Protein elution was achieved by applying a linear gradient of NaCl (0–500 mM, 250 ml) in the starting buffer (25 mM Tris-HCl, pH 8.0). Chromatographic fractions containing LPMO were pooled, concentrated (Vivaspin ultrafiltration tubes, Sartorius, Germany) and further purified by size-exclusion chromatography using a HiLoad 16/60 Superdex 75 column (GE Healthcare, Chicago, USA) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl. Enzyme purity was confirmed by SDS-PAGE (Bio-Rad, Hercules, CA, USA) and the concentration of SscLPMO10B was determined by UV spectroscopy at 280 nm using the theoretical extinction coefficient predicted with the ProtParam tool, assuming that all pairs of cysteine residues are involved in the formation of disulphide bridges [74].

The resulting LPMO sample was co-incubated with a three-fold molar surplus of Cu(I)SO₄ for 30 min at room temperature to ensure complete active site loading. Copper saturation was performed in 50 mM Tris-HCl, pH 7.5, containing 200 mM NaCl. Excess copper was removed from the enzyme preparation by desalting using a PD MidiTrap G-25 gravity flow column (GE Healthcare, Chicago, USA), equilibrated with 50 mM sodium phosphate buffer, pH 6.0, using previously described protocol [41].

HRP/Ampelix Red hydrogen peroxide production assay

Hydrogen peroxide production by SscLPMO10B in the presence of various reducing compounds was studied using the HRP/Ampelix Red assay, previously described by Kittl et al. [31]. In brief, 90 µl of LPMO solution in 50 mM sodium phosphate buffer, pH 6.0, containing HRP and Ampelix Red, was pre-incubated for 5 min at 30°C in a 96-well microtiter plate. To start the reactions, 10 µl of reductant solution was added, followed by 10 s of mixing (600 RPM) in a Varioscan LUX plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The final concentrations of LPMO, HRP, Ampelix Red and reductant were 0.5 µM, 5 U/ml, 100 µM and 1 mM, respectively. Hydrogen peroxide production was detected by following the formation of resorufin, which is the product of Ampelix Red oxidation that has strong absorbance at 563 nm.

Control reactions containing no LPMO were carried out to assess the level of hydrogen peroxide production in the presence of reductants and oxygen.

Hydrogen peroxide standard solutions were prepared in 50 mM sodium phosphate buffer, pH 6.0, and were supplied with reductant, immediately followed by HRP and Ampelix Red, to produce calibration curves. The final concentrations of HRP, Ampelix Red and reductants in the calibration samples were the same as in experimental samples (see above). The apparent hydrogen peroxide production rates were calculated from the linear part of the resorufin accumulation curves.

Hydrogen peroxide detection using Peroxy Orange 1

Hydrogen peroxide generation was also studied using Peroxy Orange 1 (PO1), a H₂O₂-selective fluorescent probe. Reaction mixtures containing 1 µM LPMO, 1 mM DHA and/or 2000 u/ml catalase were supplied with 25 µM PO1, loaded into a 96-well non-transparent microtiter plate and incubated at 30°C for 12 h. Fluorescence signals were recorded every hour using 540/560 nm excitation/emission wavelengths. Samples containing 25 µM PO1 or 25 µM PO1 and 25 µM H₂O₂ were used as negative and positive controls, respectively.

LPMO reactions with polysaccharide and oligosaccharide substrates

Lytic polysaccharide monoxygenase reactions with various soluble and insoluble substrates [1% (w/v) Avicel, 0.5%...
(w/v) PASC, 1% (w/v) CMC, 500 µM cellohexaose, 0.5% (w/v) beechwood xylan, 0.5% (w/v) konjac glucomannan, 0.5% (w/v) tamarind xyloglucan, 0.5% (w/v) wheat arabinoxylan and 1% (w/v) β-chitin) were carried out in 50 mM sodium phosphate buffer, pH 6.0 using an Eppendorf thermod mixer (Eppendorf, Hamburg, Germany) set to 30°C and 900 RPM. Experiments with Avicel were set up in the absence and in the presence of 100 µM H₂O₂, whereas reactions with other substrates were performed at standard aerobic conditions only (i.e. no exogenous hydrogen peroxide). The reaction mixtures were supplied with 1 mM reductant (ascorbic acid, gallic acid, l-cysteine or DHA). Note that the reaction mixtures featuring gallic acid and DHA contained 1% (v/v) DMSO, introduced from the reaction mixtures. If aerobic conditions only (i.e. no exogenous hydrogen peroxide) were treated with in-house produced recombinant Thermobifida fusca GH6 endoglucanase (Tf Cel6A, [75]; 2 µM final concentration) to convert oxidized LPMO products to a simple, quantifiable mixture of dimers and trimers.

Product analysis by HPAEC-PAD

The LPMO products were detected and quantified by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex ICS5000 system (Thermo Scientific, San Jose, CA, USA) equipped with a CarboPac PA200 analytical column, as previously described [41]. Data were collected and processed using Chromeleon 7.0 software. C1-oxidised cellobiose and cellotriose standards were prepared in-house according to a previously published protocol [76].

Product analysis by MALDI-TOF MS

Products generated by SscLPMO10B in the reaction with 1% (w/v) Avicel were analysed using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) UltraflexTreme mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). In this experiment, 50 mM Bis-Tris, pH 6.0, was used instead of 50 mM sodium phosphate buffer, pH 6.0, to achieve optimal co-crystallisation of the sample with matrix solution. One microlitre of the LPMO reaction samples was mixed with 2 µl of a matrix solution (9 mg/ml 2,5-dihydroxybenzoic acid in 30% acetonitrile) on an MTP 384-ground steel target plate (Bruker Daltonics). The target plate was air-dried, and the MS data were acquired using Bruker flexControl software, as described previously [1].

UV-VIS spectroscopy of LPMO reaction mixtures featuring Avicel and DHA

Samples were taken from LPMO reactions at various time points and reactions were quenched by removing the insoluble substrate as described above. Hundred microlitres of filtered reaction samples were transferred to a UV-transparent 96-well microtiter plate. UV-VIS absorbance spectra were recorded in the 230–900 nm range using 1-nm scanning steps in a Varioscan LUX plate reader (Thermo Fisher Scientific, Waltham, MA, USA).

NMR spectroscopy

The following samples (500 µL) were prepared in 5-mm LabScape Stream NMR tubes (Bruker BioSpin AG, Fällanden, Switzerland) to monitor the kinetics of ascorbic acid and DHA degradation by NMR spectroscopy: (a) 0.5 µM SscLPMO10B and 1% (w/v) Avicel with 1 mM ascorbic acid in 50 mM sodium phosphate buffer (pH 6.0) prepared in D₂O (D, 99.9%), (b) 1% (w/v) Avicel with 1 mM ascorbic acid in 50 mM sodium phosphate buffer (pH 6.0) prepared in D₂O, (c) 0.5 µM SscLPMO10B and 1% (w/v) Avicel with 1 mM DHA and 1% (v/v) DMSO (D, 99.9%; Merck) in 50 mM sodium phosphate buffer (pH 6.0) prepared in D₂O, (d) 1% (w/v) Avicel with 1 mM DHA and 1% (v/v) DMSO (D, 99.9%; Merck, Darmstadt, Germany) in 50 mM sodium phosphate buffer (pH 6.0) prepared in D₂O, (e) 10 mM ascorbic acid in 50 mM sodium phosphate buffer (pH 6.0) prepared in D₂O and (f) 10 mM DHA and 1% (v/v) DMSO (D, 99.9%; Merck, Darmstadt, Germany) in 50 mM sodium phosphate buffer (pH 6.0) prepared in D₂O.

All NMR experiments were recorded at 30°C on a Bruker Ascend 800 MHz spectrometer with an Avance III HD console and equipped with a 5-mm Z-gradient CP-TCI (H/C/N) cryogenic probe at the NV-NMR-Centre/Norwegian NMR Platform (NNP) at the Norwegian University of Science and Technology (NTNU). ¹H chemical shifts were internally referenced to the water signal, while ¹⁵N and ¹³C chemical shifts were indirectly referenced to the water signal, based on absolute frequency ratios [77]. The spectra were recorded, processed and analysed using TopSpin 3.6pl7 and TopSpin 4.0.7 software (Bruker BioSpin AG, Fällanden, Switzerland).

Time-course kinetics of the above-mentioned samples were followed using a series of 1D Nuclear Overhauser Effect Spectroscopy (NOESY) spectra with a 10-ms mixing time being recorded every 12 min for 24 h, resulting in pseudo-2D spectra with time as the second dimension. The 1D NOESY experiment was selected because it provides excellent water suppression without causing artefacts and signal reduction around the water resonance [78,79]. To assign the chemical shifts of the compounds present in the solutions, we recorded 1D-NOESY, 2D ¹³C-Heteronuclear
Single Quantum Coherence (HSQC) with multiplicity editing, 2D $^1$C-HSQC-$^1$H-$^1$H-J-total Correlation Spectroscopy (TOCSY) with 90-ms mixing time, and two-dimensional heteronuclear multibond correlation (HMBC) with three-fold low-pass J-filter for suppression of one-bond correlations, using samples (5) and (6). The signals were recorded immediately after preparation of these solutions and once again after a 24-hour incubation.

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**Conflict of interests**

The authors declare no conflicts of interest.

**Data accessibility**

The data that support the findings of this study are available in Figure 1-12 and the supplementary material of this article.

**Author contributions**

Anton A. Stepnov designed experiments, did most of the experimental work, analysed data and drafted the manuscript. Idd A. Christensen performed NMR experiments, analysed data and edited the manuscript. Zarah Forsberg, Finn L. Aachmann and Gaston Courtade contributed to designing the study, performed data analysis and edited the manuscript. Vincent G. H. Eijsink conceived and supervised the project, contributed to data interpretation and edited the manuscript. All authors read and approved the final manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. SDS-PAGE of SscLPMO10B after purification.

Figure S2. Hydrogen peroxide production by SscLPMO10B and by protein-free ultrafiltrates.

Figure S3. H2O2 detection using Peroxy Orange 1.

Figure S4. UV-VIS spectroscopy of DHA solutions in the presence or absence of Avicel.

Figure S5. Annotated 13C-HSQC spectra of (A) 10 mM ascorbate and (B) 10 mM DHA and 1% (v/v) d-DMSO after 24 hours of incubation.