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Lytic Polysaccharide Monooxygenases (LPMOs): A Comprehensive Overview of Emerging Families and Their Implications

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ABBREVIATIONS

AA	Auxiliary activity
Aa	*Aspergillus aculeatus* and *Aphanomyces astaci*
Af	*Aspergillus fumigatus*
Aj	*Aspergillus japonicus*
An	*Aspergillus nidulans* and *Aspergillus niger*
Ao	*Aspergillus oryzae*
At	*Aspergillus terreus*
Av	*Adineta vaga*
BLAST	Basic Local Alignment Search Tool
CAZy	Carbohydrate-active enzymes
CBM	Carbohydrate-binding module
CBP	Chitin-binding protein
CDH	Cellobiose dehydrogenase
CE	Carbohydrate Esterases
Cg	*Coptotermes gestroi*
dCTRs	Disordered C-terminal regions
DP	Degrees of polymerization
GH	Glycoside hydrolase
GT	Glycosyl Transferases
His (H)	Histidine
HMMs	Hidden Markov models
L2	Loop 2
L3	Loop 3

LC	Long C-terminal loop
LS	Loop short
LPMO	Lytic Polysaccharide Monooxygenase
Mt	*Myceliophthora thermophila*
Nc	*Neurospora crassa*
NCBI	National Center for Biotechnology Information
Pc	*Pycnoporus coccineus*
Phe (F)	Phenylalanine
Pi	*Phytophthora infestans*
PL	Polysaccharide Lyases
Pp	*Phytophthora parasitica*
Tc	*Trametes coccinea*
Td	*Thermobia domestica*
Tr	*Trichoderma reesei*
Tyr (Y)	Tyrosine

INTRODUCTION

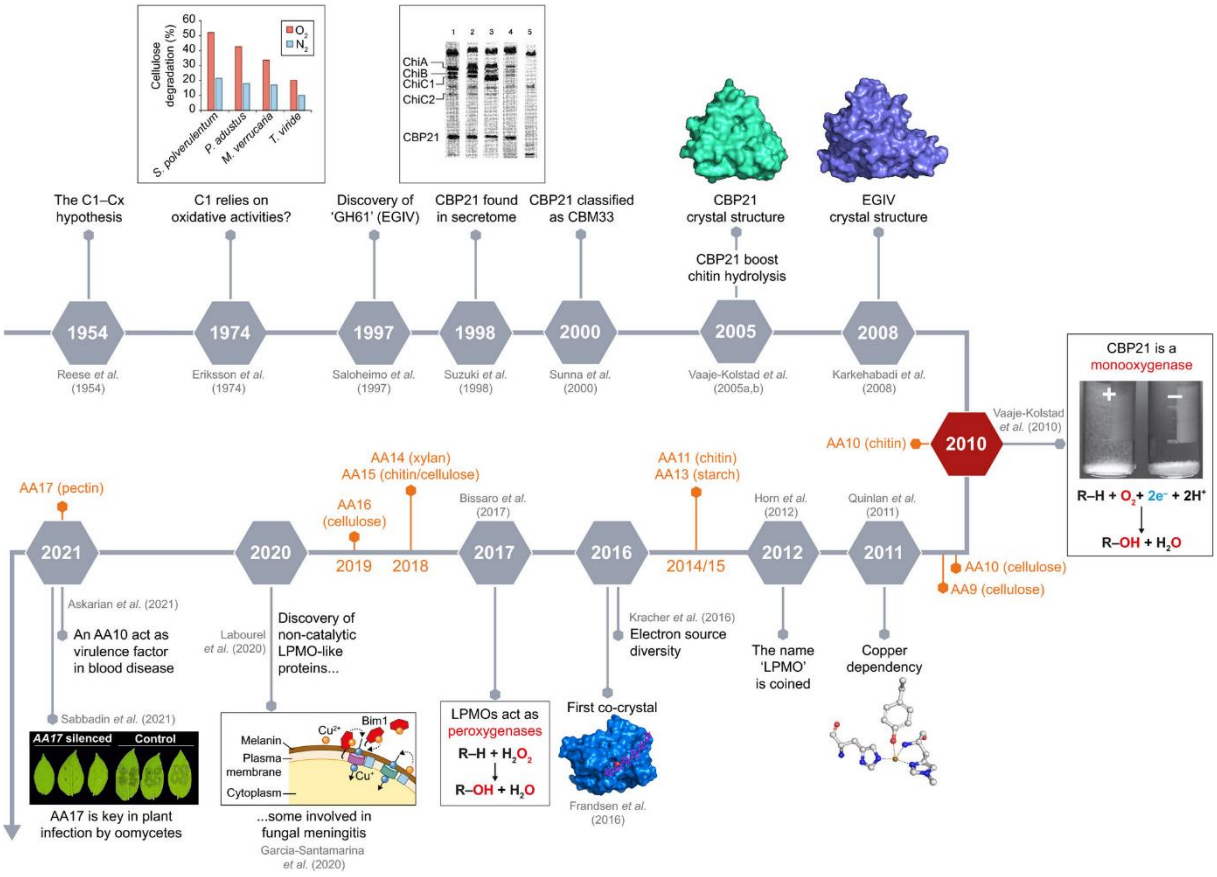
Lytic polysaccharide monooxygenases, commonly known as LPMOs, have garnered significant interest since their initial discovery in 2010. Their fascinating capacity to instigate chain breakage in inaccessible crystalline regions of recalcitrant polysaccharides, thus allowing canonical hydrolases entry, is particularly intriguing. As redox enzymes, LPMOs actively facilitate the dissolution of the glycosidic bonds within polysaccharide units. Their incorporation into enzyme cocktails, intended for polysaccharide degradation, has been a substantial boon for the bioindustry, effectively boosting product yield from biomass and thus enhancing profitability.

Despite the considerable impact and intrigue of LPMOs, a significant amount of information about this enzyme family remains unknown. In recent years, there has been a significant increase in research aimed at bridging these knowledge gaps. A primary emphasis for scientists has been the discovery of new LPMO families that target unique substrates. Such investigations may pave the way for enhanced breakdown of a more diverse array of substrates. Currently, eight families of LPMOs have been characterized in the CAZy database. However, much of the prestige in this field is derived from characterizing new families, hence there is limited research focusing on the most recent families.

Aside from the original discovery articles, there is a clear lack of comprehensive reviews of these newer LPMO families. Therefore, in this paper, I aim to present a comprehensive overview of the all the new LPMO families, outlining our current understanding and highlighting areas where knowledge is scarce. I will also conduct a comparative analysis of the key features of these families, delineating their similarities, differences, and identifying areas where our understanding is deficient. This review will serve as a foundation for my master's thesis in chemistry, providing a thorough exploration of the cutting-edge developments in the LPMO field.

BACKGROUND

LPMO research history



Figur 1 Lytic polysaccharide monooxygenase (LPMO) research timeline and milestones. The discovery of new LPMO families (along with substrate specificity) is displayed in red. The picture on the right-hand side (year 2010) shows the disruptive effect (swelling) of CBP21 on chitin in the presence of oxygen and reductant (vial labelled '+') compared with a reaction devoid of electron source ('−'). The picture on the left-hand side (year 2021) shows the loss of oomycete pathogenicity upon silencing of AA17-encoding gene. AA, auxiliary activity; CBP, chitin-binding protein; EG, endoglucanase. This figure is borrowed from (Vandhana et al., 2022).

The historical revealing and systematic classification of lytic polysaccharide monooxygenases (LPMO) is a relatively recent narrative in scientific literature, providing a rich tapestry of intriguing biochemical discoveries and theoretical underpinnings. In the mid-20th century, researchers like Reese and colleagues proposed the idea that the degradation of complex polysaccharides into simple oligomers and monomers was not a solitary task assigned to a single enzyme, but rather a cumulative effort involving multiple enzymes (Reese et al., 1950). This ground-breaking theory introduced the concept that crystalline polysaccharides

necessitated a form of enzymatic priming, which prepared them for solubilization - a pioneering idea that significantly expanded our understanding of polysaccharide breakdown.

Further emphasizing the importance of multiple enzymes in the degradation process, Erikson *et al.,* in 1974, proposed that the reduction of cellulose into simple constituents hinged on oxidative activity, marking the first association of oxidative mechanisms with polysaccharide breakdown (Eriksson et al., 1974). However, despite these early insights, the oxidative activities linked to polysaccharide degradation remained largely overlooked for several years, with scientific focus diverted to carbohydrate-binding proteins that demonstrated little to no depolymerizing activity. These proteins were classified as CBM33s, a categorization that became a placeholder in the growing knowledge base of polysaccharide degradation (Sunna et al., 2000).

In the 1990s, the field of polysaccharide degradation saw a significant stride forward when researchers identified a novel group of enzymes that appeared to be essential for cellulose breakdown. These enzymes were grouped together as the GH61 family (Saloheimo et al., 1997), a classification that advanced our understanding of the various proteins involved in the complex process of cellulose degradation.

The next pivotal point in the LPMO narrative emerged in the mid-2000s, when Vaaje-Kolstad *et al.,* postulated that the protein CBP21, a member of the CBM33 family, greatly enhanced the degradation of the polysaccharide chitin when combined with chitinases (Vaaje-Kolstad et al., 2005). In a follow-up study, the same group demonstrated that CBP21 was far more than just a binding protein; it was indeed an enzyme with the ability to cleave glycosidic bonds through an oxidative mechanism. The unique functionality of this protein required the presence of a chemical reductant, oxygen, and was dependent on a divalent metal ion (Vaaje-Kolstad et al., 2010), thereby introducing a new layer of complexity to our understanding of polysaccharide breakdown.

In a further expansion of this emerging field, several proteins from both the GH61 family and another CBM33 protein were found to display similar oxidative, catalytic activities on cellulose in 2011 (Forsberg et al., 2011; Langston et al., 2011; Phillips et al., 2011; Quinlan et al., 2011). These findings led to the incorporation of the GH61 and CBM33 families under the collective terminology of lytic polysaccharide monooxygenases or LPMOs (Horn et al., 2012). The unification of these families resulted in their reclassification in the Carbohydrate-Active

enZYmes (CAZy) database as Auxiliary Activities (AA) enzymes, referred to as AA9 and AA10 respectively (Levasseur et al., 2013).

Subsequent research led to the discovery and characterization of six additional LPMO families, namely AA11, AA13, AA14, AA15, AA16, and AA17. Each of these discoveries advanced our knowledge and understanding of LPMOs, contributing to a growing body of literature in this field (Couturier et al., 2018; Filiatrault-Chastel et al., 2019; Hemsworth et al., 2014; Sabbadin et al., 2018; Sabbadin et al., 2021b; Vu et al., 2014).

An especially notable study by Bissaro *et al.,* in 2017, challenged the long-held understanding of the nature of oxygen co-substrate in LPMOs. Their research suggested that generally excepted molecular oxygen (O_2) may not be the only oxygen donor for LPMOs and that hydrogen peroxide (H_2O_2) might act as a co-substrate as well (Bissaro et al., 2017). This theory shed new light on the complexities of LPMOs and has triggered further research into these fascinating enzymes.

Building upon these significant milestones, recent studies have explored the diversity of LPMO substrates and have suggested roles for these enzymes beyond the oxidation of polysaccharides. For instance, LPMOs may be involved in copper homeostasis (Garcia-Santamarina et al., 2020) or in cell wall remodeling processes (Labourel et al., 2020). The diverse range of LPMOs and their substrates found across microbial genomes also hint at the potential biological functions of LPMOs beyond our current understanding. This points to a future of dynamic discovery and exploration in the world of LPMO research, with profound implications for industries such as health, agriculture, biofuels, and more.

In conclusion, the ongoing exploration of LPMOs represents a fascinating journey through an ever-evolving scientific landscape. The culmination of decades of research has resulted in our current understanding of these intriguing enzymes. However, as we continue to uncover the complexities and vast potential of LPMOs, we are reminded that there is still much to learn about their roles and mechanisms. The future of LPMO research promises to be as exciting and insightful as its past, with countless opportunities for discovery and application.

The Carbohydrate-Active enZYmes Database

Launched in 1998, the Carbohydrate-Active enZYmes (CAZy) Database is a specialized repository designed for the presentation and analysis of genomic, structural, and biochemical data on Carbohydrate-Active Enzymes (CAZymes) (Cantarel et al., 2009).

The organization of the database allows for two distinct methods of accessing CAZy data. Users can either browse through sequence-based families or explore the content of genomes in carbohydrate-active enzymes. To maintain its relevance and validity, the database regularly integrates the latest published genomes. On the site, genomes are primarily organized based on the classification of the organism to which they belong—Archaea, Bacteria, Eukaryota, or Viruses—and are further alphabetized based on their scientific names (Drula et al., 2022).

CAZymes can be classified either into one of five enzyme classes or into associated modules. Enzymes falling into these classes must catalyze the degradation, biosynthesis, or modification of carbohydrates and glycoconjugates. The five classes are as follows:

1. Glycoside Hydrolases (GHs): Responsible for the hydrolysis and/or rearrangement of glycosidic bonds.
2. Glycosyl Transferases (GTs): Involved in the formation of glycosidic bonds.
3. Polysaccharide Lyases (PLs): Facilitate the non-hydrolytic cleavage of glycosidic bonds.
4. Carbohydrate Esterases (CEs): Undertake the hydrolysis of carbohydrate esters.
5. Auxiliary Activities (AAs): Comprise redox enzymes that work in concert with CAZymes.

Each enzyme class is further divided into numerous families based on sequence similarity. For a new family to be incorporated into an enzyme class, there must be published evidence verifying the activity of at least one member of the proposed family. The families in the CAZy database are regularly updated, both in terms of content and description (Drula et al., 2022). LPMOs belong to the enzyme class of Auxiliary Activities. However, it’s important to note that not all members of the AA families are LPMOs.

The Architectural Uniqueness of Lytic Polysaccharide Monooxygenases (LPMOs)

Lytic Polysaccharide Monooxygenases (LPMOs) are complex proteins that, despite their relatively small size, exhibit a unique, catalytic single-domain structure critical to their enzymatic functionality, that often have a Carbohydrate Binding Module (CBM) attached. Detailed analysis of their three-dimensional conformation reveals intricate interactions that allow these proteins to interact with and modify polysaccharides .

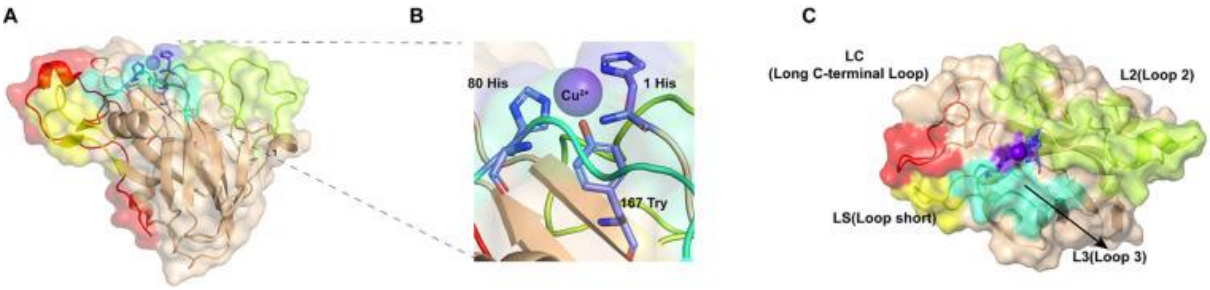


Figure 1 Topology and functional differences of the loops from AA9 LPMOs (A) Overall structure of LPMO (PDB ID: 5NNS). (B) Active center of LPMO. (C) Loops with different functional zones on the substrate-binding surface of LPMO (PDB ID: 4EIS). Figure taken form (Yu et al., 2023).

The structural architecture of a general LPMO is a β sandwich topology, which comprises 200-250 amino acids, with some LPMOs containing disulfide bonds that contribute to structural stability (**Figure 1A**) (Beeson et al., 2015). A comparison of this framework to the structure of fibronectin III reveals a striking resemblance, predominantly characterized by its β-sandwich configuration. This structure comprises 8-10 β-strands that form a robust scaffold for the active site of the enzyme (Vaaje-Kolstad et al., 2017).

Regardless of their low sequence similarity, LPMOs across all families display a common element in this β-sandwich motif, distorted to varying degrees (Beeson et al., 2015). The β-strands are interconnected via α-helices nestled within loops, contributing to the wide range of structural diversities observed among LPMOs. These loops are thought to influence the unique topology of the substrate-binding surface and the catalytic center (Courtade & Aachmann, 2019).

At the heart of LPMO structure is the active site, composed of two conserved histidine's (His) in a "histidine brace" and a Tyrosine (Tyr) or Phenylalanine (Phe), which branches to bind Cu^{2+} (**Figure 3B**) (Courtade & Aachmann, 2019). The presence of a conserved Tyr residue in most

LPMOs, oriented axially to Cu^{2+} (Jones et al., 2020), coupled with an internal conserved hydrogen bond network and water molecules, makes the active center stable and influences the regional selectivity of oxidative cleavage of glycosidic bonds (Span & Marietta, 2015).

In addition to the core structure, there are long and variable loops, namely, L2, L3, LS, and LC. These loops function as substrate-binding planes and are essential for exclusive substrate recognition (**Figure 3C**). Specifically, the region known as "loop 2" or "L2", situated between β-strand 2 and β-strand 3, plays a crucial role in identifying and determining substrate specificity. It constitutes a significant portion of the substrate-binding surface and comprises loops and short helices (Forsberg et al., 2014; Li et al., 2012; Wu et al., 2013).

LPMO catalysis

Lytic polysaccharide monooxygenases play a crucial role in the cleavage of glycosidic bonds in polysaccharides by a selective hydroxylation of a hydrogen atom at the C1 or C4 position of the substrate through an oxygen rebound mechanism with a concomitant breaking of the glycosidic bond (detailed below) (Arora et al., 2019; Phillips et al., 2011; Quinlan et al., 2011; Vaaje-Kolstad et al., 2010).

LPMO enzymatic reactions primarily encompass the removal of a hydrogen atom from a carbon atom (either at the C1 or C4 position), immediately succeeded by the cleavage of the glycosidic (C-O) bond. The enzymes can demonstrate specificity in attacking the hydrogen atom either on the C1 or C4 position. This can be seen in Figure 2. Furthermore, some LPMOs have been noted to exhibit no particular preference between the C1 and C4 positions, suggesting diverse enzymatic behaviors. Besides, instances of C6-oxidized products have been documented, albeit their significance and relationship to the chain-cleaving function of LPMOs remains an area of ongoing investigation (Bey et al., 2013; Chen et al., 2018).

In conclusion, LPMOs conduct a unique series of reactions involving the selective removal of hydrogen atoms and subsequent cleavage of glycosidic bonds. These distinctive mechanisms underline the varying substrate specificities exhibited by different types of LPMOs, thus contributing significantly to their functional diversity in biological systems (Arora et al., 2019).

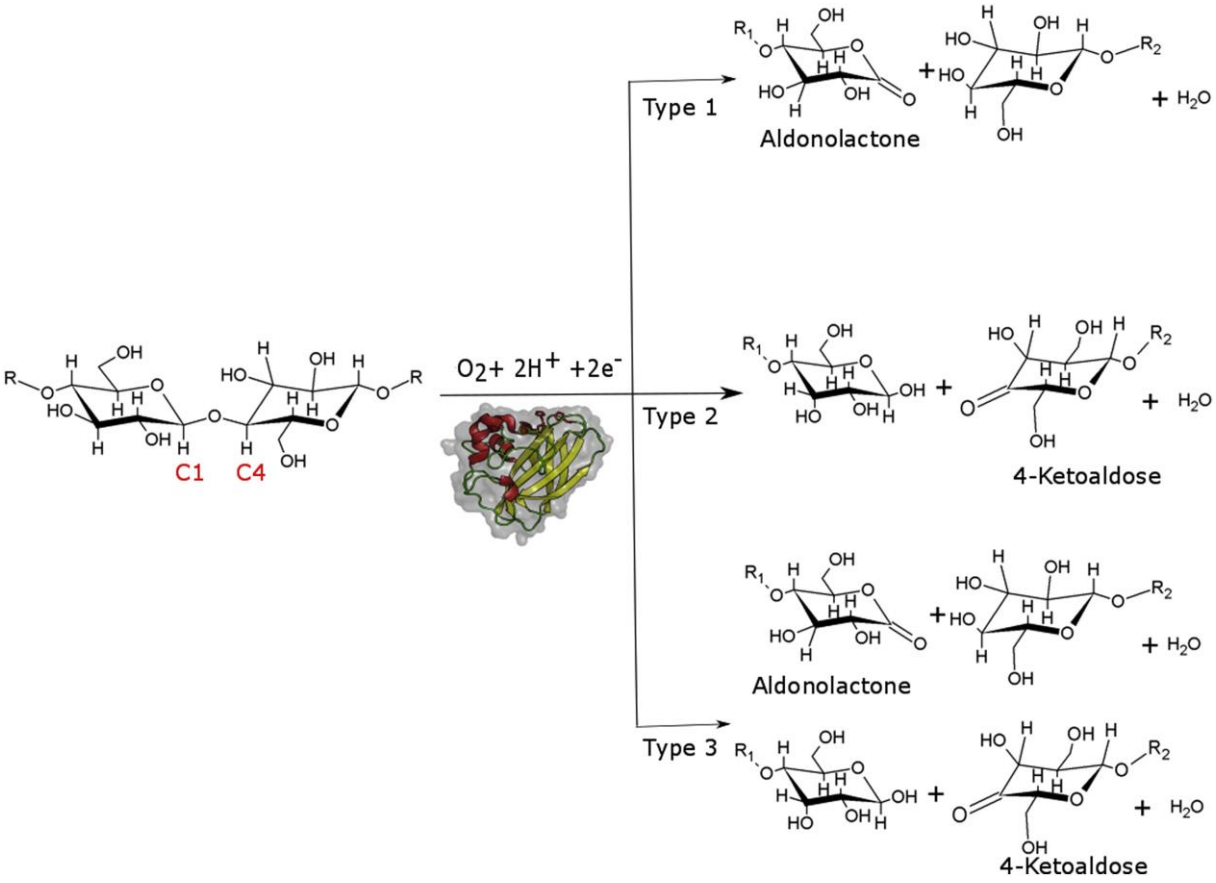


Figure 2 **LPMOs and their mechanism of action.** *Three types of LPMOs classified based on the site of attack. Image and texts taken form (Arora et al., 2019).*

The initiation of catalysis by LPMOs on glycosidic bonds hinges on the reduction of the copper ion within the enzyme's histidine brace. A transition from LPMO-Cu(II) to LPMO-Cu(I) is made possible by a reductant, a molecule proficient in electron donation (Chylenski et al., 2019). These reductants exhibit wide-ranging properties and include organic molecules like ascorbic acid, gallic acid, cystine, and reduced glutathione, along with a range of plant biomass or fungal phenolic compounds (Frommhagen et al., 2016; Kracher et al., 2016; Vaaje-Kolstad et al., 2010). It is noteworthy that photocatalytic systems can also energize LPMO reactions (Bissaro et al., 2016; Cannella et al., 2016). Furthermore, reductants could interact with co-substrates or directly participate in catalysis, a topic elaborated in subsequent sections.

In terms of co-substrates, polysaccharides frequently serve as LPMOs primary substrate, but LPMO catalysis also depends on an oxygen co-substrate. For a considerable period, O_2 was regarded as the oxygen co-substrate (Vaaje-Kolstad et al., 2010) until H_2O_2 was subsequently suggested (Bissaro et al., 2017). This sparked a debate regarding the actual natural oxygen co-substrate of LPMOs, O_2 or H_2O_2 (Kuusk et al., 2019). Regardless, it has been established that

LPMOs can employ H_2O_2 as a co-substrate, with H_2O_2-mediated reactions exhibiting faster kinetics than those driven by O_2 (Eijsink, 2019).

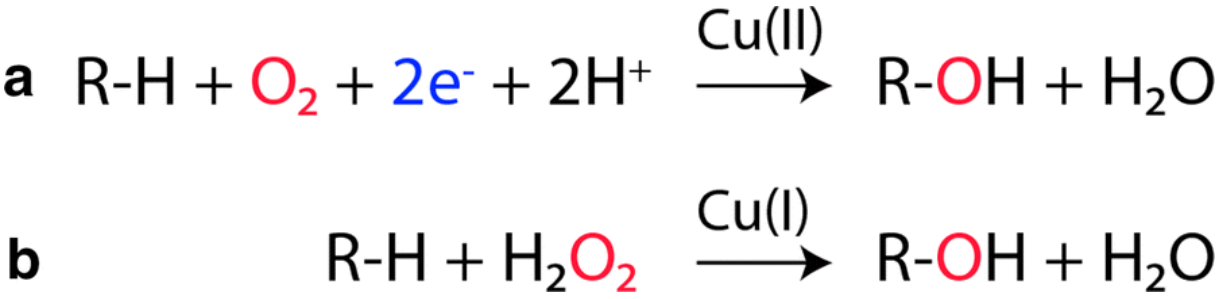


Figure 3 LPMO reaction schemes. The two panels show the reaction schemes for O_2- and H_2O_2-driven LPMO activity proposed in a) 2010 (Vaaje-Kolstad et al., 2010) and b) 2017 (Bissaro et al., 2017). The Cu(II)/Cu((I) indicated above the arrows refers to the copper ion in the active site and its oxidation state before initiation of the catalytic cycle. Figure taken form (Eijsink et al., 2019)

The exact mechanism underpinning LPMO catalysis remains ambiguous despite extensive research, further complicated by the ongoing co-substrate debate. Various mechanisms have been hypothesized and scrutinized, Fig. 4, but none have yet been definitively validated (Chylenski et al., 2019). Nevertheless, a general sequence of events can be identified, commencing with the activation of the inert LPMO molecule through the assimilation of a copper ion from the solution into the enzyme's histidine brace at the copper active site. The ensuing reactions and events remain somewhat unclear and are represented in Fig. 5.

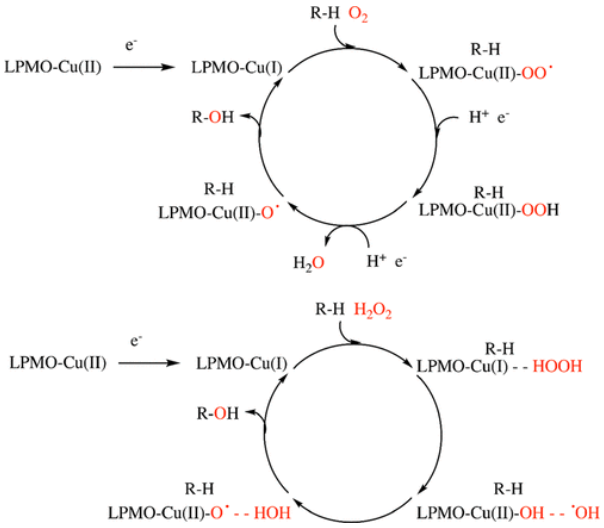


Figure 4 Schematic summaries of proposed mechanisms for hydrogen atom abstraction by an LPMO using O2 (top) or H2O2 (bottom) as the co-substrate. Figure and text taken from (Chylenski et al., 2019).

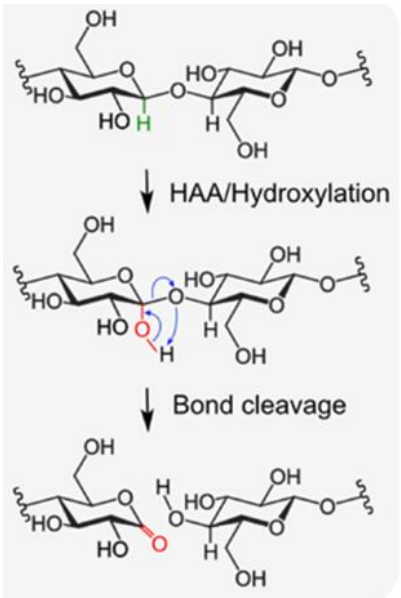


Figure 5 Schematic presentation of the mechanism of bond cleavage of the scissile glycoside bond after the C1 carbon is attacked by the LPMOs oxygen radical. Figure and text taken form (Eijsink, 2019)

Like other metallo-redox enzymes, LPMOs exhibit vulnerability to oxidative damage under non-optimal conditions, often resulting in non-linear progress curves of LPMO reactions (Chylenski et al., 2019). Oxidative damage to amino acid residues in proximity to the copper site often accompanies LPMO inactivation (Bissaro et al., 2017; Loose et al., 2018). This autocatalytic inactivation occurs irrespective of the fueling process (Chylenski et al., 2019).

When considering O_2 versus H_2O_2's role in LPMO catalysis, in the absence of a substrate, the reduced Cu(I) ion of LPMOs may interact with O_2 or H_2O_2, potentially generating harmful species such as hydroxyl radicals (Chylenski et al., 2019). In the absence of substrate binding, these species are prone to react with nearby components, possibly the copper-coordinating histidine (Bissaro et al., 2017). Interestingly, the presence of substrate seemingly protects LPMOs from oxidative inactivation, and a correlation between the LPMO's substrate affinity and its stability has been established (Chylenski et al., 2019). Further, LPMO stability is augmented at higher substrate concentrations (Courtade et al., 2018). Thus, environments replete with substrate are optimal for LPMOs to reduce autocatalytic oxidative damage. However, in industrial applications, especially during the bioprocessing of lignocellulosic biomass, maintaining high substrate concentrations is challenging due to the changing amount and nature of the substrate, thereby hastening enzyme inactivation. Hence, during the latter stages of a typical bioprocessing reaction involving a commercial cellulase cocktail containing LPMOs, when the enzyme is most needed to tackle the remaining resilient substrate, the LPMO may be inactive (Chylenski et al., 2019).

THE NEW LPMO FAMILIES

The establishment of a new LPMO family

Establishing a new Auxiliary Activities (AA) LPMO family within the Carbohydrate-Active enZymes (CAZy) system usually unfolds through a series of methodically structured stages. The process initiates with the identification of a novel LPMO, an accomplishment frequently realized via diverse approaches, ranging from genomic or metagenomic sequencing of microorganisms to advanced genetic engineering and other sophisticated biotechnological methodologies.

Upon the discovery of a new LPMO, a rigorous sequence analysis is conducted, where the enzyme's amino acid sequence is juxtaposed against the sequences of established LPMO families. A considerable divergence from known sequences might hint towards the enzyme's potential affiliation with a new, as yet unidentified, family.

Subsequent to sequence analysis, it becomes imperative to carry out a comprehensive functional characterization to discern the substrate specificity and catalytic activity of the newly discovered LPMO. Given the varied substrate preferences—such as cellulose, chitin, or hemicelluloses—and distinctive oxidative behaviors at different carbon positions (C1 or C4, or both) among LPMO families, these functional idiosyncrasies significantly contribute to determining the enzyme's unique identity.

Following the functional analysis, the focus shifts to an in-depth structural exploration of the enzyme, especially its active site and any key motifs. Sophisticated techniques such as X-ray crystallography or cryo-electron microscopy offer invaluable insights into the enzyme's structure, potentially strengthening the hypothesis of the novelty of the LPMO family.

The culmination of the process necessitates a thorough comparison of the newly identified LPMO with existing LPMO families and similar enzymes. This exhaustive comparative analysis encapsulates structural, functional, and evolutionary dimensions, shedding light on subtle nuances that may distinguish the new enzyme from its predecessors.

Upon the identification of a significant group of similar LPMOs sharing unique sequence, structural, and functional attributes, they might be grouped into a new family within the CAZy database. However, what is considered "significant" can vary, and the decision often depends on the collective agreement of experts in the field. Also, this classification process is flexible and can change over time. If new information comes up, it may lead to a change in the classification, helping us keep up-to-date with the latest knowledge about LPMOs.

The Auxiliary Activity Family 11

The Auxiliary Activity Family 11 (AA11) came to prominence following Hemsworth *et al.*'s seminal study in 2014, which established the bioactivity of an AA11 protein derived from *Aspergillus oryzae* (Hemsworth et al., 2014). This discovery solidified AA11 as the third branch of the LPMO superfamily and the inaugural addition of a novel family to the Carbohydrate-Active enZYmes (CAZy) database.

The identification of this new family utilized a "model waking approach," directed at an unclassified domain, X278, noted in AA9s, and presumed to be a chitin-binding domain. The deployment of a BLAST search against this domain revealed an AA11 candidate featuring a conserved N-terminal histidine. A subsequent BLAST search using a distinct module from the candidate yielded around 450 sequences presumably associated with a putative AA11 family. Importantly, these sequences presented no significant resemblance to the AA9 or AA10 families, prompting the formation of a discrete LPMO family (Hemsworth et al., 2014).

Predominantly, the AA11 LPMO family appears to consist of enzymes of fungal origin (Hemsworth et al., 2014; Stopamo et al., 2021), with the only exceptions being three metazoan AA11 genes identified in the multicellular organism *Adineta vaga*. Nonetheless, these genes are postulated to have evolved from fungi via horizontal gene transfer (Hemsworth et al., 2014). Consequently, AA11, along with AA9, AA13, AA14, and AA16, comprise fungal LPMO families. Like parts of the AA10 and AA15 families, AA11 exhibits chitin activity, albeit without evidence of activity on alternative substrates.

The first comprehensive exploration of this family focused on *Ao*AA11, a two-domain AA11 protein from the filamentous fungus A. *oryzae*. Despite the incomplete crystal structure, lacking two regions comprising 30 residues in total, including residues 151-161 (Hemsworth et al., 2014), our initial understanding of the AA11 family was largely extrapolated from this study. In 2021, Stopamo *et al*. unveiled the first complete crystal structure of a single domain AA11 from *Aspergillus fumigatus*, termed *Af*AA11A(Stopamo et al., 2021). In the absence of more comprehensive data, it is necessary to consider *Ao*AA11 and *Af*AA11A as representative models for the family, which exhibits substantial structural diversity.

The tertiary structure of *Ao*AA11 bears resemblance to other LPMO families. Its protein core comprises a large antiparallel β-sandwich fold, stabilized by three disulfide bonds with a copper

active site nestled in the center of a mildly concave face. The structure of both *Ao*AA11 and *Af*AA11A are shown in Figure 6. The binding surface of *Ao*AA11 accommodates residues that can form hydrogen bonds with prospective carbohydrate substrates, akin to AA10. A hydrophilic residue pathway traverses the protein core to the active site, alluding to a potential electron transport route, pending empirical confirmation (Hemsworth et al., 2014). With approximately 220 residues, the catalytic domain of *Ao*AA11 encases two histidine's forming a histidine brace that coordinates the copper ion. In addition, it houses a tyrosine sidechain close to the copper ion and a conserved secondary coordination-sphere alanine.

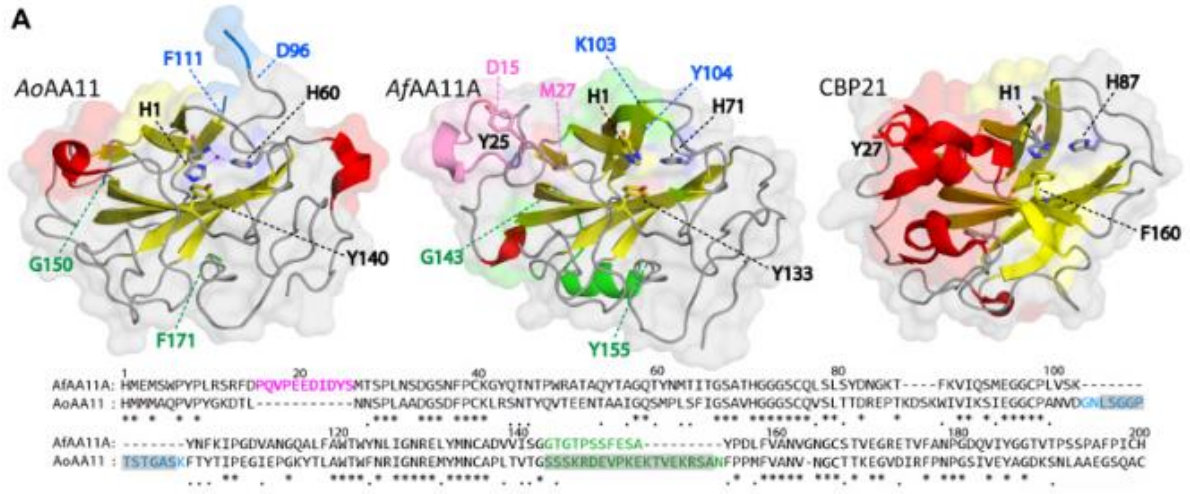


Figure 6 ***Comparison of chitin-active LPMOs.*** *A, the structures of AfAA11A, AoAA11, and CBP21, colored by secondary structure (helices red, strands yellow) and with additional coloring according to the sequence alignment underneath, showing side chains of residues involved in copper coordination and substrate binding. The 11-residue insertion in AfAA11A relative to AoAA11 that includes Tyr25 appears in pink, and residues adjacent to this insertion are labeled. The 14-residue insertion near residue 100 in AoAA11 appears in blue, and residues adjacent to the insertion are labeled in both AA11 structures; note that 11 of these residues (gray shading in the sequence) could not be modeled in the AoAA11 structure. Eight of the nine residues forming an insertion near residue 165 in AoAA11 as well as 11 preceding residues could not be modeled in the AoAA11 structure; this 19-residue region is colored green, and adjacent residues are labeled. Figure and text taken form (Stopamo et al., 2021)*

*Ao*AA11 appears to instigate primary chain cleavage, primarily yielding aldolic acid oligosaccharides with even-numbered degrees of polymerization (DP= 4, 6, 8, 10…). This behavior typically suggests that AoAA11 might predominantly act in a C1-specific manner, as C1-oxidizing LPMOs generally produce aldonic acids with even DP. However, the presence of unmodified oligosaccharides (DP = 5,6,7,8,9…) and species with a mass difference of -2 Da which implies that there could be more to *Ao*AA11's mechanism than simply C1-oxidation. This -2 Da difference might indicate the formation of C4-oxidized products (products of C4-specific LPMO action), or it could represent unopened lactones, a type of ring-shaped molecule that can form when C1-oxidized products rearrange (Hemsworth et al., 2014). In contrast,

*Af*AA11A, similar to bacterial chitin-active AA10 LPMOs, manifests significant C1-oxidizing activity, culminating in the production of lactone products (Stopamo et al., 2021). Furthermore. *Af*AA11A has also been shown to efficiently utilize H_2O_2 as a co-substrate and isn't inactivated by acting in "peroxygenase" mode, as long as H_2O_2 levels are not too high. *Ao*AA11 has been experimentally ascertained to be non-functional in the absence of an electron donor, yet the natural electron donor for AA11 remains elusive (Hemsworth et al., 2014).

The Auxiliary Activity Family 13

Established in 2014, Auxiliary Activity Family 13 (AA13) is a group of LPMOs primarily active on starch and related substrates. This revelation was put forth in a seminal 2014 paper by Vu *et al*., solidifying AA13 as the fourth recognized family in the LPMO superfamily, and the first known to be starch-active (Vu et al., 2014). The discovery of AA13 emerged from the screening of *Neurospora crassa* proteins for sequences with specific signal peptides using the SignalP 4.0 program. Vu *et al*. then selected sequences bearing N-terminal histidines a conserved feature in LPMOs, which were subsequently compared to orthologs discovered in protein databases. By identifying the presence of a second conserved histidine residue and the N/Q/E-X-F/Y motif a crucial signature sequence in the primary structure of LPMO's. This motif refers to a specific arrangement of amino acids within the protein sequence, where N/Q/E denotes asparagine, glutamine, or glutamic acid, X represents any amino acid, and F/Y denotes phenylalanine or tyrosine. From this potential LPMOs were singled out (Beeson et al., 2015). Notably, the researchers showed particular interest in one protein (NCU08746 from the fungus *Neurospora crassa*), displaying weak homology with chitin-binding domains and C-terminal carbohydrate binding module 20 (CBM20). This protein was identified as AA13 (Vu et al., 2014).

As of now, AA13 remains the only LPMO family known to act on starch and associated substrates. Although attempts to demonstrate activity on other substrates were made, no such activity could be detected (Lo Leggio et al., 2015). Four enzymes have been characterized so far within this family: *Nc*AA13 from *Neurospora crassa*, *An*AA13 from *Aspergillus nidulans*, *Ao*AA13 from *Aspergillus oryzae*, and *At*LPMO13A from *Aspergillus terreus (Lo Leggio et al., 2015; Vu et al., 2014)*. It's worth noting that *Ao*AA13 is the only enzyme within this family for which a structure has been solved (Lo Leggio et al., 2015).

The structure of *Ao*AA13 includes a β-sandwich immunoglobulin-like fold at its core, and it displays several additional helical secondary structure elements. A distinct groove, running through the enzyme's active site, likely accommodates the helical structure of an amylopectin substrate. Key structural elements of this surface groove are found in regions like the long loop preceding β2 and the loop between β5 and β6 (Lo Leggio et al., 2015). This can be seen in Fig. 8.

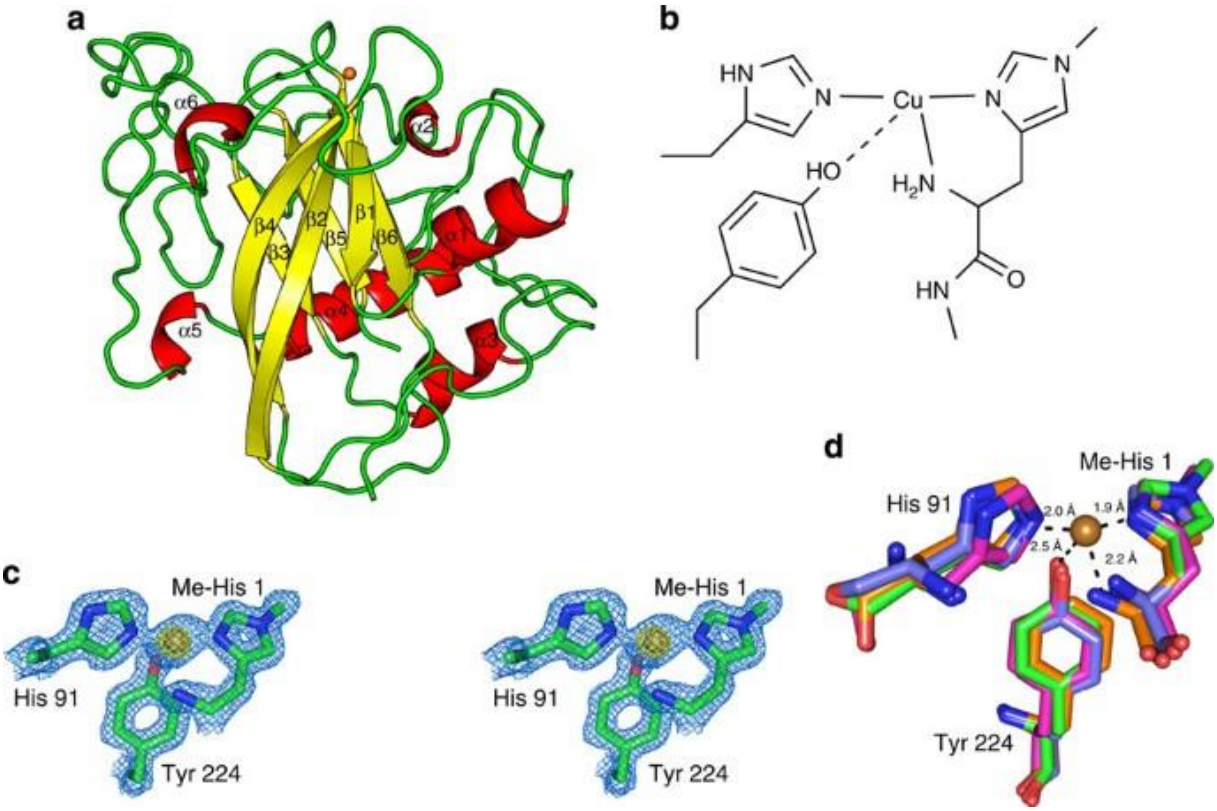


Figure 7 **Structure of Ao(AA13).** *(a) Ribbon view of overall structure with numbered secondary structure elements, copper ion shown as orange sphere; (b) line diagram of active site; (c) stereo view of the electron density map around the active site in blue (contoured at 1.5σ) with anomalous difference density in yellow (contoured at 25σ), note methylation of N-terminal histidine (the map is calculated from the final refined structure with data collected at a wavelength of 1.037 Å); (d) comparison of active site of AA13 (green) with AA9 member (Protein Data Bank (PDB) 3ZUD, magenta, r.m.s.d. for protein atoms shown of 0.73 Å), AA10 member (PDB 2YOY, orange, r.m.s.d. of 0.53 Å) and AA11 member (PDB 4MAI, purple, r.m.s.d. of 0.60 Å). Figure and text taken form (Lo Leggio et al., 2015).*

Similar to other LPMOs, the active site in AA13 encompasses a mandatory histidine brace. The copper ion within the active site is coordinated by several residues: the terminal NH_2 and the side chain of the N-terminal histidine, an additional histidine side chain (His91), and a tyrosine (Tyr224), which are all invariant across the AA13 sequence family. A loop containing a glycine approaches the copper ion from the opposite side(Lo Leggio et al., 2015). Notably, the N-terminal histidine in AA13s, when expressed in filamentous hosts, is found to be τ-N methylated.

AA13 enzymes engage in oxidative cleavage of α-1,4-linked glucose polymers in starch, utilizing copper ions and an electron donor. Specifically, AA13s introduce oxidation at the C1 position of the sugar ring, producing lactone-terminated oligosaccharides, which subsequently hydrate to form aldonic acid-terminated maltodextrins (Lo Leggio et al., 2015). Given their C1 specificity, it's suggested that these LPMOs should be able to oxidatively attack both α-1,4- and α-1,6-linkages found in amylopectin.

Interestingly, the nature of the electron donor seems to impact enzyme activity. For instance, reactions using cytosine as the electron donor display greater activity than those using ascorbic acid, a commonly used LPMO activator (Lo Leggio et al., 2015). Some studies also indicate that cellobiose dehydrogenase (CDH), a natural electron donor for cellulose-active LPMOs, may function as the natural electron donor for AA13 catalysis (Vu et al., 2014).

The Auxiliary Activity Family 14

The Auxiliary Activity Family 14 (AA14), the fifth identified LPMO family, was introduced in a pivotal 2018 paper by Couturier *et al*. Initially, this finding was considered a significant advancement in our knowledge of LPMOs diverse roles in plant biomass degradation (Couturier et al., 2018). However, a 2023 paper by Tuveng *et al.* later questioned this premise (Tuveng et al., 2023). The unveiling of the AA14 family came about through meticulous experimental procedures. Detailed studies were conducted on *Pycnoporus coccineus*, a white-rot fungus, examining its growth on diverse biomass types while integrating transcriptomic and secretomic investigations. Consequently, they found a gene displaying prominent upregulation on pine and poplar compared to maltose conditions. The subsequent sequence alignment of more than 300 proteins revealed a conserved N-terminal histidine, thereby implying the existence of a new enzyme family distinct from previously known LPMOs (Couturier et al., 2018).

Members of the AA14 family are of fungal origin and are primarily produced by white-rot and brown-rot basidiomycetes. These enzymes were initially considered active on xylans, robust polysaccharides abundant in wood biomass. This activity, however, was only evident when the enzymes were adsorbed onto crystalline cellulose, but not in a solution state (Couturier et al., 2018). Nevertheless, Tuveng *et al.*'s 2023 paper has contested the categorization of xylans as

substrates for the AA14 family, suggesting that the true substrates may still be undiscovered (Tuveng et al., 2023).

To date, three members of this family, namely, *Pc*AA14A, *Pc*AA14B, and *Tr*AA14A, have been characterized (Couturier et al., 2018; Tuveng et al., 2023). *Tr*AA14A is derived from *Trichoderma reesei*, while *Pc*AA14A and *Pc*AA14B are from *Trametes coccinea*. The elucidation of the crystal structure of *Pc*AA14B has offered insights into the unique structural attributes of the AA14 family (Tuveng et al., 2023).

*Pc*AA14A exhibits a distinctive three-dimensional structure. The core comprises an antiparallel immunoglobulin-like β-sandwich. The binding surface of the protein is not flat but rippled, with a clamp formed by two conspicuous surface loops. Its active site, composed of His1, His99, and Tyr178, showcases a surface-exposed histidine brace in the center of the binding face and a conserved tyrosine residue (Tyr240) (Couturier et al., 2018).

Couturier *et al*, believe they observed that *Pc*AA14A produces C1-oxidized products, this was also challenged by Tuveng, *et al*, that believed that the oxidation observed was from another reaction. Additionally, both *Tr*AA14A and *Pc*oAA14A have been observed to produce and consume H_2O_2, suggesting their potential roles in hydrogen peroxide formation and degradation. These enzymes host approximately one copper atom per protein molecule in their active site, a characteristic feature of LPMOs (Tuveng et al., 2023).

The transcription and the functional roles of AA14 LPMOs in fungi during wood degradation are yet to be definitively determined. Nevertheless, their genes have been identified across various tissues during fruiting body development in several basidiomycete species. Notably, a significant number of AA14 sequences have intrinsically disordered C-terminal regions (dCTRs), potentially indicating their yet-to-be-discovered roles in fungal physiology (Tuveng et al., 2023).

Despite the apparent parallels with canonical LPMOs, the true catalytic activity and biological role of AA14 proteins remain elusive. Existing data do not endorse the previously suggested oxidative catalytic activity on cellulose-associated xylan. As such, comprehensive research is warranted to elucidate the authentic functions of these intriguing enzymes in the natural world.

The Auxiliary Activity Family 15

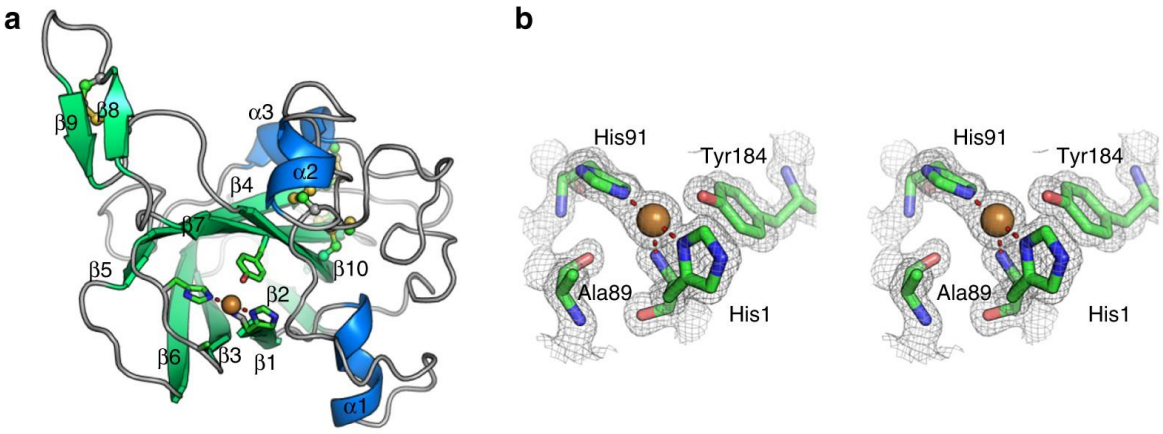


Figure **8 Structural and spectroscopic characterization of TdAA15A**. *a) The overall structure of TdAA15A is shown colored by secondary structure. The protrusion formed by strands β8 and β9 is clearly shown extending the surface surrounding the active site. Disulfide bonds are shown in ball and stick with sulfur atoms colored yellow. B) Stereo view of the electron density observed at the copper active site of TdAA15A (2mFo-Fc map contoured at 1σ). The histidine brace coordination of the copper, which is in the Cu(I) state due to photoreduction in the X-ray beam, is shown by red dashed lines. The copper ion is shown as a golden colored sphere.(Sabbadin et al., 2018)*

The sixth lytic polysaccharide monooxygenases family, auxiliary activity Family 15 (AA15), was discovered during a detailed exploration of the digestive proteome of *Thermobia domestica* (Sabbadin et al., 2018). The use of shotgun proteomics revealed the organism's ability to degrade crystalline cellulose to monomeric units. This investigation brought attention to a set of 21 Carbohydrate-Active enZymes (CAZy) with unknown function, which were notably abundant in specimens fed on crystalline cellulose (Avicel).

To uncover the identity of these enzymes, the identified sequences were cross-referenced with public databases (BlastP vs NCBI nr databases), revealing hundreds of orthologous sequences in annotated genomes across various organisms, including marine and terrestrial invertebrates, algae, and oomycetes. These sequences exhibited 20-30% amino acid sequence similarity to known LPMOs, with the most striking similarity being a conserved N-terminal histidine brace responsible for active site copper coordination. When these sequences were compared with the Hidden Markov models (HMMs) of existing LPMO families (AA9-AA11, AA13, and AA14), no significant matches were found. However, a newly created HMM, derived from multiple sequence alignment, revealed excellent hits, leading to the establishment of the new CAZy LPMO family, AA15 (Sabbadin et al., 2018).

Organisms hosting the AA15 family members span across diverse lifeforms, including viruses (14.5%) and various eukaryotes (85.5%) such as algae, oomycetes, and complex animals

(Vandhana et al., 2022). Early characterizations of AA15 LPMOs from T. *domestica* indicated activity on both cellulose and chitin (Sabbadin et al., 2018), whereas more recent investigations on two AA15 LPMOs from the termite *Coptotermes gestroi* (Cairo et al., 2021), and one from the oomycete pathogen *Aphanomyces astaci* (Sabbadin et al., 2021a), suggested exclusive activity on chitin.

To date, five members of the AA15 family (*Td*AA15A, *Td*AA15B, *Cg*AA15A, *Cg*AA15B, and *Aa*AA15A) have been characterized, with *Td*AA15A being the only one whose crystal structure has been elucidated. *Td*AA15A revealed typical LPMO features such as a β-sandwich fold, decorated with diverse loops and stabilized by five disulfide bonds. The active site harbors a characteristic histidine brace (His1 and His91), coordinating the essential copper co-factor in a T-shaped geometry. A distinctive feature of *Td*AA15A is a unique β-tongue-like protrusion linking strands 8 and 9 and shaping the surface surrounding the active site. Despite the active site's conservation throughout the family, the β-tongue-like protrusion varies among members, implying differing roles within the family (Sabbadin et al., 2018).

In terms of oxidative activity, AA15 LPMOs demonstrate exclusive ability for C1 oxidation. Their potential role in chitin remodeling is currently an active area of study (Sabbadin et al., 2018). Notably, the chitinolytic activity of AA15 LPMO from the crayfish pathogen *Aphanomyces astaci* has been highlighted (Sabbadin et al., 2021a), underlining the significant role of AA15 in the pathogenicity of oomycetes. Comprehensive *in vivo* studies to further elucidate this function are currently underway (Vandhana et al., 2022).

The Auxiliary Activity Family 16

The Auxiliary Activity Family 16 (AA16) gained recognition following Filiatrault-Chastel *et al*.'s seminal work in 2019, which characterized the bioactivity of an AA16 protein derived from *Aspergillus aculeatus* (Filiatrault-Chastel et al., 2019). The discovery of AA16, first proposed by Voshol *et al*. in 2017, added another branch to the lytic polysaccharide monooxygenase (LPMO) superfamily, increasing the number of novel families within the Carbohydrate-Active enZYmes (CAZy) database.

The identification of this family was done using a comparative secretomic approach. Five fungal strains, predominantly from *Aspergillus japonicus* and one strain of *Aspergillus niger*, were cultured with various inducers, after which the secretomes were tested for their ability to

increase the efficacy of a *Trichoderma reesei* cocktail in performing saccharification of different steam-exploded biomasses. A protein of unknown function, exclusively found in maize bran and sugar beet pulp secretomes of *A. japonicus* BRFM 1490, was found to enhance Miscanthus scarification. Sequence analysis revealed a conserved histidine post signal peptide removal, akin to what is expected in LPMOs (Filiatrault-Chastel et al., 2019).

This newly characterized LPMO family is primarily produced by filamentous fungi and so far, only three enzymes - *Aa*AA16 from *Aspergillus aculeatus*, *Mt*AA16A from *Myceliophthora thermophila* and *An*AA16A from *Aspergillus nidulans* - have been characterized. Interestingly, AA16 is found to exhibit cellulose activity (C1 oxidizing). To date, the only crystal structure available for this family is of *Mt*AA16A, unveiled by Sun *et al*. in 2023(Sun et al., 2023).

Distinct features of this family include a catalytic domain often followed by a C-terminal extension of varying lengths. In some cases, this extension is constituted by a linker and a CBM1 module or a glycosylphosphatidylinositol anchor. The tertiary structure of *Mt*AA16A demonstrates a high structural similarity with AA9, AA10, and AA11 family members. It holds the typical LPMO fold and contains one copper atom per molecule. However, it has a considerably smaller size compared to the matching structures. The copper binding site is consistent with AA9, AA11, AA13, AA15, AA17, and some AA10 members, with the histidine brace providing three equatorial ligands and an additional Tyr axial ligand (Sun et al., 2023).

*Aa*AA16A was found to display substantial activity on cellohexaose and cellulose substrates, resulting in a majority of non-oxidised products (DP2-DP5) and a small peak corresponding to C1 oxidizing products ($DP2_{ox}$-$DP4_{ox}$). *Aa*AA16A works synergistically with other cellulase degrading enzymes, suggesting potential for biotechnological applications. Conversely, *Mt*AA16A and *An*AA16A displayed no oxidizing activity on any tested carbohydrates. Instead, *Mt*AA16A demonstrated the ability to oxidize syringol-like compounds in the presence of H_2O_2.

Despite the discovery of the AA16 family in 2019, there's still a rareness of data regarding the electron pathway, the natural reductant, and whether O_2 or H_2O_2 is preferentially used. Although *Aa*AA16A has been found to produce H_2O_2 in the absence of substrate and presence of L-cysteine and ascorbate, further studies are needed to draw definitive conclusions on the catalytic mechanism of this family. Nevertheless, *Aa*AA16A and its demonstrated traits serve as crucial steppingstones towards a better understanding of the AA16 family.

The Auxiliary Activity Family 17

The discovery and establishment of Auxiliary Activity Family 17 (AA17) as a distinct LPMO group was due to the work of Sabbadin *et al*., as published in their 2021 Science paper (Sabbadin et al., 2021b). The team discovered a group of proteins lacking functional domains but possessing significant homology, and importantly, an N-terminal signal peptide for secretion. These proteins were marked by an N-terminal histidine and another conserved histidine, which could form a "histidine brace," the distinguishing characteristic of LPMOs.

When these proteins were analyzed using homology searches against the nonredundant protein sequences databases at the National Center for Biotechnology Information (NCBI), it was discovered that this new gene family was exclusive to oomycetes. Particularly, it was expanded in hemi-biotrophic and necrotrophic plant pathogenic species, with an average of 46 family members found in *Phytophthora* spp. This LPMO family, now termed auxiliary activity 17 (AA17), was then included in the Carbohydrate-Active enZYmes (CAZy) online database (Sabbadin et al., 2021b).

The AA17 family of LPMOs are predominantly produced by oomycetes, with their enzymatic activity focused on pectin. In-depth analysis through MALDI-TOF MS and ESI MS unveiled distinct oxidized and native product peaks when homogalacturonan and oligogalacturonides [degree of polymerization (DP) 10 to 15] were used as substrates. This discovery was unprecedented, as no prior LPMO activity on charged polysaccharides had been reported. AA17 LPMOs seem to specifically recognize the carboxylic groups of de-esterified pectin (Sabbadin et al., 2021b).

Three LPMOs from the AA17 family have been characterized, all from *Phytophthora infestans*: *Pi*AA17A, *Pi*AA17B, and *Pi*AA17C. Furthermore, the 3D structure of one of these, *Pi*AA17C, has been elucidated. Notably, most AA17 genes encode solely for the catalytic domain, though certain sequences from *Saprolegniales*, fish pathogens, have putative C-terminal cellulose-binding domains, protein-protein, or protein-carbohydrate interaction domains. Some sequences from *Phytophthora parasitica* even feature putative chitin-binding domains (Sabbadin et al., 2021b).

The *Pi*AA17C structure, akin to other LPMO families, reveals a central β-sandwich fold adorned with several loops and stabilized by three disulfide bonds. The active site features a

histidine brace, necessary for copper coordination. The electrostatic surface potential and residue charge distribution are distinct from typical LPMOs active on crystalline cellulose or chitin, which display a flat surface surrounding the active site. By contrast, the *Pi*AA17C active site lies within a cleft. The AA17 family seems to have the capability to bind charged polysaccharides, indicated by the conservation of several polar and negatively charged residues (Sabbadin et al., 2021b).

The AA17 LPMOs have demonstrated an exclusive ability for C4 oxidation. Sabbadin *et al.* propose that *Pi*AA17A, -B, and -C carry out a C4-oxidative cleavage of polygalacturonic acid, generating a C4-ketone in a β position relative to one carboxylic group, leading to an unstable β-keto acid that subsequently undergoes spontaneous decarboxylation and tautomerization (Sabbadin et al., 2021b).

Furthermore, it was observed that AA17s can accept electrons from ascorbic acid, but not from small phenolic compounds. This characteristic may be a reflection of the P. *infestans* life cycle, wherein the pathogen invades fresh host tissue, rich in cellular reductants like ascorbic acid, rather than lignified tissue, a source abundant in phenolic compounds (Sabbadin et al., 2021b).

Taken together, these findings illustrate the unique biochemical characteristics and biological functions of the AA17 family of LPMOs. Notably, AA17 genes are upregulated during plant infection, indicating their critical role in the pathogenicity of oomycetes. Further research is required to understand the implications of these findings more fully (Sabbadin et al., 2021b).

COMPARISON OF LPMO AA FAMILIES

Tabell 1 ***Comparison of LPMO families***

Family	Sequence	Characterized members	Resolved structure	Microbial origin	Substrate
AA9	1078	38	21	Fungi	Cellulose, hemicellulose, xylan, xyloglucan, galactoglucomannan
AA10	9999	37	27	Bacteria (mostly), fungi, archaeans, viruses	Chitin, cellulose, xylan
AA11	295	2	2	Fungi(mostly), animals	Chitin
AA13	40	4	1	Fungi	Starch
AA14	66	3	1	Fungi	Xylan (in question)
AA15	438	5	1	Fungi, viruses, archeaens, animals, plants	Cellulose, chitin
AA16	86	3	1	Fungi, viruses, oomycetes	Cellulose
AA17	421	3	1	Oomycetes	Pectin

Having examined the individual LPMO families, let's now venture into a comparative analysis of these enzymes. The LPMO families include AA9 and AA10, both established in 2010 through a recategorization of pre-existing CAZy families (Vaaje-Kolstad et al., 2010), as well as the more recent AA11, AA13, AA14, AA15, AA16, and AA17 families, which were all delineated between 2014-2021 via unique methodologies applied to extant AA LPMO families.

A significant observation lies in the research disparity between AA9 and AA10 and the six newer families. As tabulated in Table 1, the AA9 and AA10 families boast a greater number of identified LPMO gene sequences, characterized members, and resolved crystal structures. Conversely, among the newer families, only AA11 has more than one resolved structure, albeit

with the fewest characterized members. Other newer families such as AA14, AA16, and AA17 boast three characterized members each, while AA13 and AA15 present four and five members, respectively. This pales in comparison to the AA9 and AA10 families, with 21 and 27 characterized members in that order.

The distribution of these families across various species or organism groups also varies. The AA9, AA13, and AA14 families are limited to fungi, whereas AA11 is predominantly fungal, with a few metazoan AA11 genes identified in *Adineta vaga*, believed to have evolved via horizontal gene transfer. The AA10 family exhibits the broadest taxonomic coverage, encompassing bacteria, viruses, archaea, and pathogenic fungi from the Ustilaginomycetes class. AA15 enzymes are found in a variety of organisms, including viruses, algae, oomycetes, and complex animals, whereas AA16 is mainly found in fungi and oomycetes. Finally, the recently established AA17 family is exclusive to oomycetes, with a significant expansion of gene copies observed in hemibiotrophic and necrotrophic pathogens.

When considering substrate specificity, each family of LPMOs appears to have a preference for certain substrates. As of current knowledge, which is derived from characterized enzymes and hence subject to discovery of yet untested substrates, most families display narrow substrate specificity. For instance, AA11 targets chitin, AA13 starch, AA14 xylan, AA16 cellulose, and AA17 pectin. However, the AA9, AA10, and AA15 families encompass multiple substrate specificities. The AA9 family primarily targets glucose-based polymers, such as cellulose and cello-oligosaccharides, as well as certain hemicelluloses. AA10 enzymes can act on chitin, cellulose, or both, with one characterized member displaying broader substrate specificity that includes xylan. The AA15 family has shown activity on both cellulose and/or chitin.

Our next point of comparison is catalytic activity, in reference to the oxidative mechanisms of LPMOs. Some act on the C1 position of the sugar molecule, others at the C4 position, and some display mixed regioselectivity. Specifically, AA11, AA13, AA15, and AA16 are known to act exclusively on the C1 position. AA17 is unique in its action on the C4 position, while AA10 can act on both C1 and C4 positions, and AA9 can act on C1, C4, and also C6.

To conclude, while all LPMO families demonstrate some common structural motifs - primarily the immunoglobulin-like β-sandwich fold and a copper-coordinating histidine brace at the active site, their variations underscore the adaptability of these enzymes to perform different functional roles across diverse substrates and conditions.

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