Current understanding of substrate specificity and regioselectivity of LPMOs

Xiaoli Zhou and Honghui Zhu

Abstract
Renewable biomass such as cellulose and chitin are the most abundant sustainable sources of energy and materials. However, due to the low degradation efficiency of these recalcitrant substrates by conventional hydrolases, these biomass resources cannot be utilized efficiently. In 2010, the discovery of lytic polysaccharide monooxygenases (LPMOs) led to a major breakthrough. Currently, LPMOs are distributed in 7 families in CAZy database, including AA9–11 and AA13–16, with different species origins, substrate specificity and oxidative regioselectivity. Effective application of LPMOs in the biotransformation of biomass resources needs the elucidation of the molecular basis of their function. Since the discovery of LPMOs, great advances have been made in the study of their substrate specificity and regioselectivity, as well as their structural basis, which will be reviewed below.

Keywords: Lytic polysaccharide monooxygenase, LPMO, Substrate specificity, Regioselectivity

Introduction
Biocatalytic degradation of renewable biomass resources is a potential way to address energy and environmental crises. Despite the abundance, the crystalline structure of cellulose and chitin hinders the accessibility of hydrolases, and thus the effective saccharification by traditional glycoside hydrolase systems. In 1950, Reese et al. postulated that the process of cellulolytic organisms degrading cellulose involves two steps (Reese et al. 1950). Firstly, the 'C1' degrades native cellulose into shorter linear polyanhydroglucose chains, which are then hydrolyzed by Cx into soluble, small molecules. In 1974, Eriksson et al. reported the presence of an oxidase in the extracellular enzyme system of Sporotrichum pulverulentum, which boosted the degradation of cellulose by the mixture of endo- and exo-glucanases (Eriksson et al. 1974). However, this oxidase has not been clearly characterized for a long time.

The first structure of Cel61B (a member of GH61 family) was resolved in 2008, revealing its difference from other glycoside hydrolases, suggesting that it may have different enzyme activities (Karkehabadi et al. 2008). Until 2010, Vaaje-Kolstad et al. reported that the bacterial CBP21 protein (a member of CBM33 family) is actually an enzyme that catalyzes oxidative depolymerization of chitin (Vaaje-Kolstad 2010). Shortly thereafter, the cellulose oxidative activities of GH61 family members were characterized (Quinlan et al. 2011). Then these Cu-dependent enzymes were named as lytic polysaccharide monooxygenases (LPMOs), and the GH61 and CBM33 families were reclassified as AA9 (Auxiliary Activity family 9) and AA10, respectively. Currently the LPMOs are distributed in 7 Auxiliary Activity families in CAZy database (www.cazy.org), with various origins and substrate specificities: AA9s, AA11s, AA13s, AA14s and AA16s are mainly from eukaryota with cellulose-, chitin-, starch-, and xylan-active, respectively; AA10s are from bacteria, eukaryota, viruses or archaea, with cellulose- or chitin activity; AA15s are from eukaryota (including insect) or viruses, with cellulose- or chitin-activity. The currently reported cleavage of chitin, starch and xylan substrates is C1-oxidized, while the cleavage of cellulosic substrates
is C1- or C4-oxidized, or both. The information on currently characterized LPMOs are summarized in Table 1.

Despite the low sequence identities, the catalytic domains of these LPMOs share some common structural features (Fig. 1), as recently reviewed (Beevon et al. 2015; Hemsworth et al. 2013a; Span and Marletta 2015; Vaaje-Kolstad et al. 2017). The core of the catalytic domain is a β sandwich of seven to nine β-strands. Loops connecting these β-strands constitute the ‘flat’ substrate binding surface, which is believed to interact with flat surfaces of crystalline substrates. The region located between β1 and β2 of LPMO9 (between β1 and β3 of LPMO10), denoted L2, includes a variable number of loops and short helices. Some LPMOs have an insertion between β3 and β4 denoted L3, which interacts with L2. In AA9 and AA13 LPMOs, there are LS (loop short) on the opposite side of L2. Besides, AA9 members have a long C-terminal loop, termed LC. As discussed below, the variable length and amino acid constitution of these loops might contribute to the substrate specificity and regioselectivity. The N-terminal histidine and a second conserved histidine coordinate a copper ion, forming the ‘histidine brace’. The N-terminal histidine of some fungal LPMOs is methylated at the Ne2, and the significance of this methylation is unclear.

Studies have shown that adding LPMOs to cellulase cocktails can improve the degradation efficiency of cellulose biomass and reduce the required enzyme amount (de Gouvea et al. 2019; Dimarogona et al. 2013; Harris et al. 2010; Hemsworth et al. 2015; Zhang et al. 2019). It is speculated that this synergy is due to the oxidative cleavage of polysaccharide crystalline regions by LPMOs, which provides more accessible sites for glycoside hydrolases (Fig. 2). Further elucidating the biological functions and catalytic mechanisms of these enzymes will bring more exciting possibilities for their application in the utilization of renewable biomass resources. The catalytic mechanism of LPMOs has been in scientific debate. One view is that, the catalytic center Cu (II) is activated by reduction into Cu (I) by two external electrons (Kjaergaard et al. 2014; Kracher et al. 2016). The Cu (I) activates dioxygen, leading to hydrogen abstraction from one of the carbons in the scissile glycoside bond. Then the hydroxylation of the resulting substrate radical leads to bond cleavage via an elimination reaction. In other studies, however, it has been proposed that, instead of dioxygen, H2O2 is the preferred co-substrate for LPMOs, in a per-oxigenase reaction where a single priming reduction to Cu(I) is needed (Bissaro et al. 2017). The catalytic mechanism of LPMOs has been extensively reviewed (Forsberg 2019; Tandrup et al. 2018; Walton and Davies 2016) and not discussed in depth here. The focus of this review is to give an insight into the current understanding of the substrate specificity, oxidation regioselectivity and their structural basis of LPMOs.

**Substrate specificity**

AA9 (former GH61) and AA10 (former CBM33) were originally found to act on crystalline cellulose and chitin substrates, respectively. As more related proteins are characterized, the broad substrate spectrum of LPMO superfamily is revealed. Besides insoluble substrates (such as cellulose, chitin, starch and xylan), the soluble oligosaccharides like xyloglucan, glucomannan and β-(1→3), (1→4)-d-glucan have been found to be oxidized by some LPMOs (Isaksen et al. 2014; Kojima et al. 2016). Biochemical characterization and structural studies, especially the complex structures of LPMOs and soluble oligosaccharide substrates, provide us much for in-depth understanding of LPMOs (Frandsen et al. 2016; Simmons et al. 2017). Detailed sequence and structure comparisons have revealed that the substrate binding surfaces of LPMOs with different substrate specificities have diverse characteristics in terms of amino acid composition and topological features. Since the L2, L3, LS and LC loops constitute the majority of the substrate binding surface, and their amino acids composition are highly variable, these loops are believed to affect substrate recognition and specificity.

**Amino acids composition on the substrate binding surface**

There are usually several aromatic amino acids on the substrate binding surface loops of LPMO9s (Fig. 3a, b). From structural studies and MD simulations, it was found that the spatial distribution of these aromatic amino acids facilitates stacking interactions with the sugar units of cellulose substrates, although the enzymes may bind to the surface of the cellulose fibers in different directions (Liu et al. 2018; Wu et al. 2013). In Wu’s study, 100 ns MD simulations of PchGH61D on cellulose showed that the three tyrosines on substrate binding surface tightly bonded with polysaccharide chains in the substrate (the interaction energies were −10.86 kcal/mol for Y28, −10.17 kcal/mol for Y75 and −9.5 kcal/mol for Y198, respectively) and are the main contributors to substrate binding. While LPMO10s generally only have one aromatic amino acid involved in substrate binding, LPMO11s and LPMO13s do not even have aromatic amino acids on substrate binding surface (Fig. 3a), and their polar amino acids are more abundant, possibly binding to substrates by polar interactions (Forsberg et al. 2014a; Hemsworth et al. 2014). Structural studies and site-directed mutagenesis revealed that binding of CBP21 to chitin is mediated primarily by conserved, solvent-exposed, hydrophilic residues, which arranged in a patch on the substrate binding surface (Aachmann et al. 2012;
| Family | Organism         | Protein names                      | Associated CBMs | PDB code | Substrates          | Regioselectivities | References                                      |
|--------|------------------|------------------------------------|-----------------|----------|---------------------|-------------------|------------------------------------------------|
| AA9    | Aspergillus nidulans | AN1602                             | CBM1            | –        | PASC                | C4                | (Jagadeeswaran et al. 2018; Jagadeeswaran et al. 2016) |
|        | Aspergillus nidulans | AN3046                             | –               | –        | PASC                | C1                | (Jagadeeswaran et al. 2016)                      |
|        | Gloeophyllum trabeum NBRC 6430 | LPMO9A-2, GTLPMO9A-2              | C-terminal domain with unknown function | –        | PASC                | C1 / C4           | (Kojima et al. 2016)                            |
|        | Gloeophyllum trabeum KUC 8013 | Cel61G, GTGH61, GTLPMO9B, LPMO98 | –               | 5NNS     | PASC                | C1                | (Hegnar et al. 2019)                            |
|        | Heterobasidion irregularis TC 32-1 | HiLPMO98                           | –               | 5NNS     | PASC                | C1                | (Liu et al. 2018)                               |
|        | Lentinus similis  | LsAA9A                             | –               | 5ACF     | PASC                | C1 / C4(PASC)     | (Frandsen et al. 2016)                         |
|        | Neurospora crassa OR74A | PMO-2, NCPO-2, NCPO9D, GH61-4, NCU01050, LPMO99D | –               | 4EIR     | PASC                | C4                | (Li et al. 2012; Petrovic et al. 2019)           |
|        | Neurospora crassa OR74A | LPMO-03328, NCPO9F, GH61-6, NCU03328, LPMO99F | –               | 4Q8      | Microcrystalline cellulose | C1               | (Kittl et al. 2012) (Tan et al. 2015)             |
| Family        | Organism          | Protein names | Associated CBMs | PDB code | Substrates | Regioselectivities | References                                                                 |
|--------------|-------------------|---------------|-----------------|----------|------------|-------------------|---------------------------------------------------------------------------|
| *Neurospora crassa* OR74A | PMO-01867 LPMO-01867 | CBM1          | –               | Microcrystalline cellulose | C1          | Kittl et al. 2012  |
|              | LPMO-01867 NcLPMO9J |                | NcLPMO9JPASCA  | Steam-exploded spruce | C1          | Li et al. 2012; Vu et al. 2014a |
|              | GH61-10            |                | NCU02916LPMO9C | C1/C4    | Li et al. 2012; Vu et al. 2014a |
|              | B1 3N4.070 LPMO9J  |                |                 | C1/C4    | Vu et al. 2014a |
| *Neurospora crassa* OR74A | PMO-3 NcLPMO9M GH61-13 NcPMMO-3 NCU07898 LPMO9M | CBM1          | 4EIS           | PASC     | C1/C4      | (Agger et al. 2014; Borisova et al. 2015; Courte et al. 2016; Isakson et al. 2014; Karnaouri et al. 2017; Kittl et al. 2012; Kojima et al. 2016; Kracher et al. 2018; Nekuniite et al. 2016b; Varanai et al. 2018; Westereng et al. 2016) |
| *Neurospora crassa* OR74A | PMO-02916 LPMO-02916 NcLPMO9C GH61-3 NCU02916 LPMO9C | CBM1          | 4D7U 4D7V      | PASC Avicel Steam-exploded spruce Cello-oligosaccharides Xyloglucan β-(1→3,1→4)-D-Glucan Glucomannan | C4          | (Agger et al. 2014; Borisova et al. 2015; Courte et al. 2016; Isakson et al. 2014; Karnaouri et al. 2017; Kittl et al. 2012; Kojima et al. 2016; Kracher et al. 2018; Nekuniite et al. 2016b; Varanai et al. 2018; Westereng et al. 2016) |
| *Neurospora crassa* OR74A | GH61-2 NCU07760     | CBM1          | –              | PASC     | C1/C4      | (Vuong et al. 2017; Westereng et al. 2013; Westereng et al. 2011; Wu et al. 2013) |
| *Neurospora crassa* OR74A | GH61-1 NcLPMO9A NCU02240 | CBM1          | 5FOH 5FOH      | PASC     | C4          | (Petrovic et al. 2019; Vu et al. 2014a) |
| *Neurospora crassa* OR74A | NCU08336             | CBM1          | –              | PASC     | C1          | (Vuong et al. 2017; Westereng et al. 2013; Westereng et al. 2011; Wu et al. 2013) |
| *Neurospora crassa* OR74A | PMO-08760 LPMO-08760 NcLPMO9E GH61-5 NCU08760 LPMO9E | CBM1          | –              | PASC     | C1          | (Karnaouri et al. 2017; Kittl et al. 2012; Kojima et al. 2016; Kracher et al. 2018; Nekuniite et al. 2016b; Varanai et al. 2018; Westereng et al. 2016) |
| *Pestalotiopsis* sp. NCi6 | PsLPMO8            | –             | –              | PASC     | C4          | (Patel et al. 2016) |
| *Pestalotiopsis* sp. NCi6 | PsLPMOA            | –             | –              | PASC     | C1/C4      | (Patel et al. 2016) |
| *Phanerochaete chrysosporium* K-3 | GH61D              | –             | 4BSQ           | PASC Avicel | C1          | (Danneels et al. 2019; Danneels et al. 2017; Vuong et al. 2017; Westereng et al. 2013; Westereng et al. 2011; Wu et al. 2013) |
| Family                          | Organism                      | Protein names            | Associated CBMs | PDB code | Substrates                                      | Regioselectivities | References |
|--------------------------------|-------------------------------|--------------------------|-----------------|----------|------------------------------------------------|-------------------|------------|
| **Podospora anserina**         | S mat+                        | Pa_4_1020 PalPMO9H       | CBM1            | –        | PA SC Gluco-oligosaccharides, Xyloglucan, Glucosan, Lichenan, β-(1 → 3,1 → 4)-d-Glucan CMC | C1/C4             | (Bennati-Granier et al. 2015; Chalak et al. 2019; Fauvel et al. 2017; Garajova et al. 2016; Villares et al. 2017) |
| **Podospora anserina**         | S mat+                        | Pa_4_7570 PalPMO9D       | –               | –        | PA SC                                       | C1               | (Bennati-Granier et al. 2015) |
| **Podospora anserina**         | S mat+                        | Pa_1_16300 PalPMO9E      | CBM1            | –        | PA SC                                       | C1               | (Bennati-Granier et al. 2015; Chabbert et al. 2017; Garajova et al. 2016) |
| **Podospora anserina**         | S mat+                        | Gh61B                    | CBM1            | –        | PA SC                                       | C1               | (Bey et al. 2013) |
| **Thermoascus aurantiacus**    | TaAA9                         | TaAA9                    | –               | –        | PA SC                                       | C1/C4            | (Cannella et al. 2016; Harris et al. 2010; Kitaoku et al. 2018; Muller et al. 2015; Petrovic et al. 2018; Quinlan et al. 2011; Singh et al. 2019) |
| **Myceliophthora thermophila** | MtLPMO9J MYCTH_79765          | –                        | –               | –        | PA SC Cello-oligosaccharides, Xyloglucan     | C4               | (Kadowaki et al. 2018) |
| **Myceliophthora thermophila** | MtPMO3 MYCTH_92668 MtLPMO9D   | –                        | –               | –        | RAC PA SC                                   | C1               | (Frommhagen et al. 2018; Span et al. 2017; Vu et al. 2014a) |
| **Myceliophthora thermophila** | MYCTH_112089 MYCTH112089      | –                        | –               | –        | PA SC                                       | C1               | (Vu et al. 2014a) |
| **Myceliophthora thermophila** | MtLPMO9A LPMO9A               | –                        | –               | –        | RAC PA SC Xyloglucan β-(1 → 3,1 → 4)-d-Glucan | C1/C4            | (Frommhagen et al. 2016; Frommhagen et al. 2015; Gusakov et al. 2017) |
| **Myceliophthora thermophila** | MYCTH_103537 MtLPMO9L         | –                        | –               | –        | PA SC Avicel                                 | C1               | (Zhou et al. 2019a) |
| Family                      | Organism                          | Protein names | Associated CBMs | PDB code          | Substrates                  | Regioselectivities | References                                      |
|----------------------------|-----------------------------------|---------------|-----------------|------------------|-----------------------------|-------------------|------------------------------------------------|
| **Myceliophthora thermophila/Thermothelomyces thermophilus** | MtLPMO9B                          | –             | –               | RAC               | C1                          | (Frommhagen et al. 2016; Frommhagen et al. 2017a; Frommhagen et al. 2018) |
| **Myceliophthora thermophila/Thermothelomyces thermophilus** | MtLPMO9C                          | –             | –               | RAC, β-(1→3,1→4)-o-Glucan, Xyloglucan | C4                          | (Frommhagen et al. 2016; Frommhagen et al. 2017b) |
| **Myceliophthora thermophila/Thermothelomyces thermophilus** | MtLPMO9                           | CBM1          | –               | PASC              | C1/C4                       | (Karnaouri et al. 2017) |
| **Trichoderma reesei**      | LPMO9A, HjLPMO9A, TrCel61A        | CBM1          | 5O2W, 5O2X      | PASC              | C1/C4                       | (Hansson et al. 2017; Tanghe et al. 2015) |
| **Trichoderma reesei**      | HjLPMO9B, HjGHK61B, Cel61B        | –             | 2VTC            | PASC              | C1/C4                       | (Karkehabadi et al. 2008) |
| **Collariella virescens**   | CvAA9A                            | –             | 5NLT            | PASG              | Glucomannan, Mannohexaose, Xylohexaose | (Simmons et al. 2017) |
| **Aspergillus fumigatus**   | AfAA9B                            | –             | 5X6A, 6H1Z, 6HA5, 6HAQ | –                | –                           | (Lo Leggio et al. 2018) |
| **Fusarium graminearum**    | FgLPMO9A                          | –             | –               | PASC              | Xyloglucan, C1/C4           | (Nekiunaite et al. 2016b) |
| **Geotrichum candidum**     | GcLPMO9A                          | –             | –               | PASC              | Xyloglucan, C1/C4           | (Ladeveze et al. 2017) |
| **Geotrichum candidum**     | GcLPMO9B                          | –             | –               | PASC              | Xyloglucan, C1/C4           | (Ladeveze et al. 2017) |
| **Malbranchea cinnamomea**  | McAA9A                            | –             | –               | PASC              | Xyloglucan, Glucomannan, Cellohexaose, C1/C4 | (Huttner et al. 2019) |
| **Malbranchea cinnamomea**  | McAA9B                            | –             | –               | PASC              | Xyloglucan, G4 (Xyloglucan) | (Huttner et al. 2019) |
| **Malbranchea cinnamomea**  | McAA9F                            | –             | –               | PASC              | Xyloglucan, Cellohexaose, G4 (Xyloglucan) | (Huttner et al. 2019) |
| Family          | Organism                  | Protein names                  | Associated CBMs | PDB code       | Substrates       | Regioselectivities | References                                      |
|-----------------|---------------------------|--------------------------------|----------------|----------------|-----------------|-------------------|------------------------------------------------|
| Malbranchea cinnamomea | McAA9H                    | –                              | –              | PASC           | Xylan           | C1 (PASC)         | (Huttner et al. 2019)                           |
| Thielavia terrestris  | TtLPMO9E                   | –                              | –              | PASC           | Avicel          | C1 /C4 (Xylan)    | (Cannella et al. 2016; Gusakov et al. 2017; Kim et al. 2017; Mollers et al. 2017; Westereng et al. 2015) |
| Chaetomium thermophilum  | CtLPMO1                   | –                              | –              | PASC           | Celloheptaose   | C1 /C4            | (Chen et al. 2018)                                |
| Bacillus amylophilaeiens   | ChhB                      | –                              | –              | 2YOW           | α and β chitin  | C1                | (Gregory et al. 2016; Hemsworth et al. 2013b)  |
| Bacillus licheniformis    | ChhB                      | –                              | –              | 5LW4           | α and β chitin  | C1                | (Courtade et al. 2015; Forsberg et al. 2014b)  |
| Bacillus thuringiensis  | Lpmo10A                   | –                              | –              | 5WSZ           | α and β chitin  | C1                | (Zhang et al. 2015)                              |
| Bacillus thuringiensis  | Cbp                       | CBM5 Fibronectin-type III-like domains | –              | β Chitin       | C1              | (Manjeet et al. 2019; Manjeet et al. 2013)   |
| Cellvibrio japonicus Ueda107 | CjpLPMO10A CJA_2191       | CBM5 CBM73                      | 5FJQ           | α and β chitin | C1              | (Forsberg et al. 2016)                           |
| Cellvibrio japonicus Ueda107 | CjpLPMO10B CJA_3139       | CBM10                           | –              | PASC Avicel BMCC Filter paper | C1 | (Gardner et al. 2014) |
| Enterococcus faecalis V583 | EFAA10A                   | –                              | –              | 4A02 4ALE 4ALQ 4ALR 4AL6 4ALT | α and β chitin | (Gudmundsson et al. 2014; Vaaje-Kolstad et al. 2012) |
| Hahella chejuensis KCTC 2396 | HcAA10-2 HCH_00807       | CBM2                           | –              | Avicel         | C1              | (Ghatge et al. 2015)                              |
| Family                  | Organism                     | Protein names                  | Associated CBMs | PDB code | Substrates                | Regioselectivities | References                                                                 |
|-------------------------|------------------------------|--------------------------------|-----------------|----------|---------------------------|--------------------|-----------------------------------------------------------------------------|
| *Jonesia denitrificans* | DSM 20603                    | Jden_1381 JdLPMO10A LPMO10A    | CBM5, GH18      | 5AA7     | α and β chitin            | C1                 | (Back et al. 2017; Mekasha et al. 2016)                                     |
| Listeria monocytogenes  |                              |                                |                 | 5VG0     |                           |                    |                                                                             |
| Serratia marcescens     | BJL200                       | Cbp21 CBP21 Cbp SmAA10A SmLPMO10A |                 | 2BEM     | α and β chitin            | C1                 | (Pasapaliari et al. 2015)                                                  |
| Serratia marcescens     | ATCC 23877                   | SAMO570 SamLPMO10B             |                 | 5L2V     | α and β chitin            | C1                 |                                                                             |
| Streptomyces ambofaciens | ATCC 23877                  | Cbp SmAA10A SmLPMO10A          |                 | 2BEN     |                           |                    |                                                                             |
| Streptomyces ambofaciens | ATCC 23877                  | SAML1174 SamLPMO10C            | CBM2            | 4OY7     | PASC Avicel β Chitin      | C1/C4              | (Forsberg et al. 2014a)                                                    |
| Streptomyces coelicolor | A3(2)                       | ScLPMO10B ScCO0643 SCF91.03c  |                 | 4OY8     | PASC Avicel β Chitin      | C1                 | (Courtade et al. 2018; Forsberg et al. 2014a, Forsberg et al. 2011)        |
| Streptomyces coelicolor | A3(2)                       | ScLPMO10C Ce6S2 ScAA10C SCO1188 SGG11A.19 | CBM2, 4OY7 | 4OY7     | PASC Avicel β Chitin      | C1                 |                                                                             |
| Streptomyces griseus subsp. griseus NBRC 13350 | | SGR_6855 SglLPMO10F | –                 | 4OY7     | PASC Avicel β Chitin      | C1                 | (Nakagawa et al. 2015)                                                     |
| Streptomyces lividans 1326 |                              | SLLPMO10E SLL_3182             |                 | 5FTZ     | β Chitin                  | C1                 | (Chaplin et al. 2016)                                                      |
| Teredinibacter turnerae T7901 |                            | TaAA10A TERTU_0046            | CBM10, 6W7     | 4GBO     | PASC Avicel β Chitin      | C1/C4              | (Fowler et al. 2019)                                                       |
| Thermobifida fusca YX   | Tfu_1268 Tfu_1 268 Tfu_1 268 Tfu_1 268 | TflPMO10A                      |                 | 4GBO     | PASC Avicel β Chitin      | C1/C4              | (Forsberg et al. 2014a; Kruger-Zerhusen et al. 2017; Russo et al. 2019)    |
| Vibrio cholerae O1 biovar B | Tor str 1N6961              | GbpAVcGbpAVcAA10BVCA0811 GbpAVcAD2 GbpAVcAD2 GbpAVcAD2 GbpAVcAD2 |                 | 2XXW     | β Chitin                  | C1                 | (Loose et al. 2014; Wong et al. 2012)                                      |
| Tectaria macrodonta     | Tma12                        |                                |                 | 6F7      | Colloidal crab chitin     | –                  | (Shukla et al. 2016; Yadav et al. 2019)                                     |
| Family | Organism | Protein names | Associated CBMs | PDB code | Substrates | Regioselectivities | References |
|--------|----------|---------------|-----------------|----------|------------|-------------------|------------|
|        | Anomala cuprea entomopoxivirus CV6M | Fusolin ACV034 | – | 4YN1 4YN2 4X29 4X27 4OW5 | – | – | (Chiu et al. 2015) |
|        | Micromonospora aurantiaca | MaLPMO10B | CBM2 | 5OPF | PASC β Chitin | C1 /C4 C1 (chitin) | (Forsberg et al. 2018) |
|        | Micromonospora aurantiaca | MaLPMO10D | CBM2 | – | PASC β Chitin | C1 /C4 C1 (chitin) | (Forsberg et al. 2018) |
|        | Bacillus cereus | BcLPMO10A | CBM5 Fibronectin-type III-like domain | – | α and β chitin | C1 | (Mutahir et al. 2018) |
|        | Aspergillus oryzae RIB40 | AoLpmo11 | X278 | 4MAH 4MAi | β Chitin | C1 | (Hemsworth et al. 2014) |
|        | Fusarium fujikuroi | FfAA11 | X278 | – | α and β chitin | C1 | (Wang et al. 2018) |
|        | Aspergillus nidulans FGSC A4 | AnAA13 AN5463.2 | CBM20 | – | Retrograded starch | C1 | (Lo Leggio et al. 2015) |
|        | Aspergillus oryzae RIB40 | AoAA13 | – | 4OPB 5LSV 5T7J 5T7N | – | – | (Frandsen et al. 2017; Lo Leggio et al. 2015) |
|        | Aspergillus terreus NIH2624 | AtLPMO13A ATEG_07286 | CBM20 | – | Wheat starch | – | (Nekiunaite et al. 2016a) |
|        | Magnaporthe oryzae | MoLPMO13A | CBM20 | – | Binding to wheat starch | – | (Nekiunaite et al. 2016a) |
|        | Neurospora crassa OR74A | NcAA13 NCU08746 | CBM20 | – | Amylose Amylopectin Cornstarch | C1 | (Vu et al. 2014b; Vu et al. 2019) |
|        | Myceliophthora thermophila | MtAA13 | – | – | Amylose Amylopectin Cornstarch | – | (Vu et al. 2019) |
|        | Trametes coccinea CIRM-BRM 310 | PcAA14A | – | – | Xylan | C1 | (Couturier et al. 2018) |
|        | Trametes coccinea CIRM-BRM 310 | PcAA14B | – | 5NO7 | Xylan | C1 | (Couturier et al. 2018) |
|        | Thermobia domestica | TdAA15A | – | 5MSZ | β Chitin PASC | C1 | (Sabbadin et al. 2018) |
|        | Thermobia domestica | TdAA15B | – | – | α and β chitin | C1 | (Sabbadin et al. 2018) |
|        | Aspergillus aculeatus ATCC 15872 | ASPACDRAFT_74022 AaAA16 | – | – | PASC | C1 | (Filiatrault-Chastei et al. 2019) |
Fig. 1 The overall structures and substrate binding surfaces of LPMOs. The loop regions are colored in red (L2), green (L3), yellow (LS) and blue (LC). The catalytic center histidines are shown in sticks. The structures representing different families are: NcuLPAM09C (PDB ID 4d7u) (Borisova et al. 2015), CBP21 (PDB ID 2bem) (Vaaje-Kolstad et al. 2005b), AoLPMO11 (PDB ID 4mah) (Hemsworth et al. 2014), AoAA13 (PDB ID 4OPB) (Lo Leggio et al. 2015), PcAA14B (PDB ID 5no7) (Couturier et al. 2018), TdAA15A (PDB ID 5msz) (Sabbadin et al. 2018).
Vaaje-Kolstad et al. (2005b). MD simulations of CBP21 on crystalline chitin substrates have also shown that although the only tyrosine Y54 on the substrate-binding surface is a key factor, the hydrogen bonding formed between substrate and the residues E55, T111, H114, Q57, and D182 was very important for substrate binding (Bissaro et al. 2018).

Within the AA10 family, the amino acid composition of the substrate-binding surface of different substrate-specific LPMOs is also diverse. The Gln-Thr pair (Q78 and T133 in CjLPMO10A) is presumed to be a determinant of chitin activity, since it is conserved in chitin-active LPMO10s, whereas in cellulose-active LPMO10s, the corresponding sites are Phe and Trp (Forsberg et al. 2016). Li et al. suggested that, compared with chitin-active SmAA10A, an insertion in the cellulose-active ScAA10C that contains four aromatic residues could account for cellulose specificity (Li et al. 2012). In previous work, we found a motif on L2 with different amino acid composition in different substrate-specific LPMO10s (Fig. 3c) (Zhou et al. 2019b). In cellulose-active LPMO10s, this motif mainly consists of non-polar amino acids (Y[W]NWF[N]G[A]V[N]L[Y]). While in chitin-active LPMO10s, this motif mainly consists of polar amino acids (Y[W]EPQSVE). We speculated that the different amino acid composition of this motif may lead to differences in substrate binding surface electrostatic potential, which in turn affects
substrate specificity. Jensen et al. constructed a mutation library of five sites on the substrate binding surface of ScLPMO10C, three of which are located in this motif region (Y79, N80, F82), and the other two are located in the adjacent loops (Y111, W141). Substrate specificity of the mutant M18 (Y79/N80D/F82A/Y111F/W141Q) significantly changed from wild-type cellulose-preference to chitin-preference, demonstrating the role of these residues in substrate specificity (Jensen et al. 2019).

The complex structures of the LsAA9A and soluble oligosaccharide substrates showed that in addition to the Y203 stacking, the hydrogen bond network formed between the +2 subsite and the polar residues (N28, H66 and N67) plays an important role in substrate binding, and this may be a determinant of soluble oligosaccharide...
activity, as sequence and structure alignments found that there is no corresponding residue forming a hydrogen bond network in LPMOs that can only act on crystalline substrates (Frandsen et al. 2016).

**The topological features of substrate binding surface**

The crystal structure of BaAA10A shows a cavity near the catalytic Cu center, and the authors speculated that it is for dioxygen binding (Fig. 3a) (Hemsworth et al. 2013b). Shortly thereafter, through structural comparisons, Forsberg et al. found that this cavity is absent in the cellulose-active LPMO10s (Forsberg et al. 2014a). Therefore, the cavity was presumed to accommodate N-acetyl group of chitin substrates, and may be a structural feature that determines substrate specificity. However, one exception is the chitin-active CjLPMO10A, which shows similar features to cellulose-active LPMO10s without this cavity (Forsberg et al. 2016).

LPMOs that can act on oligosaccharides, such as LsAA9A, NcLPMO9C and NcLPMO9D, have a more contoured substrate binding surface than LPMOs that can only act on crystalline substrates (Borisova et al. 2015; Frandsen et al. 2016; Li et al. 2012). The ridge near substrate binding subsites +1 and +2 was proposed to allow LPMOs binding to more contoured substrates such as oligosaccharides (Fig. 3a).

In AoAA13, the surface loops (the long loop preceding β2, the loop between β2 and β3, the long loop preceding β4 and the loop between β5 and β6) form a shallow groove, crossing the copper active site (Fig. 3a) (Lo Leggio et al. 2015). It was speculated that, compared with the flatter substrate binding surface of LPMO9s, which is more suitable for the binding of flatter crystalline cellulose substrates, the groove on the surface of AoAA13 might be more suitable for the binding of the contoured surface of resistant starch. It is worth noting that no crystal structures of the currently characterized LPMO13s have been resolved so far, and the structurally characterized AoAA13 has not been reported to have starch activity.

Similarly, the substrate binding surface of PcAA14B, an xylan-active LPMO, has a rippled shape with a clamp formed by two prominent surface loops, which are equivalent to the L2 and L3 regions of AA9 (Figs. 1 and 3a). The extended L3 loop of PcAA14B forms a protrusion through the cystines (C67–C90). Although there is no enzyme–substrate complex structure, these loops constitute a large part of the substrate binding surface, and it is speculated that this clamp is a structural feature of LPMO14s required for the xylan substrate binding (Courtier et al. 2018).

From the sequence alignment of PaLPMO9H and NcLPMO9C, it was speculated that the L3 loop, which is a common feature of these two enzymes, might be a prerequisite for xyloglucan specificity (Bennati-Granier et al. 2015). NMR (nuclear magnetic resonance) studies on enzyme–substrate interactions also showed that L3 of NcLPMO9C did participate in the binding of xyloglucan substrate (Courtade et al. 2016). However, as more LPMOs are characterized, some enzymes have been found to have xyloglucan-activity, but L3 is absent, such as GtLPMO9A-2. It was presumed that the extended L2 of the xyloglucan-active GtLPMO9A-2 compensate for the lack of L3 (Kojima et al. 2016).

**The appended modules**

Similar to GHs (glycoside hydrolases), a considerable part of LPMOs are modular, with domains of non-catalytic CBMs (carbohydrate-binding modules), GHs or other unknown functions appended to the catalytic domain. Domain similarity network analysis has shown the correlation between the additional domains and the substrate specificity of the full enzymes (Book et al. 2014; Zhou et al. 2019b). CBM truncation studies have been reported for both LPMO9s and LPMO10s (Chaklak et al. 2019; Courtade et al. 2018; Crouch et al. 2016; Forsberg et al. 2016; Laurent et al. 2019). Comparison of the performance of LPMOs with and without CBMs have shown that, deletion of CBMs reduced LPMO’s binding capacity to crystalline substrates, especially at low substrate concentrations. Therefore, CBMs may affect substrate specificity through promoting the binding of LPMOs to the appropriate substrates.

**Oxidative regioselectivity**

LPMO9s have been shown to oxidize either the C1, C4 or both the C1 and C4 carbon of the scissile bond of cellulose substrates. According to the oxidative regioselectivity, LPMO9s have been classified into three types: PMO1s are the strict C1-oxidizers; PMO2s are the strict C4-oxidizers; PMO3s are the mixed C1/C4-oxidizers; and a subtype of PMO3, PMO3’s, are the C1-oxidizers (Vu et al. 2014a). Cellulose-active LPMO10s are strict C1-oxidizers or mixed C1/C4-oxidizers, whereas no strict C4-oxidizing LPMO10 has been reported. LPMOs acting on chitin (LPMO10s, 11s and 15s), starch (LPMO13s) and xylan (LPMO14s) have only been shown to oxidize the C1-carbon. It is speculated that the oxidative regioselectivity may be determined by the precise positioning of the enzyme on the substrates, so factors that affect the relative position of the enzyme’s active center Cu and the C1 or C4 carbon of the scissile glycosidic bond may affect regioselectivity (Fig. 4).
Amino acid composition and arrangement on substrate binding surface

Due to the contribution of L2 to the substrate binding surface and the diversity of its amino acid composition, many studies on the regioselectivity of LPMOs have focused on this region. By sequence alignment, Vu et al. found that PMO3s had a 12-amino acid insertion on L2, including a conserved tyrosine, compared to other subgroups of LPMO9s. Deletion of this sequence caused the loss of C4-oxidizing function of NCU07760, indicating the importance of this sequence for C4 regioselectivity of PMO3. However, although the conserved tyrosine in this insertion is a feature of PMO3, mutation of this residue into glycine did not change the regioselectivity of NCU07760 (Vu et al. 2014a).

Sequence and structural information show that the number and distribution of aromatic residues on the surfaces of LPMOs are different. Therefore, it is speculated that LPMOs may bind to the substrates in different directions, resulting in different regioselectivity (Li et al. 2012). Recently, Danneels et al. studied the oxidative regioselectivity of LPMO9s in detail (Danneels et al. 2019). One part of the research was the mutation of aromatic amino acids on the substrate binding surfaces of PcLPMO9D, ScLPMO9C and HjLPMO9A. They found that the properties of these aromatic amino acids affect C1/C4-oxidation ratios. In another work, Liu et al. used molecular dynamics simulations to study the binding mode of HiLPMO9B to the substrate, and found that multiple surface-exposed hydrophobic residues, including the tyrosine on L2, are important for substrate binding in this C1-specific LPMOs. Besides, acidic amino acids on L2 and LC participate in substrate binding. In both the two binding modes obtained with different binding directions, the catalytic center Cu is more biased towards the C1 carbon of the glycosidic bond, suggesting that the arrangement of amino acids on substrate binding surface may affect regioselectivity by affecting the relative position of the catalytic center Cu and the substrate (Liu et al. 2018).

Similar speculation has been made for LPMO10s. On the substrate-binding surface of chitin-active C1-specific LPMO10s, the conservative amino acids involved in the formation of hydrogen bonds with the polysaccharide substrate are arranged on opposite sides of the catalytic center Cu, and thus direct the orientation of the substrate relative to the Cu. This directed binding makes the enzyme prone to act on C1 carbon of the scissile glycosidic bond (Hemsworth et al. 2013b). Forsberg et al. mutated a subset of coevolutionary residues of C1/C4-oxidizing MaLPMO10B into the corresponding residues of C1-oxidizing LPMO10s, and the resulting mutants lost the C4-oxidizing activity. They found that, the residues located near the catalytic Cu that are involved in substrate positioning (especially the N85 of MaLPMO10B) are the major determinants of regioselectivity (Forsberg et al. 2018).

Accessibility to the surface-exposed axial copper coordination site

A conserved alanine in LPMO10s active site has been postulated to provide steric congestion at the solvent-facing axial position of active center Cu (Hemsworth et al. 2013b). Subsequent research showed that the loop hosting this alanine adopts different conformations in C1- and C1/C4-oxidizers, making the solvent-facing axial position of C1/C4-specific ScLPMO10B more open than C1-specific ScLPMO10C (Forsberg et al. 2014a). Similarly, structural comparisons revealed that, strictly C1-oxidizing LPMO9s have a conserved tyrosine, preventing optimal axial access to the copper ion, whereas C4-oxidizing LPMO9s have an open access to this position. The mixed C1/C4-oxidizing LPMO9s show an intermediate situation (Borisova et al. 2015). Thus, the accessibility of surface-exposed axial position of Cu, or the ability to bind a ligand in the axial position, could be a determinant of C4-oxidizing activity. However, recent studies suggested that, mutations affecting accessibility of this axial position did not change the regioselectivities of PcLPMO9D and MaLPMO10B (Danneels et al. 2019; Forsberg et al. 2018).

The appended CBM modules

The CBM domains seem to affect the binding of LPMOs to substrates, thereby affecting the precise positioning of the enzymes on the substrates’ surfaces, that is, the relative position of C1 or C4 carbon to the catalytic center Cu, and thus the regioselectivity of the enzymes. Removing or replacing the endogenous CBMs of LPMO9s and LPMO10s have been reported to alter the regioselectivity
of these enzymes. For instance, deleting CBM1 of PaLP-
MO9H significantly increased the proportion of C1-oxi-
dized products (Laurent et al. 2019). Crouch et al.
replaced the endogenous CBM2a domain of TblP-
MO10 with the CBM10 of CjLPMO10B, and found that the
ratio of non-oxidized to oxidized products of the mutant
increased significantly. The authors speculated that the
non-oxidized products are the oligosaccharides derived
from C1-oxidation near the reducing end of cellulose,
which may be due to the grafted CBM affecting the
localization of the enzyme on the substrate (Crouch et al.
2016). But the impact of CBMs on the regioselectivity
of LPMOs is also controversial, e.g., removing the CBM
domains did not significantly change the regioselectivity
of MalP-
MO10B, NcLPMO9C and HjLPMO9A (Dan-
neels et al. 2019; Forsberg et al. 2018; Laurent et al. 2019).

N-Glycan on substrate binding surface
Fungal-derived LPMOs are generally glycosylated on the
surface, but their function is unclear. Sequence and struc-
tural information show that C1/C4-specific LPMO9s
often have an N-glycan at the planar active surface,
which is a feature different from the other two groups (Li
et al. 2012). Mutation studies showed that removing this
N-glycan can alter the C1/C4-oxidation ratios of HjL-
MO9A. The authors suggested that this is because N-gly-
can affects the structural features of the substrate binding
surface, which in turn affects the substrate binding and
oxidative force accurate directions (Danneels et al. 2019).

Structures of substrates
The regioselectivity of LPMOs appears to be substrate-
dependent. The most typical examples are the LPMO10s
with both cellulose- and chitin-activity. They are C1/
C4-specific for cellulose oxidation and C1-specific for
chitin oxidation. Recently, a multifunctional LPMO10,
KpLPMO10A has been reported that besides chitin-
and cellulose-activity, it can also act on xylan to produce
C4-oxidized products (Correa et al. 2019). In addition,
it is reported that, PaLPMO9H is C4-specific on mixed-
linkage glucans, and C1/C4-specific on glucomannan
(Fanuel et al. 2017). LsAA9A and CvAA9A are reported to
be C4-specific for shorter oligosaccharides and C1/
C4-specific for longer polysaccharides (Simmons et al.
2017).

Conclusions
Elucidating the molecular basis of substrate specific-
ity and oxidative regioselectivity of LPMOs will be more
helpful for their application in the biotransformation of
renewable biomass. Researches indicate that the sub-
strate binding and regioselectivity of LPMOs are pre-
cisely regulated. This precise regulation is based on the
complex synergistic modules and amino acid networks
that evolved from interactions with complex and diverse
substrate structures in nature. However, the character-
ized LPMOs are only a small part of the sequences that
have been found so far. More enzymatic and structural
characterization is needed to provide more information.
Structural-based mutation studies and MD simulations
will bring in-depth understanding of the molecular basis
of the function of LPMOs. In addition, given the com-
plexity and structural characteristics of the substrates,
it is necessary to develop more effective enzyme activity
detection methods to avoid the neglect of weak enzyme
activity.

Abbreviations
LPMO: Lytic polysaccharide monooxygenase; CBM: Carbohydrate-binding
module; PDB: Protein data bank; NMR: Nuclear magnetic resonance; AA: Auxil-
iary Activity; GH: Glycoside hydrolases; MD: Molecular dynamic.

Acknowledgements
The authors are thankful to the Guangdong Province Science and Technology
Innovation Strategy Special Fund (2018B020206001); the GDAS’ Special Project
of Science and Technology Development (2018GDASCX-0909); and the Sci-
ence and Technology Plan Project of Guangdong Province (2016A010105013,
2019B030316017); and the National Natural Science Foundation of China
(31400681).

Authors’ contributions
XZ, HZ developed the manuscript. XZ reviewed and corrected the manuscript
for grammatical and syntax errors. HZ reviewed the manuscript and provided
comments to enhance the quality of manuscript. Both authors read and
approved the final manuscript.

Funding
This work was funded by the Guangdong Province Science and Technology
Innovation Strategy Special Fund (2018B020206001); the GDAS’ Special Project
of Science and Technology Development (2018GDASCX-0909); and the Sci-
ence and Technology Plan Project of Guangdong Province (2016A010105013,
2019B030316017); and the National Natural Science Foundation of China
(31400681).

Availability of data and materials
Not applicable.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Received: 7 January 2020   Accepted: 21 February 2020
Published online: 02 March 2020

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