

REVIEW

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Current understanding of substrate specificity and regioselectivity of LPMOs

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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 poly-anhydroglucose 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

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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 (Beeson 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 β 1 and β 2 of LPMO9 (between β 1 and β 3 of LPMO10), denoted L2, includes a variable number of loops and short helices. Some LPMOs have an insertion between β 3 and β 4 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, H_2O_2 is the preferred co-substrate for LPMOs, in a peroxxygenase 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 \rightarrow 3), (1 \rightarrow 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 *Pch*GH61D 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;

Table 1 Overview of characterized LPMOs

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
AA9	<i>Aspergillus nidulans</i>	ANI602	CBM1	–	PASC Cellohexaose	C4	(Jagadeeswaran et al. 2018; Jagadeeswaran et al. 2016)
	<i>Aspergillus nidulans</i>	AN3046	–	–	PASC Xyloglucan	C1	(Jagadeeswaran et al. 2016)
	<i>Gloeophyllum trabeum</i> NBRC 6430	LPMO9A-2 GtLPMO9A-2	C-terminal domain with unknown function	–	PASC CMC Xyloglucan Glucomannan	C1/C4	(Kojima et al. 2016)
	<i>Gloeophyllum trabeum</i> KUC 8013	Cel61G GtGH61 GtLPMO9B LPMO9B	–	–	PASC Xyloglucan	C1/C4	(Hegnar et al. 2019)
	<i>Heterobasidium irregulare</i> TC 32-1	HiLPMO9B	–	5NNS	PASC	C1	(Liu et al. 2018)
		HiLPMO9H	CBM1	–	PASC	C1	(Liu et al. 2017)
		HiLPMO9I	CBM1	–	PASC Glucomannan	C4	(Liu et al. 2017)
	<i>Lentinus similis</i>	LsAA9A	–	5ACF 5ACG 5ACH 5ACI 5ACJ 5N04 5N05 5NKW 5NLN 5NLO 5NLP 5NLQ 5NLR 5NLS	PASC Cello-oligosaccharides	C1/C4(PASC) C4 (Cello-oligosaccharides)	(Frandsen et al. 2016)
	<i>Neurospora crassa</i> OR74A	PMO-2	–	4EIR	PASC	C4	(Li et al. 2012; Petrovic et al. 2019)
		NgPMO-2	–	5TKF	Xyloglucan		
		NcLPMO9D	–	5TKG	Glucomannan		
		GH61-4	–	5TKH			
		NCU01050	–	5TKI			
		LPMO9D	–				
	<i>Neurospora crassa</i> OR74A	PMO-03328	–	4QJ8	Microcrystalline cellulose	C1	(Kittl et al. 2012) (Tan et al. 2015)
		LPMO-03328 NcLPMO9F	–				
		GH61-6	–				
		NCU03328	–				
		LPMO9F	–				
			–				

Table 1 (continued)

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
	<i>Neurospora crassa</i> OR74A	PMO-01867 LPMO-01867 NcLPMO9J GH61-10 NCU01867 B13N4.070 LPMO9J	CBM1	–	Microcrystalline cellulose	C1	(Kittl et al. 2012)
		PMO-3 NcLPMO9M GH61-13 NgPMO-3 NCU07898 LPMO9M	–	4EIS	PASC	C1/C4	(Li et al. 2012; Vu et al. 2014a)
		PMO-02916 LPMO-02916 NcLPMO9C GH61-3 NCU02916 LPMO9C	CBM1	4D7U 4D7V	PASC Avicel Steam-exploded spruce Cello-oligosaccharides Xyloglucan β -(1 \rightarrow 3;1 \rightarrow 4)-D-Glucan Glucomannan	C4	(Agger et al. 2014; Borisova et al. 2015; Courtade et al. 2016; Isaksen et al. 2014; Karnaouri et al. 2017; Kittl et al. 2012; Kojima et al. 2016; Kracher et al. 2018; Nekiunaite et al. 2016b; Vairnai et al. 2018; Westereng et al. 2016) (Vu et al. 2014a)
		GH61-2 NCU07760	CBM1	–	PASC	C1/C4	(Petrovic et al. 2019; Vu et al. 2014a)
	<i>Neurospora crassa</i> OR74A	GH61-1 NcLPMO9A NCU02240	CBM1	5FOH	PASC	C4	(Vu et al. 2014a)
	<i>Neurospora crassa</i> OR74A	NCU00836	CBM1	–	PASC	C1	(Vu et al. 2014a)
	<i>Neurospora crassa</i> OR74A	PMO-08760 LPMO-08760 NcLPMO9E GH61-5 NCU08760 LPMO9E	CBM1	–	PASC	C1	(Kracher et al. 2016; Vu et al. 2014a)
	<i>Pestalotiopsis</i> sp. NCi6 <i>Pestalotiopsis</i> sp. NCi6 <i>Phanerochaete chrysosporium</i> K-3	PslPMOB	–	–	PASC	C4	(Patel et al. 2016)
		PslPMOA	–	–	PASC	C1/C4	(Patel et al. 2016)
		GH61D PcGH61D	–	4B5Q	PASC	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)
		PcLPMO9D	–	–	Avicel		

Table 1 (continued)

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
	<i>Podospora anserina</i> S mat+	Pa_4_1020 PaLPMO9H	CBM1	–	PASC Glucan-oligosaccharides Xyloglucan Glucomannan Lichenan β -(1→3,1→4)-D-Glucan CMC	C1/C4	(Bennati-Granier et al. 2015; Chalak et al. 2019; Fanuel et al. 2017; Garajova et al. 2016; Villares et al. 2017)
	<i>Podospora anserina</i> S mat+	Pa_4_7570 PaLPMO9D	–	–	PASC	C1	(Bennati-Granier et al. 2015)
	<i>Podospora anserina</i> S mat+	Pa_1_16300 PaLPMO9E	CBM1	–	PASC	C1	(Bennati-Granier et al. 2015; Chabbert et al. 2017; Garajova et al. 2016)
	<i>Podospora anserina</i> S mat+	Gh61B Pa_7_3160 PaLPMO9B	CBM1	–	PASC	C1/C4	(Bey et al. 2013)
	<i>Podospora anserina</i> S mat+	Pa_6_7780 PaLPMO9F	–	–	PASC	C1	(Bennati-Granier et al. 2015)
	<i>Podospora anserina</i> S mat+	Gh61A Pa_2_6530 PaLPMO9A	CBM1	–	PASC	C1/C4	(Bennati-Granier et al. 2015; Bey et al. 2013)
	<i>Thermoascus aurantiacus</i>	Gh61A TaAA9 TaAA9A TaGH61 TaGH61A TaLPMO9A	–	2YET 3ZUD	PASC	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)
	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MtLPMO9J MYCTH_79765	–	–	PASC Cello-oligosaccharides Xyloglucan	C4	(Kadowaki et al. 2018)
	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MtPMO3 MYCTH_92668 MtLPMO9D	–	5UFV	RAC PASC	C1	(Frommhagen et al. 2018; Span et al. 2017; Vu et al. 2014a)
	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MYCTH_112089 MYCTH12089	–	–	PASC	C1	(Vu et al. 2014a)
	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MtLPMO9A LPMO9A	–	–	RAC PASC Xyloglucan Xylan β -(1→3,1→4)-D-Glucan	C1/C4	(Frommhagen et al. 2016; Frommhagen et al. 2015; Gusakov et al. 2017)
	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MYCTH_103537 MtLPMO9L	–	–	PASC Avicel	C1	(Zhou et al. 2019a)

Table 1 (continued)

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MtLPMO9B	–	–	RAC	C1	(Frommhagen et al. 2016; Frommhagen et al. 2017a; Frommhagen et al. 2018)
		MtLPMO9C	–	–	RAC β-(1→3,1→4)-D-Glucan Xyloglucan	C4	(Frommhagen et al. 2016; Frommhagen et al. 2017b)
	<i>Myceliophthora thermophila</i> / <i>Thermothelomyces thermophilus</i>	MtLPMO9	CBM1	–	PASC	C1/C4	(Karnaouri et al. 2017)
		LPMO9A HjLPMO9A TrCel61A	CBM1	5O2W 5O2X	PASC	C1/C4	(Hansson et al. 2017; Tanghe et al. 2015)
<i>Trichoderma reesei</i>	<i>Trichoderma reesei</i>	HjLPMO9B HjGH61B Cel61B	–	2VTC			(Karkehabadi et al. 2008)
		CvAA9A	–	5NLT	PASC Gluco-oligosaccharides Xyloglucan β-(1→3,1→4)-D-Glucan Glucomannan Mannohexaose Xylohexaose	C1/C4	(Simmons et al. 2017)
<i>Aspergillus fumigatus</i>	<i>Aspergillus fumigatus</i>	AfAA9B	–	5X6A 6H1Z 6HA5 6HAQ	–	–	(Lo Leggio et al. 2018)
		FgLPMO9A	–	–	PASC Xyloglucan	C1/C4	(Nekiunaite et al. 2016b)
		GcLPMO9A	–	–	PASC Xyloglucan	C1/C4	(Ladeveze et al. 2017)
		GcLPMO9B	–	–	PASC Xyloglucan	C1/C4	(Ladeveze et al. 2017)
<i>Malbranchea cinnamomea</i>	<i>Malbranchea cinnamomea</i>	McAA9A	–	–	PASC Xyloglucan Glucomannan Cellohexaose	C1/C4 C4 (Xyloglucan)	(Huttner et al. 2019)
		McAA9B	–	–	PASC Xyloglucan	C1/C4 C4 (Xyloglucan)	(Huttner et al. 2019)
		McAA9F	–	–	PASC Xyloglucan	C1/C4 C4 (Xyloglucan)	(Huttner et al. 2019)A
			–	–	Cellohexaose		

Table 1 (continued)

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
AA10	<i>Malbranchea cinnamomea</i>	MCAA9H	–	–	PASC Xylan	C1 (PASC) C1/C4 (Xylan)	(Huttner et al. 2019)
	<i>Thielavia terrestris</i>	TtLPMO9E	–	–	PASC Avicel	C1 C1/C4(Cannella et al. 2016; Mollers et al. 2017)	(Cannella et al. 2016; Gusakov et al. 2017; Kim et al. 2017; Mollers et al. 2017; Westering et al. 2015)
AA10	<i>Chaetomium thermophilum</i>	CtPMO1	–	–	PASC Celloheptaose	C1/C4	(Chen et al. 2018)
	<i>Bacillus amyloliquefaciens</i>	ChbB BaAA10A BaCBM33 Rbam17540 BAMF_1859	–	2YOW 2YOX 2YOY 5UU	α and β chitin	C1	(Gregory et al. 2016; Hems-worth et al. 2013b)
	<i>Bacillus licheniformis</i>	ChbB Cbp BlICBP BIAA10A BL00521 BL00145 BLPMO10A	–	5LW4	α and β chitin	C1	(Courtade et al. 2015; Forsberg et al. 2014b)
	<i>Bacillus thuringiensis</i> ACCC10066	Lpmo10A BtLPMO10A	–	5WSZ	α and β chitin	C1	(Zhang et al. 2015)
	<i>Bacillus thuringiensis</i> ATCC33679	Cbp BtCBP BtLPMO10A-FL	CBM5 Fibronectin-type III-like domains	–	β Chitin	C1	(Manjeet et al. 2019; Manjeet et al. 2013)
	<i>Cellvibrio japonicus</i> Ueda107	CjLPMO10A CJA_2191	CBM5 CBM73	5FIQ	α and β chitin	C1	(Forsberg et al. 2016)
	<i>Cellvibrio japonicus</i> Ueda107	CjLPMO10B CJA_3139	CBM10	–	PASC Avicel BMCC Filter paper	C1	(Gardner et al. 2014)
	<i>Enterococcus faecalis</i> V583	EFAA10A EF0362 EFCBM33A EfaCBM33	–	4A02 4ALC 4ALE 4ALQ 4ALR 4ALS 4ALT	α and β chitin	C1	(Gudmundsson et al. 2014; Vaaje-Kolstad et al. 2012)
	<i>Hahella chejuensis</i> KCTC 2396	HcAA10-2 HCH_00807	CBM2	–	Avicel	C1	(Ghatge et al. 2015)

Table 1 (continued)

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
	<i>Jonesia denitrificans</i> DSM 20603	Jden_1381 JdLPMO10A LPMO10A	CBM5 GH18	5AA7 5VG0 5VG1	α and β chitin	C1	(Bacik et al. 2017; Mekasha et al. 2016)
		GbpA LmLPMO10 Lmo2467	CBM5 Fibronectin-type III-like domain	5L2V	α and β chitin	C1	(Paspallari et al. 2015)
	<i>Serratia marcescens</i> BJL200	Cbp21 CBP21 Cbp	–	2BEM 2BEN 2LHS	α and β chitin	C1	(Aachmann et al. 2012; Vaaje-Kolstad et al. 2005a; Vaaje-Kolstad et al. 2005b; Vaaje-Kolstad 2010)
		SmAA10A SmLPMO10A	–	–	β Chitin	C1	(Valenzuela et al. 2017)
	<i>Streptomyces ambifaciens</i> ATCC 23877	SAML0570 SamLPMO10B	–	–	PASC Flax pulp fibers	C1	(Valenzuela et al. 2017)
		SAML1174 SamLPMO10C	CBM2	–	PASC Avicel β Chitin	C1/C4	(Forsberg et al. 2014a)
	<i>Streptomyces coelicolor</i> A3(2)	ScLPMO10B SCO0643 SCF91.03c	–	4OY6 4OY8	PASC Avicel β Chitin	C1	(Courtade et al. 2018; Forsberg et al. 2014a; Forsberg et al. 2011)
		ScLPMO10C CelS2	CBM2	4OY7	PASC Avicel	C1	(Nakagawa et al. 2015)
	<i>Streptomyces griseus</i> subsp. <i>griseus</i> NBRC 13350	SGR_6855 SgLPMO10F	–	–	α and β chitin	C1	(Chaplin et al. 2016)
		SiLPMO10E SLI_3182	–	5FTZ	β Chitin	C1	(Fowler et al. 2019)
	<i>Teredinibacter turnerae</i> T7901	TtAA10A TERTU_0046	CBM10	6RW7	Avicel	C1/C4	(Forsberg et al. 2014a; Krueger et al. 2019)
		Tfu_1268 E7	–	4GBO	β Chitin PASC Avicel	C1/C4	(Forsberg et al. 2014a; Krueger et al. 2019)
	<i>Thermobifida fusca</i> YX	TfLPMO10A E8	CBM2 FN3	–	PASC Avicel	C1	(Forsberg et al. 2014a; Krueger et al. 2019)
		TfAA10B Tfu_1665	–	–	β Chitin	C1	(Loose et al. 2014; Wong et al. 2012)
	<i>Vibrio cholerae</i> O1 biovar <i>El Tor</i> str. N16961	GbpA VcGbpA VcAA10B VCA0811	GbpAD2 GbpAD3 GbpAD4	2XWX	β Chitin	C1	(Shukla et al. 2016; Yadav et al. 2019)
		Tma12	–	6IF7	Colloidal crab chitin	–	
	<i>Tectaria macrodonta</i>						

Table 1 (continued)

Family	Organism	Protein names	Associated CBMs	PDB code	Substrates	Regioselectivities	References
AA11	<i>Anomala cuprea entomopox-virus</i> CV6M	Fusolin	–	4YN1	–	–	(Chiu et al. 2015)
		ACV034		4YN2			
				4X29			
				4X27			
				4OW5			
AA13	<i>Micromonospora aurantiaca</i>	MaLPMO10B	CBM2	5OPF	PASC β Chitin	C1/C4 C1 (chitin)	(Forsberg et al. 2018)
		MaLPMO10D	CBM2	–	PASC β Chitin	C1/C4 C1 (chitin)	(Forsberg et al. 2018)
		BcLPMO10A	CBM5 Fibronectin-type III-like domain	–	α and β chitin	C1	(Mutahir et al. 2018)
AA11	<i>Aspergillus oryzae</i> RIB40	Aol.pmo11	X278	4MAH 4MAI	β Chitin	C1	(Hemsworth et al. 2014)
		FfAA11	X278	–	α and β chitin	C1	(Wang et al. 2018)
		AnAA13 AN5463.2	CBM20	–	Retrograded starch	C1	(Lo Leggio et al. 2015)
AA13	<i>Aspergillus oryzae</i> RIB40	AoAA13	–	4OPB 5LSV 5T7J 5T7N	–	–	(Frandsen et al. 2017; Lo Leggio et al. 2015)
		AtLPMO13A ATEG_07286	CBM20	–	Wheat starch	–	(Nekiunaite et al. 2016a)
		MoLPMO13A	CBM20	–	Binding to wheat starch	–	(Nekiunaite et al. 2016a)
AA14	<i>Magnaporthe oryzae</i>	NcAA13 NCU08746	CBM20	–	Amylose Amylopectin Corn starch	C1	(Vu et al. 2014b; Vu et al. 2019)
		MtAA13	–	–	Amylose Amylopectin Corn starch	–	(Vu et al. 2019)
		PcAA14A	–	–	Xylan	C1	(Couturier et al. 2018)
AA15	<i>Trametes coccinea</i> CIRM-BRFM 310	PcAA14B	–	5NO7	Xylan	C1	(Couturier et al. 2018)
		TdAA15A	–	5MSZ	β Chitin PASC	C1	(Sabbadin et al. 2018)
		TdAA15B	–	–	α and β chitin	C1	(Sabbadin et al. 2018)
AA16	<i>Aspergillus aculeatus</i> ATCC 16872	ASPACDRAFT_74022 AaAA16	–	–	PASC	C1	(Filiatrault-Chastel et al. 2019)

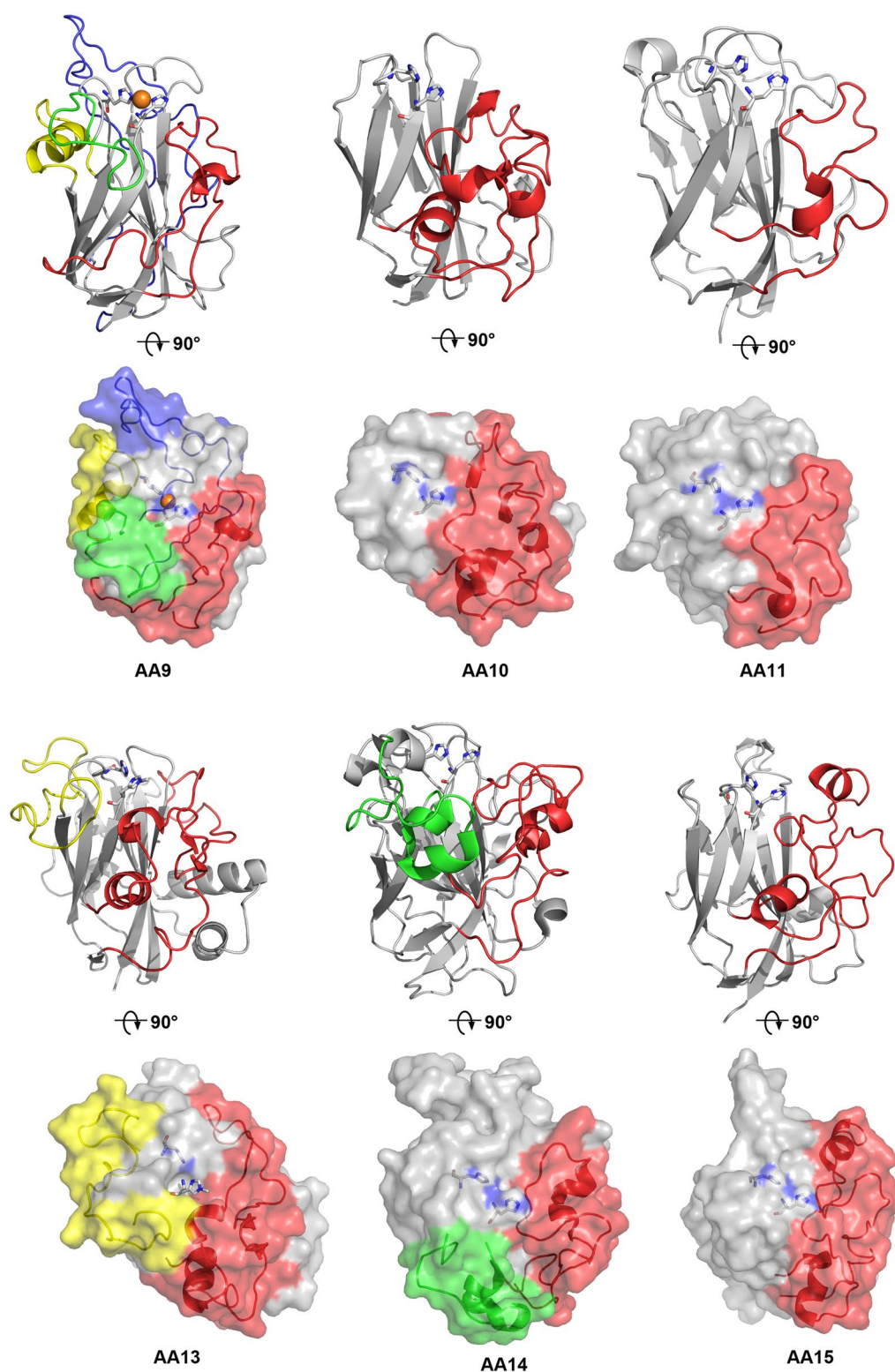


Fig. 1 The overall structures and substrate binding surfaces of LPMOs. The loop regions are colored in red (L2), green (L3), yellow (L5) and blue (L6). The catalytic center histidines are shown in sticks. The structures representing different families are: NcLPMO9C (PDB ID 4d7u) (Borisova et al. 2015), 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)

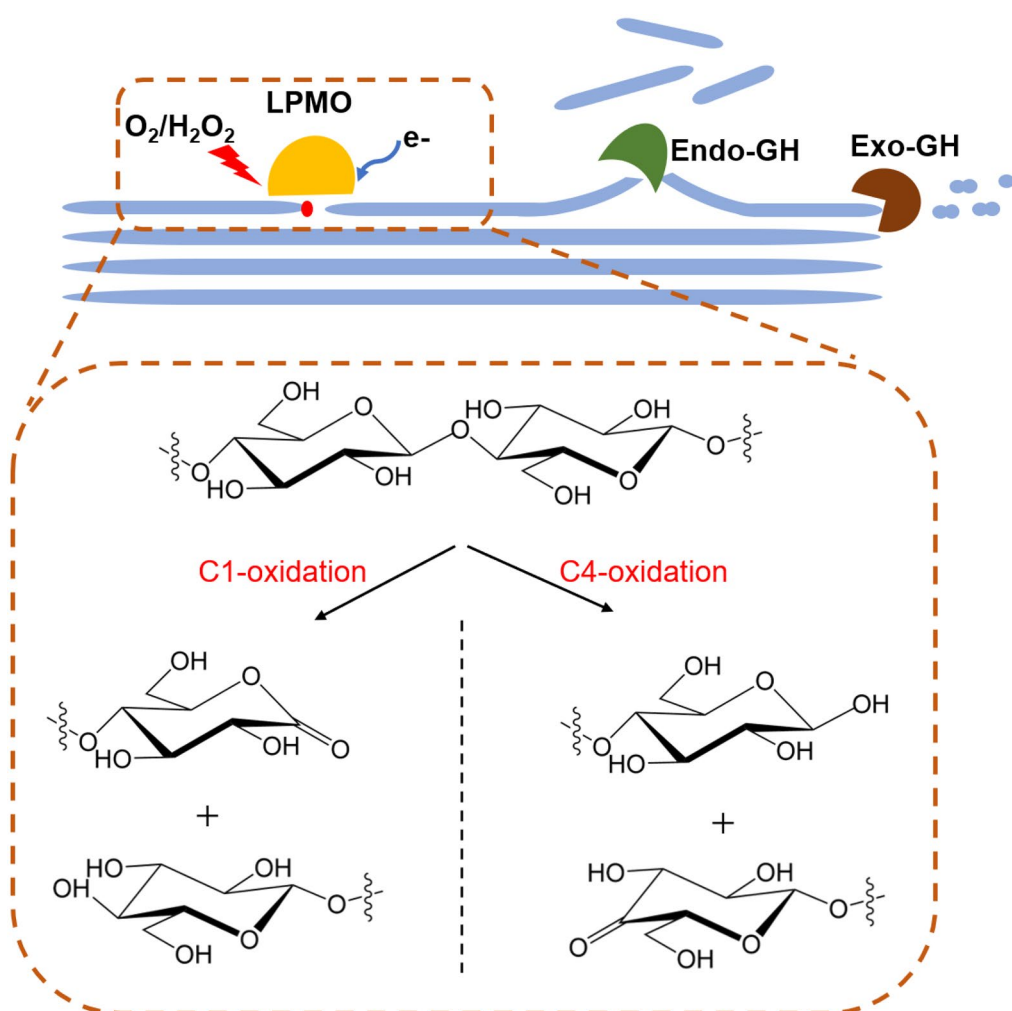


Fig. 2 Schematic representation of the LPMOs reaction. The upper part of the figure is a model of the synergistic degradation of polysaccharide substrates by LPMO and glycosidases. Exo-glycosidases (Exo-GH) act on chain ends to generate soluble sugars. LPMOs and endo-glycosidases (Endo-GH) act on crystalline and amorphous regions within the polysaccharide chain, respectively, providing more accessible sites for exo-glycosidases. The enlarged dotted box is a schematic diagram of the action mechanism of the LPMOs. Oxidation at C1 results in the formation of a lactone at the reducing end. C4 oxidation produces a ketoaldehyde at the non-reducing end

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

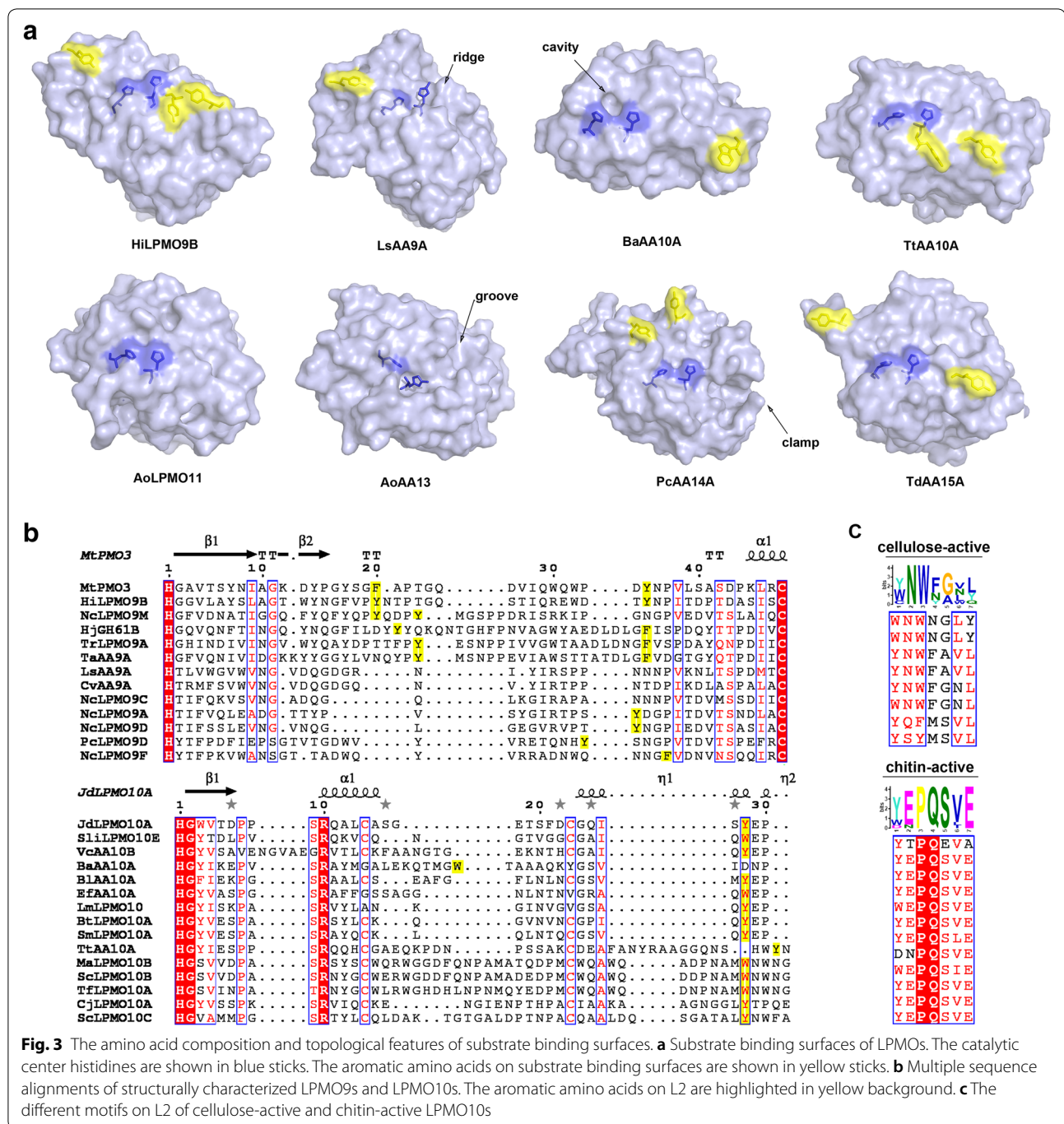


Fig. 3 The amino acid composition and topological features of substrate binding surfaces. **a** Substrate binding surfaces of LPMOs. The catalytic center histidines are shown in blue sticks. The aromatic amino acids on substrate binding surfaces are shown in yellow sticks. **b** Multiple sequence alignments of structurally characterized LPMO9s and LPMO10s. The aromatic amino acids on L2 are highlighted in yellow background. **c** The different motifs on L2 of cellulose-active and chitin-active LPMO10s

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 β 2, the loop between β 2 and β 3, the long loop preceding β 4 and the loop between β 5 and β 6) 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 (Couturier 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 (Chalak 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).

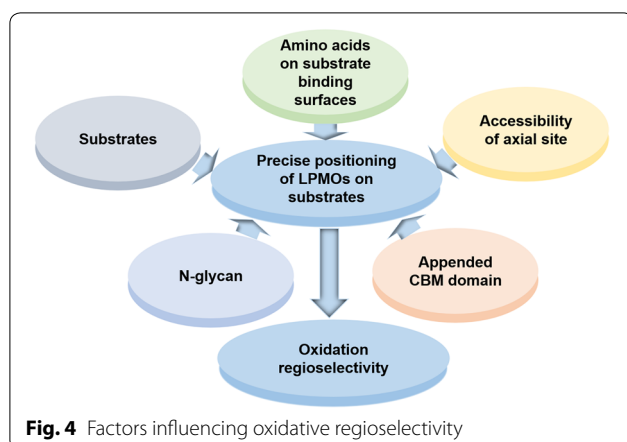


Fig. 4 Factors influencing oxidative regioselectivity

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-oxidized products (Laurent et al. 2019). Crouch et al. replaced the endogenous CBM2a domain of TbLPMO10 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 MaLPMO10B, NcLPMO9C and HjLPMO9A (Danneels 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 structural 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 HjLPMO9A. The authors suggested that this is because N-glycan 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 (Faniel 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 specificity and oxidative regioselectivity of LPMOs will be more helpful for their application in the biotransformation of renewable biomass. Researches indicate that the substrate binding and regioselectivity of LPMOs are precisely 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 characterized 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 complexity 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: Auxiliary Activity; GH: Glycoside hydrolases; MD: Molecular dynamic.

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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.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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