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LPMO as a Key Enzyme in the Sustainable Conversion of Lignocellulosic Biomass

T. Rezić,[a*] *M. Andlar*,[a] and *A. Vrsalović Presečki*[b]

[a] University of Zagreb, Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10 000 Zagreb, Croatia
[b] University of Zagreb, Faculty of Chemical Engineering and Technology, Trg Marka Marulića 19, 10 000 Zagreb, Croatia

Abstract

The importance of the lytic polysaccharide monooxygenase (LPMO) enzyme in the preparation of lignocellulosic raw materials for production in biorefineries has been confirmed in numerous investigations. Therefore, LPMO enzymes were investigated to explore the enzyme-substrate interaction with the aim of successful biomass conversion in biorefinery processes. After reductive activation of LPMOs active site, they cleave the substrate and prepare it for biomass degradation by hydrolytic enzymes. In this paper, the role of LPMO in lignocellulosic biomass conversion is described based on the recent studies of: LPMO enzyme structure, LPMO substrate preferences, and the LPMO reaction mechanism. These findings are important for the selection of suitable bioprocess conditions with the aim of LPMO activation/stabilisation in biorefinery production processes.

Keywords

Lytic polysaccharide monooxygenase, substrate, lignocellulosic biomass, reaction mechanism, enzymatic hydrolysis

1 Introduction

Fossil fuels are being replaced increasingly by renewable and sustainable alternatives such as lignocellulosic biomass. Products made from this type of biomass (*e.g.*, wood biomass residues and agricultural crops) meet the environmental and social criteria for sustainable production. However, the cost of lignocellulosic production is significantly higher than the cost of producing sugar- and starch-based feedstocks.[1]

To meet the economic sustainability of production from lignocellulosic biomass, new technological approaches must be used. In order to be used as a substrate for production, agricultural wastes must be prepared for production by pretreatment. This usually consists of a very complex matrix of cellulose, hemicellulose, and lignin. Because lignin acts as a barrier that impedes cellulose and restricts enzyme access, numerous pretreatment processes, which include physical, chemical, and biological methods, have been developed for the successful removal of lignin and preparation of feedstock for enzymatic hydrolysis for the production of bioethanol and other biochemicals. The purpose of pretreatment is to open the structure of a complex matrix such as lignocellulose, thereby reducing the crystallinity of the material and making the cellulose accessible, separating the components of the lignocellulosic biomass, and removing the lignin.[2,3]

The advantages of biologically mediated pretreatment using enzymes are high specificity of enzymes for cellulosic substrates, and carrying out the process in aqueous media. In addition, biological pretreatment is more environmentally friendly, and does not require the use of toxic and corrosive chemicals. The combination of different types of pretreatments results in a more efficient decomposition of the complex structure of lignocellulosic biomass. The combination of wet grinding with discs and enzymatic hydrolysis has proven to be a good alternative to production during the saccharification process in biorefineries. This method is not only effective for residues obtained from sugarcane processing, but also for other lignocellulosic raw materials. Enzymatic hydrolysis also results in a significant reduction in the degree of cellulose polymerisation.[4]

Integration of delignification (pretreatment) and solid-state fermentation can also be used for the improvement of biological processes. Such processes rely on the use of fungi that release a complex of hemicellulose- and lignin-degrading enzymes, whose action on the lignocellulosic matrix leads to an increase in cellulose availability for solid-state fermentation.[5]

The degradation of lignocellulose by fungi depends on a series of endo- and exo-acting enzymes that cleave lignocellulosic matrix like glucan chains in cellulose into soluble cellobiose molecules. Although promising as an environmentally friendly alternative, biological pretreatment technologies are too slow for industrial use. However, for biomass processing and biofuel production, industry uses commercial enzyme mixtures in which lytic polysaccharide monooxygenase enzymes (LPMOs) are essential components.[6] LPMOs prepare the terrain for the conversion of polysaccharide substrates, and together with hydrolytic enzymes increase efficiency of the lignocelluloses biomass degradation.[7]

Investigations have discovered different substrates for the LPMO action, like cellulose, glucans, xylan, starch, chitin, xyloglucan. As mentioned previously, LPMO has prominent

* Corresponding author: Prof. Tonči Rezić, PhD
Email: trezic@pbf.hr

role in the prearrangement of the lignocelluloses biomass substrates, especially in the crystalline cellulose degradation by primary degradation of the cellulose molecules. On the other hand, importance of LPMO has been confirmed by the phylogenetic investigation confirming the presence of the LPMO genes in the majority of lignocelluloses degrading organisms like fungi and bacteria.[8,9]

LPMO has been extensively studied, and some of the research published over the past decade is reflected in this paper with the intention of highlighting the importance of LPMO in the processing of lignocellulosic biomass for biorefinery production. The structure of LPMO enzyme, LPMO substrate preferences, and LPMO reaction mechanism are important for the selection of suitable bioprocess conditions. In addition, the importance of bioprocess conditions for appropriate LPMO activation and stabilisation in biorefinery production is highlighted.

2 Enzymatic hydrolysis and biodegradation of lignocellulosic biomass

Enzymatic hydrolysis of lignocellulosic raw materials to fermentable sugars is an important step in bioproduction. The enzymatic hydrolysis of the lignin, hemicellulose, and cellulose content of lignocellulosic biomass to their monomeric sugars suitable for the production of value-added biochemicals has been affected in many ways. Hydrolysis usually requires multiple enzymes with different capabilities to degrade the complex lignocellulosic structure.[10] In particular, synergistic action of lignocellulosic enzymes-cellulases, hemicellulases, lignases (ligninolytic enzymes), and most recently LPMO, is required for effective degradation.[11] The goal of enzymatic hydrolysis is to depolymerise the polysaccharides in the water-insoluble solid fraction that remains after pretreatment. After most pretreatments, the majority of these remaining polysaccharides consist of cellulose.[12]

Complete enzymatic hydrolysis of lignocellulosic feedstocks requires different types of pectinolytic, hemicellulolytic, ligninolytic, and cellulolytic enzymes. Enzymes used in the lignocellulose degradation are usually produced by the fungi as exo-cellular enzymes. These enzymes are important for the fungal metabolism and adequate utilisation of lignocellulosic substrates. Culture supernatants of these fungi can be collected and tested in the hydrolysation processes.[13] In the previous investigation, the ability of the culture supernatants of fungi *Neurospora crassa*, *Sclerotium rolfsii*, and *Trametes versicolor* to hydrolyse thermally pretreated sugar beet pulp (SBP) was investigated. The supernatant of *Sclerotium rolfsii*, a facultative plant pathogen from soil grown on SBP as a carbon source, showed the highest hydrolytic activity. A relatively low loading rate of the culture supernatant (enzyme) was sufficient to hydrolyse a large portion of the pectin and hemicellulose in SBP. The addition of *Trichoderma reesei* cellulases resulted in almost complete hydrolysis of the cellulose. This investigation also revealed the importance of the multi-enzyme complexes from fungi supernatants for efficient polysaccharides and oligosaccharides degradation.[14]

The theoretically possible number of linear and branched isomers of a single reducing hexameric oligosaccharide yields 10^{12} unique structures.[15] Variety of the oligosaccharide and the additional sugars linkage to the other organic molecular structures results in the numerous different carbohydrate conjugates. Since all of these carbohydrates must be both synthesised and degraded, the number and, more importantly, the complexity of the enzymes that perform these activities is enormous. Systematisation of the enzymes involved in the conversion of the carbohydrate and its glycoconjugates have been summarised in the CAZy database. Enzymes described in the CAZy database are grouped under the term Carbohydrate-Active enZYmes or CAZymes, and the database is open to new CAZymes members. To this day, new groups and members of the database are classified according to investigation results on enzyme action and the mechanisms of enzyme action on different substrates.

Studies on the enzymatic degradation of lignocellulosic biomass have discovered the importance of the synergistic activity of many enzymes, which is the key to successful degradation. The enzymes cellobiose dehydrogenase (CDH) and LPMO enzymes are believed to have the ability to directly degrade cellulose.[17] The rate of oxidative cleavage of cellulose catalysed by the CDH and LPMO enzymes requires a high rate of electron transfer and reducing equivalents generated by the enzymes.[18] There are still many opportunities to further improve the most efficient commercial cellulase mixture for hydrolysis of lignocellulosic feedstocks (*e.g.*, Celluclast, Viscozyme, Pectinex). It has been shown that after pretreatment of raw materials, some hemicellulose and lignin remain in the solid cellulose fraction. Lignin and hemicellulose are interlocked with the cellulose fraction, and therefore hemicellulolytic and ligninolytic enzyme activities can influence glucose release during hydrolysis.[19]

3 Lytic polysaccharide monooxygenase enzyme (LPMO)

In the CAZy database, LPMOs belongs to the families 9, 10, 11, 13, 14, and 15 of enzymes with auxiliary activities (AA). LPMO family AA9 belongs to the previous class GH61, and consists only of fungal enzymes. AA10, formerly known as CBM33, are proteins found in different forms of life, such as bacteria, archaea even in eukaryotes. Family AA11 is composed mainly of fungi, while AA13 is the most recent addition in the group of enzymes with auxiliary activities. The AA14 group belongs to enzymes that break down xylan, and have the ability to oxidise carbon atoms at C1 position. Family AA15 is widespread among crustaceans, molluscs, chelicerae, algae, and oomycetes, none of which were previously known to contain LPMOs.[20]

The genomes of biomass-degrading fungi typically encode multiple LPMO genes (with numbers up to more than 40), and contribute to the wide occurrence of LPMOs. LPMOs are abundant and exhibit diversity in sequence, indicating different functional roles of LPMOs. It seems that transcription and expression of fungal LPMOs is upregulated in the presence of different substrates, as well as other growth

conditions. Lignocellulose biomass degradation is mostly investigated; however, new investigation suggests importance of LPMOs in other biological functions, like development of viral diseases.[21]

3.1 LPMO enzyme structure and substrate preferences

All known LPMOs have two strictly conserved histidine residues in a constellation known as a "histidine brace" which enables coordination of the copper atom in the active site. In order to act on a planar substrate such as cellulose, the active site exposed to the solvent is located on a flat "binding surface" oriented toward the substrate surface during catalysis.[22] Thus, LPMOs are not dependent on the accessibility of individual glucan chains. LPMO substrates are polymeric and many of them are insoluble, which poses challenges to the study of enzyme-substrate interactions.

In 2010, the activity of new enzyme classes called LPMO was demonstrated on the crystalline β-chitin and α-chitin by CBP21.[23] Study demonstrated that, in synergy with *Serratia marcescens* chitinases, the activity of LPMO decreases with the substrate crystallinity enhancement.[24] Further studies revealed activity of *Streptomyces coelicolor* LPMO on cellulose, and a new group of enzymes in the CAZy database was formed named AA9s (LPMOs active on cellulose).[25] Subsequent studies have discovered LPMO activity on substrates like crystalline cellulose, soluble cello-oligomers, and xyloglucan from hemicellulose. Those activities were demonstrated by NcLPMO9C enzyme produced by *Neurospora crassa*.[26] NcLPMO9C cleaved β-1,4-glucan and demonstrated its ability to accept substitutions at different positions in the β-glucan backbone. Activity was also correlated with the backbone β-glucan substituents and its positions.[27]

Myceliophora thermophila AA9 LPMO (MtLPMO9A) showed activity on xylan-coated cellulose, cleaving the bonds in xylan and cellulose β-1,4-glucosyl bonds.[28] All of these substrates share similar features, namely, the β-1,4 bonds connecting the individual units in the sugar backbone. *Vu* and co-workers found that LPMOs are not limited to cleavage of β-1,4-bonds by demonstrating the *N. crassa* family AA13 LPMO activity on starch (*i.e.*, α-1,4-bonds).[29] Later, a starch-active *Aspergillus nidulans* fungal LPMO was also discovered.[30] Thus, LPMO substrates are actually more diverse than originally thought.

Crystal structure of LPMO and especially structure of active sites with copper and nearly located aromatic and hydrophilic amino acids give insight into the substrate binding of LPMOs.[31,32] *Eibinger et al.*[33] found that LPMOs copper reduction (*e.g.,* by ascorbic acid) is essential for the successful binding of the substrates. Experimental evidence suggests that copper reduction, co-substrate activation, and interaction with carbohydrate substrates occur close to the active site, and confirm precisely timed reaction steps. According to insoluble nature of the majority of substrates, the standard biochemical methods for tracking LPMOs properties is challenging.[34,35] Thermal stability of LPMOs can be examine by the combination of fluorimetric assay, spectroscopic methods and/or differential scanning fluorimetry. This methodology was applied for the investigation of the thermal stability of fungal *N. crassa* LPMOs during interactions with different carbohydrate substrates.[36] Copper-saturated LPMOs stability from the bacteria *Bacillus amyloliquefaciens* was also examined.[37] This investigation has led to attempts of enzyme engineers to stabilise LPMOs by introducing additional disulphide bridges into *Streptomyces coelicolor* LPMO. This strategy increased thermal stability of LPMO.[38]

3.2 Reaction mechanism

Analysis of the reaction products revealed that LPMOs hydrolyse the C4 or C1 carbons of glucosyl moieties adjacent to the glycosidic bond, resulting in strand breaks that are starting points for hydrolytic enzymes.[39] Detailed catalytic mechanism by which LPMOs facilitate substrate cleavage remains to be experimentally elucidated.

Investigations conducted up to now reveal that, in the first step, LPMOs active site copper has to be reduced by low molecular weight reducing agents or by the fungal flavocytochrome cellobiose dehydrogenase.[40,41] The second step has been proposed to involve the recruitment of molecular oxygen as a co-substrate, and the formation of the copper bound superoxide or oxyl intermediate.[42] The timing for providing the second electron for the reaction differs depending on the proposed reactive oxygen species.[43] Recently, hydrogen peroxide as a co-substrate was reported to lead to higher turnover rates than with oxygen.[44]

From the first discovery of LPMO and its abilities to decompose lignocellulosic biomass, its mechanism of action was investigated as well as the substrate preferences. Investigations revealed that LPMO enzymes are copper-dependent, and that their activity depends on substrate properties. Initially, it was thought that the enzyme is active on chitin and cellulose, further investigations revealed activity on starch, xyloglucan from hemicellulose soluble cello-oligosaccharides.[45] Similarities in the active site architecture between different LPMOs suggest similarities in the reaction mechanisms; on the other hand, characterisation of the reaction products revealed differences. Some LPMOs can oxidise carbohydrates molecule on the C1 position, and some others on the C4 position and generate different products 1,5-δ-lactonesa and 4-ketoaldose, respectively. Concentration of oxygen and H_2O_2 as the co-substrates is also important for enzyme stability and activity.[46] Another LPMO-directed H_2O_2 cleavage mechanism for enzymatic oxidative cleavage of polysaccharides was proposed by *Bissaro* and co-authors.[44] Briefly, LPMO active site Cu(II) is firstly reduced to LPMO active site Cu(I) ('priming reduction'). Cu(I) then reacts with H_2O_2 in the presence of substrate. This reaction leads to the cleavage of a H_2O and the formation of a Cu(II) oxyl intermediate (which could also be Cu(III) oxo). Oxyl and oxo intermediates can cleave hydrogen atoms from the substrates.[47] Simultaneously, re-bound mechanism is a result of Cu(II) associated hydroxide reaction with the substrate radical. This reaction leads to the substrate hydroxylation and regeneration of the Cu(I) centre, which can enter a new catalytic cycle. As a result of the proposed reaction mechanism, polysaccharide substrates bonds are cleaved, and molecules of the substrates are rearranged and prepared for attachment of cellulolytic enzymes.[44]

LPMO activity was first demonstrated using ascorbic acid as an electron source. Since then, several natural electron sources have been identified, including enzymes (such as cellobiose dehydrogenase CDH), simple phenols, quinones used by GMC (glucose-methanol-choline) oxidoreductase (such as glutamate dehydrogenase GDH), and even light-activated photosynthetic pigments. Electrons reduce a catalytic copper ion, activating LPMO by forming a reactive Cu(II) superoxide.[48–50] The latter initiates degradation by breaking down cellulose chains and reducing crystallinity (Fig. 1).

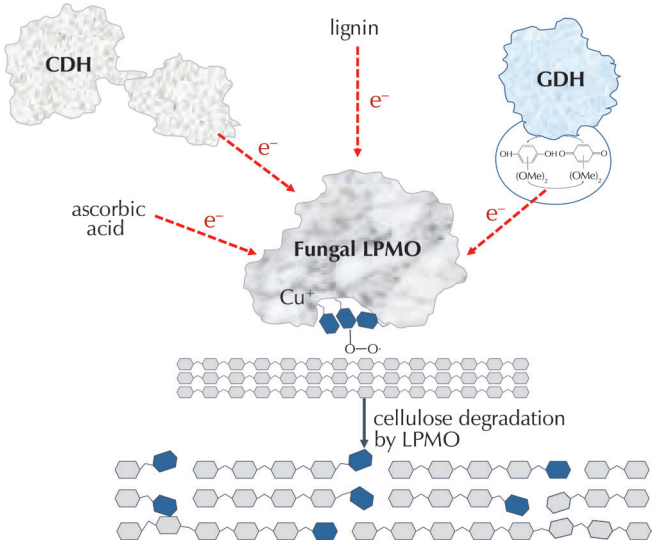

Fig. 1 – Reaction mechanism and potential electron donors for LPMO[50]

Slika 1 – Reakcijski mehanizam LPMO enzima i potencijalni donori elektrona[50]

3.3 Application of LPMO in the biorefinery

Modern biotechnology advancements have enabled the production of lignocellulosic ethanol on three continents, capping 60 years of research into the enzymatic degradation of cellulose. Investments by the U.S. Department of Energy, and major enzyme producers, such as Novozymes (Norway), have led to a 20-fold reduction in the cost of using enzymes. This has given producers confidence in the commercial viability of a biotechnology process to convert lignocellulose to ethanol. Companies GranBio and Raizen of Brazil, Poet & DSM and Abengoa of the U.S. began production in 2014. The first company to start commercial-scale operations at a demonstration plant in Crescentino in northern Italy was M&G. This was supported by a grant from the BIOLYFE project, funded by the European Union's 7th Framework Programme, which included the installation of an E85 fuelling station and possibly the world's first cars fuelled with ethanol produced using LPMOs.[51]

Enzymes are critical for the degradation of lignocellulosic biomass to monomeric sugars in both natural and industrial processes, but to achieve the goal of industrial production of lignocellulosic biofuels, significant improvement in efficiency was required. The discovery of biocatalysts targeting the highly recalcitrant cellulose surfaces has not only introduced a new enzymatic paradigm, but also provides new opportunities for refining commercial enzyme mixtures used for biomass processing and biofuel production. LPMOs are essential components of current commercial cellulolytic enzyme cocktails, although proper aeration and provision of electrons at industrial scale are considered major challenges, as is the instability of LPMOs.[52] The inclusion of an oxidative enzyme in the cellulolytic cocktail means that process conditions must be re-optimised. The performance and stability of LPMO can be controlled by regulating the supply of H_2O_2, a cheap and easy-to-use bulk industrial chemical, in combination with the consumption of sub-stoichiometric amounts of reducing agent.[53] Further process improvements can be achieved by better control of the activation-inactivation equilibrium resulting from the combined effects of H_2O_2 amounts, reductant amounts, LPMO concentration, and substrate availability.[54,55] Although such control is seemingly straightforward for the reactions shown in Fig. 1, the situation is more complex when working with copolymer biomasses that are potentially full of redox-active compounds such as lignin and metals. Nevertheless, the true potential of LPMOs in the biorefining of lignocellulosic biomass has yet to be exploited optimally, considering the possibility of rate increases through the use of H_2O_2. In particular, substrate availability is critical for LPMO stability and can be influenced by other enzymes in a cellulolytic enzyme cocktail, which could open up new binding sites for LPMO by removing LPMO-disrupted chains from the substrate surface.[51]

4 Conclusions

The structure of LPMO and the mechanism of LPMO action on cellulosic substrates are important for successful implementation in the biorefinery process and degradation of lignocellulosic biomass. Previous research has elucidated the structure of LPMO and investigated the mechanisms of action on pretreated cellulosic substrates, as well as cellulose and hemicellulose. The importance for the industrial application of LPMO lies in tuning the reaction conditions and optimisation of process conditions for LPMO activation. On the other hand, the operational stability of LPMO needs to be improved by selecting a suitable co-substrate and electron source to enable the efficient use of LPMO in the biorefinery.

List of abbreviations
Popis kratica

LPMO – lytic polysaccharide monooxygenase
– litička polisaharidna monooksigenaza

CAZy – database of Carbohydrate-Active enZYmes
– baza podataka enzima s aktivnošću na kompleksne ugljikohidrate i glukokonjugate

SBP – sugar beet pulp
– pulpa šećerne repe

CDH – cellobiose dehydrogenase
– celobioza dehidrogenaza

AA – enzymes with auxiliary activities
– enzimi s pomoćnom aktivnošću

GH61 – family 61 of the glycoside hydrolases
– glikozid hidrolaze

CBM33 – family 33 of carbohydrate binding module
– proteinska struktura karakteristična za vezanje ugljikohidrata

NcLPMO9C – LPMO produced from *Neurospora crassa*
– litička polisaharidna monooksigenaza iz Neurospora crassa

GMC – glucose-methanol-choline oxidoreductase
– glukoza-metanol-kolin oksidoreduktaza

GDH – glutamate dehydrogenase
– glutamat dehidrogenaza

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SAŽETAK

LPMO – ključni enzim u održivoj pretvorbi lignocelulozne biomase

Tonči Rezić,[a*] *Martina Andlar* [a] i *Ana Vrsalović Presečki* [b]

Važnost enzima litičke polisaharidne monooksigenaze (LPMO) u pripremi lignoceluloznih sirovina za proizvodnju u biorafinerijama potvrđena je u brojnim istraživanjima. Stoga su istražene i interakcije LPMO enzima i supstrata s ciljem primjene tih istraživanja u uspješnoj pretvorbi lignocelulozne biomase u biorafinerijama. Nakon redukcije aktivnog mjesta LPMO enzima dolazi do vezanja i razgradnje supstrata te pripreme za djelovanje hidrolitičkih enzima. U ovom radu opisana je uloga LPMO enzima u pretvorbi lignocelulozne biomase na temelju nedavnih istraživanja: strukture LPMO enzima, specifičnosti supstrata na koje djeluje i mehanizma reakcije. Ova istraživanja važna su za odabir prikladnih uvjeta bioprocesa s ciljem aktivacije/stabilizacije LPMO enzima tijekom proizvodnje u biorafinerijama.

Ključne riječi
Litička polisaharidna monooksigenaza, supstrat, lignocelulozna biomasa, reakcijski mehanizam, enzimska hidroliza

[a] *Sveučilište u Zagrebu, Prehrambeno-biotehnološki fakultet, Pierottijeva 6, 10 000 Zagreb*
[b] *Sveučilište u Zagrebu, Fakultet kemijskog inženjerstva i tehnologije, Trg Marka Marulića 19, 10 000 Zagreb*

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