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Methodology

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**A sensitive, accurate, and high-throughput gluco-oligosaccharide oxidase based
HRP colorimetric method for lytic polysaccharide monooxygenase activity assay**

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30 **Abstract**

31 **Background:** The AA9 (auxiliary activities) family of lytic polysaccharide monooxygenases
32 (AA9 LPMOs) are ubiquitous and diverse group of enzymes amongst the fungal kingdom. They
33 catalyze the oxidative cleavage of glycosidic bonds in lignocellulose and exhibit great potential for
34 secondary biorefinery applications. Screening of AA9 LPMOs for desirable properties is crucial
35 for biorefinery industrial applications. However, robust, high-throughput and direct method for
36 AA9 LPMO activity assay, which is prerequisite for screening of LPMOs with excellent properties,
37 is still lacking. Here, we have described a gluco-oligosaccharide oxidase (GOOX) based
38 horseradish peroxidase (HRP) colorimetric method for AA9 LPMO activity assay.

39 **Results:** We cloned and expressed a GOOX gene from *Sarocladium strictum* in *Trichoderma*
40 *reesei*, purified the recombinant SsGOOX, validated its properties, and set up a SsGOOX based
41 HRP colorimetric method for cellobiose concentration assay. Then we expressed two AA9
42 LPMOs from *Thielavia terrestris*, TtAA9F and TtAA9G in *T. reesei*, purified the recombinant
43 proteins, and analyzed their product profiles and regioselectivity towards phosphoric acid swollen
44 cellulose (PASC). TtAA9F was characterized as a C1 type (class 1) LPMO, while TtAA9G was
45 characterized as a C4 type (class 2) LPMO. Finally, the SsGOOX based HRP colorimetric method
46 was used to quantify the total concentration of reducing lytic products from LPMO reaction, and
47 consequently, the activities of both C1 and C4 types of LPMOs were analyzed. These LPMOs
48 could be effectively analyzed with limits of detection (LoDs) lower than 30 nmol/L, and standard
49 curves between A_{515} and LPMO concentrations with determination coefficients greater than 0.994
50 were obtained.

Conclusions: A novel, sensitive and accurate assay method that directly targets the main activity of both C1 and C4 type of AA9 LPMOs was established. This method is easy to use and could be performed on a microtiter plate ready for high-throughput screening of AA9 LPMOs with high properties.

Keywords: Lytic polysaccharide monooxygenase, Enzyme activity assay, Gluco-oligosaccharide oxidase, Horse radish peroxidase, *Trichoderma reesei*, *Thielavia terrestris*, *Sarocladium strictum*

Background

Lytic polysaccharide monooxygenases (LPMOs) constitute a large class of copper-dependent enzymes that catalyze the oxidative cleavage of the glycosidic bonds in recalcitrant polysaccharides, such as cellulose, chitin, and xylan, in the presence of an external electron donor, thereby making these polymers more accessible to hydrolytic enzymes by reducing the crystallinity [1, 2]. Initially, the LPMOs e.g., GH61 family enzymes, were classified as glycoside hydrolases as they produced small amounts of reducing sugars while acting on cellulosic substrates [3]. However, major breakthrough was achieved by Vaaje-Kolstad et al, as they revealed that these enzymes were in fact lytic polysaccharide mono-oxygenases (LPMOs) which led to discovery of a number of LPMOs species, their exact nomenclature and classification[4]. Currently, LPMOs are divided into seven auxiliary activity (AA) families in the Carbohydrate Active enzyme (CAZy) database, i.e., families AA9-AA11 and AA13-AA16. Among them, AA9 family LPMOs are produced by fungi, active towards crystalline cellulose and exhibit potential applications in secondary biorefinery industry [5-7]. As, the AA9 LPMOs are a diverse group of enzymes and also vary in their activity, thus, it is required to screen LPMOs with higher activity for their efficient application in secondary biorefinery.

74 The prerequisite for large scale screening of LPMOs with high enzymatic activities is to establish
75 a convenient, sensitive, and high-throughput assay method. AA9 LPMOs catalyze the cleavage of
76 crystalline cellulose, releasing a small amount of reducing sugars which although detected by
77 traditional methods such as 3,5-dinitrosalicylic acid (DNS) method, yet not considered sensitive
78 enough. In order to solve this problem, advanced quantitative analyzing methods based on
79 ingenious equipment, such as high performance anion exchange chromatography (HPAEC),
80 Ultra-high performance liquid chromatography (UHPLC), or chromatography equipment
81 combined with a mass spectrum, have been established [8-10]. Though, these methods are
82 accurate and can reveal detailed insights of the product formation, but require expensive
83 instruments, are generally time-consuming and labor-intensive [11]. In this context, some new
84 methods have been set up to determine the activity of LPMOs. When LPMOs are incubated with
85 an external electron donor such as ascorbic acid in the absence of cellulose substrate, hydrogen
86 peroxide is produced linearly depending upon the concentration of LPMOs. Thus, a fast and easy
87 method for LPMO activity assay was established by quantifying the formation of hydrogen
88 peroxide with Amplex Red/horseradish peroxidase reaction[12]. In another study,
89 2,6-dimethoxyphenol (2,6-DMP) or hydrocoerulignone were used as chromogenic substrates to
90 assay LPMO activity[11, 13]. 2,6-DMP or hydrocoerulignone are converted to colored product
91 coerulignone by LPMOs with H₂O₂ as a co-substrate. Thus, activity of LPMOs was determined by
92 measuring the absorbance of the product coerulignone. These two methods are considered
93 sensitive, are easy to use, and do not require any expensive equipment, therefore can be adapted
94 for high-throughput screening of LPMOs. However, as they are based on two side activities, i.e.,
95 the H₂O₂ generating activity and the peroxidase-like activity, which may not correlate with the

LPMOs activity on polymeric substrates such as cellulose.

Recently, reduced phenolphthalein (rPHP) (a chromogenic agent for blood test used in forensic science), proposed to be an oligosaccharide mimic was used as a chromogenic substrate for LPMOs activity assay [14]. In the presence of a proper co-substrate, the colorless rPHP turns into pink PHP upon LPMO catalysis and the LPMO activity can be analyzed spectrophotometrically. This is also a very sensitive and potentially a high-throughput method, albeit doubt still remains about the correlation of the rPHP oxidizing activity and the cellulose oxidative lytic activity because the performance of co-substrates differs in these two reactions [14]. Wang et al developed a suitable method for the activity assay of C1 type LPMOs which is based on the binding of Ni^{2+} to carboxyl group of insoluble lytic products [15]. In their reaction system, C1 LPMOs catalyze the lytic oxidation of PASC and generate insoluble aldonic acids which in turn capture the solute Ni^{2+} and thereby reduce its concentration. The decrease in Ni^{2+} concentration quantified with pyrocatechol violet as a complexometric indicator, which is correlated with the LPMO activity. Although, this method is simple and the assayed activity is directly related with the natural activity of LPMOs, yet it has some drawbacks due to its limitation to only C1 type LPMOs and relatively higher error may result while measuring the decrease in Ni^{2+} concentration.

Here, we have designed a gluco-oligosaccharide oxidase (GOOX) based method for LPMO activity assay. GOOXs catalyze the oxidation of gluco-oligosaccharides efficiently, producing a stoichiometric amount of H_2O_2 that can be easily quantified through horseradish peroxidase (HRP) colorimetric assay [16-18]. We expressed the GOOX gene from *Sarocladium strictum* (previously known as *Acremonium strictum* T1 [16]) in *Trichoderma reesei*, characterized the purified enzyme (SsGOOX) and set up the SsGOOX based method for reducing sugars as well as LPMO activity

assay. Based on sequence alignment, a putative C1 type LPMO (sequence number THITE-2142696 in GenBank, designated as TtAA9F in this work) and a putative C4 type LPMO (sequence number THITE-170174, designated as TtAA9G) from *Thielavia terrestris* were expressed in *T. reesei*. Later on, they were purified, regioselectivity characterized, and used for the activity assay. The approach of LPMO activity assay is provided in Scheme 1. The reaction mixture for AA9 LPMO activity assay contains phosphoric acid swollen cellulose (PASC), LPMO and ascorbic acid (Asc). Upon LPMOs action, reducing sugars are released and after a certain duration ascorbic acid oxidase is added to exhaust Asc and terminate the LPMO reaction. The reducing sugars are subsequently oxidized by SsGOOX addition, leading to equimolar production of H₂O₂ which is quantified by the HRP colorimetric assay.

Scheme 1 Schematic diagram of the GOOX based HRP colorimetric method for LPMO assay. The action of C1 LPMO towards PASC releases native oligosaccharides and aldonic oligosaccharide derivatives, and only native oligosaccharides bear a reducing aldehyde end. The action of C4 LPMO releases native oligosaccharides and gemdiol oligosaccharide derivatives, and both kinds of products bear a reducing aldehyde end. The native oligosaccharides released by C1 LPMO cleavage, and the two products released by C4 LPMO cleavage are then oxidized by GOOX, generating a stoichiometric amount of H₂O₂ which is finally quantified with HRP colorimetric assay. RE: reducing aldehyde end in oligosaccharides.

Results

Heterologous expression and purification of SsGOOX

Figure 1 SDS-PAGE (A) and Western blot (B) analysis of the recombinant SsGOOX protein. M:
molecular marker; **S1** and **W1:** culture supernatant of the parent strain *T. reesei* QM9414; **S2** and **W2:**
culture supernatant of the recombinant strain; **S3** and **W3:** purified recombinant SsGOOX.

Mature peptide region of the SsGOOX gene encodes a 474 amino acid protein that contains a FAD (flavin adenine dinucleotide) binding domain. Its theoretical molecular weight is about 52.5 kDa, and its theoretical pI (isoelectric point) is 4.66. *T. reesei* transformants were verified with PCR amplification, DNA sequencing and western blot analysis for the secreted recombinant protein using His-tag antibody. The selected transformant for recombinant SsGOOX production was cultivated in 50 ml recombinant protein production medium for 6 days. After harvesting, the recombinant SsGOOX was purified to homogeneity using Ni²⁺ affinity chromatography and gel filtration chromatography. SDS-PAGE and Western blot analysis of the culture supernatant and purified SsGOOX are shown in Fig. 1. SDS-PAGE results confirmed that the molecular weight of the recombinant SsGOOX protein is similar to its theoretical value.

Properties of the recombinant SsGOOX protein

Properties of the purified recombinant SsGOOX were studied with cellobiose as the substrate. The HRP colorimetric assay was applied to monitor the production of H₂O₂, with 4-amino-antipyrine (4-AAP) and 3,5-dichloro-2-hydroxybenzenesulfonic acid (DCHBS) as the chromogenic substrates. Upon oxidation of cellobiose via SsGOOX, a stoichiometric amount of H₂O₂ was produced, which was then used by HRP to convert AAP and DCHBS into a pink substance with maximum absorbance at 515 nm (A₅₁₅). Concentrations of the chromogenic substrate 4-AAP and DCHBS used in the HRP colorimetric assay were determined as 0.1 mM and 2.0 mM, respectively, based on the experiments of A₅₁₅ dependence on 4-AAP and DCBHS concentrations (Fig. S1).

The kinetic parameters of SsGOOX oxidation towards cellobiose were correlated with the substrate concentration dependence for velocity. Similar to the results of Vuong et al[18], the catalytic reaction of recombinant SsGOOX towards cellobiose exhibited substrate inhibition (Fig. 2). We simulated the recombinant SsGOOX reaction towards cellobiose with both the normal substrate inhibition model (equation 1) and a modified Hill's model (equation 2)[19]. Where, K_S is the dissociation constant for substrate binding, $V_{\max}$ is the maximum reaction velocity, V_i is the reaction velocity in the presence of inhibition, K_i is the substrate inhibition constant, and n_H is the Hill coefficient.

$$v = \frac{V_{\max} \cdot [S]}{K_S + [S] + \frac{[S]^2}{K_i}} \quad (1)$$

$$v = \frac{V_{\max} + V_i \cdot \left(\frac{[S]^2}{K_i^2} \right)}{1 + \frac{K_S^{n_H}}{[S]^{n_H}} + \frac{[S]^2}{K_i^2}} \quad (2)$$

The initial parameters were set referring to the parameters determined by Vuong et al[18], i.e., i.e., $V_{\max}$: 6.75 $\mu\text{M}/\text{min}$; V_i : 2.16 $\mu\text{M}/\text{min}$; K_i : 2.51 mM; K_S : 0.048 mM; n_H : 2.2. As depicted in Fig. 2, the modified Hill's model simulated the experimental data better, with a coefficient of determination value (R^2 value) of 0.995. Therefore, the kinetics parameters were determined using the modified Hill's model and listed in Table S1. The determined k_{cat} , K_S and K_i are respectively 729 min^{-1} , 0.0177 mM and 0.995 mM, different from but still comparable to those ($k_{\text{cat}} = 420 \text{ min}^{-1}$, $K_S = 0.048 \text{ mM}$, $K_i = 2.51 \text{ mM}$) determined by Vuong et al[18]. The differences between the parameter values of this work and Vuong et al might be due to the different approaches applied in parameter correlation. They first determined the K_S and $V_{\max}$ values by simulating the

experimental data in low substrate concentration region of the curve with Michaelis-Menten equation, and then determined other parameters using the modified Hill's equation. In this work, we directly determined all the parameters by simulating all the experimental data with the modified Hill's equation. Another reason for difference in kinetic parameter value may be the usage of different host strains for recombinant SsGOOX production, i.e., *P. pastoris* was used by Vuong et al, while *T. reesei* was used in this work[18].

Figure 2 Simulation of the experimental data using the normal substrate inhibition model (the dashed line) and the modified Hill's model (the solid line). Error bars show standard deviation (n=3; independent experiments).

During pH effect and temperature effect experiments, the concentration of cellobiose was set at 0.1 mM to ensure an excess of substrate (higher than K_s value). Effects of temperature and pH on recombinant SsGOOX are shown Fig. S2. The optimal temperature of recombinant SsGOOX is 50°C, which is consistent with the previously reported results[16]. Because the recombinant SsGOOX still exhibits about 70% of the maximal activity at 30°C, for ease of handling, in the following assays, the reaction was carried out at room temperature. However, the recombinant SsGOOX has an optimum pH of 9.0 as compared to native GOOX of *Acremonium strictum* T1 which has an optimum pH of 10.0 [16]. Moreover, the activity of the recombinant SsGOOX is less affected. As we found that at pH values 8.0 and 7.0, the recombinant SsGOOX still retains 97% and 85% activity, respectively, while at pH10.0 it has only 71% activity. We propose that difference between pH effects in our experiment and that of Lin et al might be due to different

glycosylation pattern of proteins in *T. reesei* and *Acremonium strictum* T1. As, SsGOOX is coupled with HRP in the detection of cello-oligosaccharides, therefore, pH value in the reaction system was controlled at 7.0 with phosphate buffer in the following assays. With respect to thermo-stability, SsGOOX is rather stable at 50 and 55°C, which means that it retains almost 100% activity when incubated for an hour at these temperatures. However, higher temperature, i.e., 60°C can result in its quick deactivation (Fig. S3).

Detection of reducing sugar

We propose that SsGOOX is highly effective in oxidizing oligosaccharides under appropriate reaction conditions. Indeed, in our reaction condition, up to 0.32 mM cellobiose has been completely oxidized within 25 min, as shown in Fig. 3. Initially, when cellobiose concentration was low, A_{515} increased gradually during the first 25 min and reached to a maximum value which remained constant thereafter, indicating complete exhaustion of cellobiose. Therefore, the duration of assay based on the concentrations of cellobiose or oligosaccharides was determined as 25 min. When the initial cellobiose concentration was high (for example, 0.32 mM), A_{515} value decreased over time slightly after reaching the peak value possibly due to further oxidation of the pink substance by excessive H_2O_2 . To observe this phenomenon, we added H_2O_2 manually and tested its effect on the stability of the pink substance and witnessed slight fading of the color (data not shown).

Figure 3 Cellobiose exhaustion upon recombinant SsGOOX oxidation over time indicated by HRP reaction. ○: 0.01mM ; △: 0.04mM ; □: 0.08mM ; ●: 0.32mM.

Figure 4 The relationship between A_{515} and cellobiose concentration in SsGOOX based HRP colorimetric method for cellobiose concentration assay. The standard curve of low cellobiose concentration range is shown in the insert.

We determined the detection range of cellobiose concentration for SsGOOX based assay. At low cellobiose concentrations (0-0.08 mM), A_{515} value was proportional to cellobiose concentration and a standard curve was obtained with a R^2 value of 0.996 (Fig. 4). In a broader cellobiose concentration range, the slope of the curve decreased at high cellobiose concentration region, which might be due to the absorbance property of the pink substance, substrate inhibition, or further oxidation of the pink substance by excessive H_2O_2 .

Heterologous expression and purification of LPMOs

As, the LPMOs from *Thielavia terrestris* are among the earliest reported ones which exhibited boosting effect on cellulase activity towards lignocellulose deconstruction [20]; thus, we choose two LPMOs from *T. terrestris* in order to verify the applicability and reliability of SsGOOX based method for the LPMO activity assay. The coding sequences of these two LPMOs are THITE-2142696 and THITE-170174 in GenBank, and the corresponding LPMOs are designated as TtAA9F and TtAA9G, respectively. The success of the recombinant expression of LPMOs in *T. reesei* culture supernatant was verified by SDS-PAGE and western blot analysis (Fig. 5). TtAA9F contains cellulose-binding domain 1 (CBM1), while TtAA9G is CBM free. The molecular weights of recombinant TtAA9F and TtAA9G were approximately 40 kDa and 35kDa, respectively, as estimated according to SDS-PAGE analysis. The molecular mass deduced according to the amino acid sequences of the recombinant proteins should respectively be 32.5 kDa and 26 kDa.

Therefore, glycosylation exists on both of the recombinant proteins of *T. reesei*. The recombinant proteins were both successfully purified to homogeneity through Ni²⁺ affinity chromatography and subsequent gel chromatography, as shown in Fig. 5.

Figure 5 SDS-PAGE and Western blot analysis of recombinant TtAA9F (A) and recombinant TtAA9G (B). **M:** molecular weight marker; **S1:** SDS-PAGE analysis of the culture supernatant of the parental strain; **S2:** SDS-PAGE analysis of the culture supernatant of the recombinant strain; **S3:** SDS-PAGE analysis of the purified recombinant protein; **W1:** Western blot analysis of the culture supernatant of the parental strain; **W2:** Western blot analysis of the culture supernatant of the recombinant strain; **W3:** Western blot analysis of the purified recombinant protein.

Activity and regioselectivity of the recombinant LPMOs

Activities of TtAA9F and TtAA9G towards PASC using Asc as the electron donor were determined through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis and high performance anion exchange chromatography with pulsed amperometric analysis (HPAEC-PAD). MALDI-TOF MS analysis of the supernatant of the reaction mixture revealed that cello-oligosaccharides and corresponding derivatives were generated in normal LPMO reactions, while in the control reactions there was no addition of Asc, thus there were no cello-oligosaccharides or derivatives released. As shown in Fig. 6, the characteristic molecular ion peaks corresponding to cello-oligosaccharides or their derivatives with various degrees of polymerization (DP) presented in the reaction supernatant of both LPMOs, indicating that the purified recombinant proteins were active LPMOs and they acted in an endo-type oxidative cleavage mode. At the same time, the two LPMOs produced distinctively different

270 product profiles. As an example, the molecular ion peaks in DP6 region were zoomed in and
271 shown as Fig. 6C and 6D. For TtAA9F, there were three main molecular ion peaks in this region,
272 peak 1013 that corresponds to sodium adduct of native hexasaccharide, peak 1029 that possibly
273 corresponds to sodium adduct of aldonic acid form of hexasaccharide, potassium adduct of native
274 hexasaccharide, or sodium adduct of gemdiol form of hexasaccharide, and peak 1051 that possibly
275 corresponds to sodium adduct of sodium salt of aldonic form of hexasaccharide (Fig. 6C). Peak
276 1029 and peak 1051 dominate this region, indicating that TtAA9F acts in a C1 oxidative mode[21].
277 Therefore, upon TtAA9F action, PASC is oxidatively cleaved, generating a small part of native
278 cello-oligosaccharides and a large part of aldonic acid form of cello-oligosaccharides. The
279 molecular ion peak profile of the product of TtAA9G action is significantly different. As shown in
280 Fig. 6D, there are two main molecular ion peaks in the DP6 region, peak 1013 that corresponds to
281 sodium adduct of hexasaccharide and peak 1011 that corresponds to sodium adduct of ketoaldose
282 derivate of hexasaccharide, or the sodium adduct of lactone derivate of hexasaccharide. The
283 abundance of peak 1011 exceeds 20% of that of peak 1013 in the product of TtAA9G, while this
284 peak is negligible in the product of TtAA9F. Therefore, peak 1011 most probably corresponds to
285 sodium adduct of ketoaldose derivate of hexasaccharide rather than sodium adduct of lactone
286 derivate of hexasaccharide, because the hydrated gemdiol derivatives generated by C4 oxidation are
287 more easily dehydrated as a result of sample preparation for MALDI-TOF MS analysis than the
288 aldonic acid derivatives generated by C1 oxidation. In addition, peak 1029 and peak 1051 that
289 probably correspond to sodium adducts of aldonic acid derivatives are very small. Therefore,
290 recombinant TtAA9G is most probably a C4 LPMO which oxidatively cleaved PASC, releasing
291 native cello-oligosaccharides and cello-oligosaccharide gemdiols.

Figure 6 MALDI-TOF-MS analysis of the products generated by two recombinant LPMOs with ascorbic acid as an electron donor. A: Products generated by the recombinant TtAA9F; **B:** Products generated by the recombinant TtAA9G; **C:** magnified DP6 peaks of A; **D:** magnified DP6 peaks of B.

We further identified native cello-oligosaccharides and their oxidative derivatives released from the reactions of TtAA9F and TtAA9G towards PASC by HPAEC-PAD (Fig. 7). The samples for HPAEC-PAD assay were prepared in three different modes: i) sample prepared with normal LPMO reaction using Asc as electron donor (the middle blue line); ii) sample prepared with LPMO reaction without Asc addition (the lower green line); iii) sample prepared with normal LPMO reaction using Asc as electron donor, and the lytic products further oxidized by SsGOOX (the upper purple line). The products generated by TtAA9F were detected as a series of native cello-oligosaccharides with degree of polymerization (DP) from 4 to 6 and aldonic acid derivatives of cello-oligosaccharides with DP from 1 to 6. The cello-oligosaccharides were eluted at retention times ranging from 7.6 min to 9.5 min, while the aldonic acid derivatives were eluted at retention times ranging from 7.3 min to 13.4 min. In control reaction which lacked Asc addition, there was no cello-oligosaccharide or oxidized derivative released. When the cello-oligosaccharides and oxidized derivatives generated by LPMO reaction were further oxidized by SsGOOX, the peaks corresponding to native cello-oligosaccharides disappeared, while those corresponding to C1-oxidized derivative of cello-oligosaccharide remained and slightly increased in peak area (Fig. 7A). Therefore, the peak profile of HPAEC-PAD analysis, along with the alteration of the peak profile after SsGOOX oxidation strongly supported that TtAA9F was a C1 type LPMO.

As for the products generated by TtAA9G, the peaks corresponding to native cello-oligosaccharides from DP1-DP6 were eluted at retention times ranging from 3.6 min to 9.6

min, while the peaks corresponding to C4-oxidized oligosaccharide derivatives emerged at retention times ranging from 21.3 min to 23.3 min. There was no obvious formation of aldonic acid derivative of cello-oligosaccharide. When the products generated by TtAA9G were further oxidized by SsGOOX, the native cello-oligosaccharides were transformed into aldonic acid derivatives, as indicated by the disappearance or emergence of the corresponding peaks, while the C4-oxidized cello-oligosaccharide derivatives were transformed into 4-ketoaldonic acids or gemidiol aldonic acids and the retention time of the corresponding peaks increased significantly (Fig. 7B). Therefore, based on the HPAEC-PAD analysis, TtAA9G was determined as a C4 LPMO which oxidatively cleaved PASC, releasing native cello-oligosaccharides and gemidiol derivatives of cello-oligosaccharide, both of which bear a reducing end.

Figure 7 HPAEC-PAD analysis of the products generated by reactions of TtAA9F (A) and TtAA9G (B) towards PASC. Control (lower line): control reaction without Asc addition; **Normal reaction (middle line):** normal LPMO reaction with Asc as the external donor; **Normal reaction+GOOX (upper line):** after normal LPMO reaction, GOOX was added to oxidize the reducing sugars. Non-oxidized oligosaccharides from DP2 to DP5 and the corresponding aldonic acid oligosaccharides (oxidized at C1) were used as standard.

Removal of residual Asc with ascorbic acid oxidase

Asc is a commonly used external electron donor for LPMO reaction. However, as previously reported, reducing agents such as Asc interfere with the HRP colorimetric assay [22]. In our LPMO activity assay method, the HRP colorimetric assay was applied to detect H₂O₂ generated along with the oxidation of cello-oligosaccharides or derivatives by SsGOOX. Therefore, we first

evaluated the effect of Asc on SsGOOX based HRP colorimetric assay and found a way to eliminate this effect. As shown in Fig. 8, the existence of 1 mM Asc in the cellobiose detection system indeed affected the detection results significantly. Fortunately, the interference of Asc could be easily eliminated by ascorbic acid oxidase. With the addition of 5 U/ml or above ascorbic acid oxidase and reaction for 1 min, Asc in the cellobiose detection system was efficiently oxidized and the detection results were similar to that of control reaction without Asc addition (Fig. 8). To ensure the complete oxidization of residual Asc, in the following assays, more ascorbic acid oxidase (20 U/ml) was used and the reaction time for Asc oxidization was prolonged to 5 min.

Figure 8 Elimination effect of ascorbate oxidase on residual Asc in SsGOOX based HRP colorimetric assay. The reaction time for ascorbic acid oxidation was 1 min, the cellobiose concentration for detection was set at 0.1 mM, and the absorbance without ascorbic acid addition was defined as 100%.

LPMO activity assay

For LPMO activity assay, the GOOX based cello-oligosaccharide detection method as set up above was applied to detect the release of cello-oligosaccharides after LPMO action. Activities of both the C1-type TtAA9F and the C4-type TtAA9G were assayed. Different amounts of LPMO were added into the reaction mixture with PASC as the substrate and Asc as the external electron donor, and reaction mixture without Asc addition was set as control. After 30 min of incubation, the amount of reducing cello-oligosaccharides and derivatives released in the supernatant of the reaction mixture was analyzed using the GOOX based detection method. The released amount of reducing sugars was linearly correlated with LPMO concentration ($R^2 > 0.994$); when the

concentrations of TtAA9F and TtAA9G were in the ranges of 0 to 3.6 μ M and 0 to 6.4 μ M, respectively (Fig. 9). Activities of both the C1 type and C4 type LPMOs can be analyzed through GOOX based assay HRP colorimetric method, and more importantly, this method directly targets the main activity of LPMOs.

To determine the lower limit of detection (LoD), we assayed the activities of both LPMOs with a series of low concentrations, and found the lowest detectable concentrations of 15 nM for TtAA9F and TtAA9G (Fig. 9C and 9D). Therefore, the A_{515} readings with deduction of the blank average of 60 independently operated blanks without LPMO, 60 independently diluted and assayed 15 nM TtAA9F samples, and 60 independently diluted and assayed 15 nM TtAA9G samples were used to determine the LoDs of our method. The LoDs were calculated according to Eq. (3) as suggested by Armbruster & Pry (2008) [23].

$$\text{LoD} = \text{LoB} + 1.645 \cdot (\text{SD}_{\text{low concentration sample}}) \quad (3)$$

Where, LoB was calculated according to Eq. (4).

$$\text{LoB} = \text{mean}_{\text{blank}} + 1.645 \cdot (\text{SD}_{\text{blank}}) \quad (4)$$

The LoBs (in A_{515} reading and with deduction of the average blank A_{515} reading) for TtAA9F and TtAA9G were calculated as 0.000735 and 0.000713, respectively. The LoDs (in A_{515} reading) for TtAA9F (C1 type) and TtAA9G were 0.00140 and 0.00162, respectively. The LoDs in LPMO concentration for TtAA9F and TtAA9G were calculated as 28.6 nM 27.9 nM, respectively, according to the standard curves for LPMO activity analysis (Fig. 9A and 9B). The purified TtAA9F and TtAA9G samples were respectively diluted to concentrations of 28.6 nM and 27.9 nM. Each dilution was independently repeated for 60 times, and the diluted samples were assayed with our method and compared with the corresponding LoBs. The results revealed that all the

assayed values were higher than the LoBs (Fig. 9E and 9F). Therefore, the LoDs of C1 type LPMO and C4 type LPMO in the SsGOOX based HRP colorimetric assay were verified as 28.6 nM and 27.9 nM, respectively.

Figure 9 Detection of the LPMO activity with the SsGOOX based HRP colorimetric assay

method. A: The A_{515} -concentration curve of TtAA9F, a linear relation was found in the range 0-3.6 μ M; **B:** The A_{515} -concentration curve of TtAA9G, the linear range was 0-6.4 μ M; **C:** Comparison of the raw assay readings of 12 blanks and 12 independently diluted samples containing 15 nM TtAA9F; **D:** Comparison of the raw assay readings of the blanks and the 15 nM TtAA9G samples; **E:** Comparison of the LoB and 60 independently assayed results of 28.6 nM TtAA9F samples; **F:** Comparison of the LoB and 60 independently assayed results of 27.9 nM TtAA9G at samples.

There is a noteworthy difference between the ratios of the total cello-oligosaccharides or the derivatives to the reducing cello-oligosaccharides or the derivatives released by C1-type LPMOs and C4-type LPMOs, i.e., both geminal diols and native cello-oligosaccharides generated by C4 LPMOs bear a reducing end, while only native cello-oligosaccharides generated by C1 LPMOs bear a reducing end. Therefore, in principle, in C4 LPMO activity assay with our method, all the soluble products released can be detected, while in C1 LPMO activity assay, only native cello-oligosaccharides can be detected, leading to the underestimation of C1 LPMO activity. However, the underestimation of C1 LPMO activity can be corrected with the ratio of the amount of total soluble lytic products to the amount of native soluble cello-oligosaccharide released by

C1-LPMO, which was designated as T/R ratio in this work and was estimated as 4.75 according to calculation of the peak areas in Fig. 7A. Although this coefficient may not be applicable to other C1 LPMOs, however it could provide a reference, or this coefficient may be independently determined for other C1 LPMOs.

In a batch enzyme reaction, initially the reaction speed is highest and known as “initial reaction speed” which remains constant for a considerable period and then the reaction speed starts declining due to consumption of the substrate, deactivation of the enzyme, or accumulation of the inhibitors. To obtain more accurate analysis, the “initial reaction time” for LPMOs during which the reaction speed remains constant should be determined. For TtAA9F, when the amount of enzyme used was 3.6 μ M, the initial reaction time was 30 min during which the reducing cello-oligosaccharide concentration increased linearly over time. As for TtAA9G, the initial reaction time was also 30 min when the amount of enzyme used was 6.4 μ M (Fig. 10). Therefore, we assume that a 30 min reaction time is appropriate for LPMO activity assay.

Based on the analysis results, we provided a definition of LPMO activity, that is, one unit of LPMO activity was defined as the amount of enzyme that released one nmol of cello-oligosaccharides and derivatives in one minute. According to this definition, the specific activity of TtAA9G was determined as 121.6 U/mg, while the specific activity of TtAA9F was determined as 270.3 U/mg by multiplying with the T/R ratio 4.75. The clear definition of LPMO activity and specific activity is important for the comparison of the catalytic efficiencies of AA9 LPMOs of different origin.

Figure 10 Determination of the initial speed range for LPMO activity assay. A: TtAA9F; B:

425 TtAA9G.

426 LPMO activity assay protocol

427 Based on the above results, we suggest the following assay protocol for testing LPMO activity.

428 **Step 1:** Set up the LPMO reaction system of 196 μ l in 1.5 ml Eppendorf tubes which consist of
429 100 μ l PASC suspension (with concentration of 90 g/L for C1 LPMO activity assay, or 30 g/L for
430 C4 LPMO activity assay), 10 μ l LPMO solution, and 76 μ l 100 mM phosphate buffer (pH7.5).

431 Set a reaction system with 10 μ l pure water substituting for 10 μ l LPMO solution as control.

432 Pre-incubate the reaction mixture at 50°C for 3 min and start the reaction by adding 10 μ l 20 mM
433 freshly prepared Asc and incubate at 50°C for 30 min.

434 **Step 2:** Place the reaction mixture immediately on ice after removing it from the water bath, add 4
435 μ l ascorbate oxidase solution (1000 U/ml) into the reaction mixture, and vortex for 5 minute to
436 remove Asc. Centrifuge the reaction mixture on a mini centrifuge at 12,000 r/min for 5 min and
437 transfer the supernatant to fresh tubes.

438 **Step 3:** Prepare the SsGOOX based HRP colorimetric assay on a 96-well microtiter plate. The
439 total reaction volume is 200 μ l, including 20 μ l of the supernatant from the LPMO reaction, 20 μ l
440 of 1 mM 4-AAP, 20 μ l of 20 mM DCHBS, 20 μ l of 1 μ M GOOX, 20 μ l of 500 U/ml HRP and 100
441 μ l of sodium phosphate buffer (100 mM sodium phosphate, pH7.0). The solutions of 4-AAP and
442 DCHBS solutions should be stored separately and used within 12 h.

443 **Step 4:** Incubate the 96-well plate at room temperature for 25 min and then record A_{515} on a
444 microtiter plate reader.

445 **Step 5:** Calculate the total amount of reducing oligosaccharides or derivatives released according to

a pre-established A₅₁₅-cellubiose concentration standard curve with the SsGOOX based HRP colorimetric assay method (for example, the insert of Fig. 4 in this work). For C4 LPMO activity assay, the amount of total oligosaccharides or derivatives equals the total amount of reducing oligosaccharides or derivatives, while for C1 LPMO, the amount of total oligosaccharides or derivatives equals the amount of reducing oligosaccharides times the T/R ratio. This ratio was determined as 4.75 according to the HPAEC-PAD analysis results of the products released by TtAA9F. For other C1 LPMOs it can be determined based on the HPAEC-PAD analysis of a certain C1 LPMO.

Discussions

A robust and easy method for AA9 LPMO activity assay is important not only for screening of AA9 LPMOs with high activity, but also for studying their properties and the catalytic mechanisms involved. Currently, there are two main approaches for LPMO activity assay. One approach is to determine the amount of cello-oligosaccharides or derivatives released from LPMO reaction through HPAEC-PID or HPLS-MS analysis[24-26]. This approach is time-consuming and has low-throughput. For example, analysis of one sample using HPAEC usually requires 25 min. The second approach utilizes the side reaction activities which generate H₂O₂ or catalyze the oxidation of chromogenic substrates such as 2,6-DMP or rPHP, and analyze the LPMO activity using a colorimetric method[11, 12, 14]. Although, this approach is considered potentially high-throughput but the assayed side reaction activities may not accurately correlate with the main activity that oxidatively cleaves the cellulosic substrate. Our work sets up a GOOX based HRP colorimetric method for LPMO activity assay, which is accurate, sensitive, potentially high-throughput, and more importantly it directly targets the main activity of LPMOs.

GOOXs belong to CAZy family AA7, catalyze the oxidation of gluco-oligosaccharides and simultaneously generate a stoichiometric amount of H₂O₂. Some of them have proved to be highly effective towards gluco-oligosaccharides and can completely oxidize the substrate in proper reaction conditions[16-18]. Our results also support the efficacy of GOOX action towards gluco-oligosaccharides and corresponding derivatives bearing a reducing end. After treatment with SsGOOX for 30 min, an overwhelming majority of cello-oligosaccharides or gemdiols was oxidized and transformed into corresponding aldonic acids (Figure 7). Therefore, GOOX oxidation coupled with the HRP colorimetric assay can be used for the assay of oligosaccharide concentration, cellulase activity, as well as LPMO activity. Ferrari et al[27] have developed a colorimetric method for the assay of cellulase and chitinase activity through a mutant chito-oligosacchride oxidase from *Fusarium graminearum*. Their assay presented superiority in sensitivity and ease of handling over the DNS method. AA9 LPMOs oxidize β -1,4-glucan in two manners. C1 LPMOs (class 1) oxidize pyranose ring of glucose moieties at C1 position, generating aldonic acids and native cello-oligosaccharides, of which only native cello-oligosaccharides bear reducing end; while C4 LPMOs (class 2) oxidize at C4 position, generating geminal diols and native cello-oligosaccharides, both of which bear reducing end[28]. In theory, analytic methods targeting the reducing sugars such as DNS method can be used for the assay of AA9 LPMO activity. However, the amount of reducing sugars released by AA9 LPMO is relatively much smaller than that released by cellulase, therefore the sensitivity of DNS method is inadequate for an accurate assay. In contrast to the DNS method, the GOOX based HRP colorimetric method is far more sensitive in the detection of reducing sugars, and thus exhibits great potential for accurate assay of reducing sugars released by LPMOs. We expressed a *S. strictum* GOOX gene in *T. reesei*

by using the strong constitutive *pdg* promoter, and obtained purified recombinant SsGOOX [29].

Albeit with slight differences, the properties of recombinant SsGOOX are by and large similar to those of native or *P. pastoris* expressed SsGOOX[18]. The purified recombinant SsGOOX was used for cellobiose assay along with the HRP colorimetric system and an A₅₁₅-cellobiose concentration standard curve was obtained. The standard curve was comparable to that of Ferrari et al [27]. In order to avoid complexity and keep the operational simplicity we used cellobiose to establish the standard curve. Actually, SsGOOX has broad substrate specificity and catalyze the oxidation of cello-oligosaccharides with different DP with kinetic parameters comparable to that of cellobiose [17]. This broad substrate specificity could also be verified with HPAEC-PAD analysis (as performed in this work, Figure 7). Therefore, we suggest that SsGOOX based assay method can detect all the soluble cello-oligosaccharides or derivates which bear a reducing aldehyde group and can be used for cellulases or AA9 LPMO activity assay, where the units of activity are generally defined according to the rate of oligosaccharide releasing.

For AA9 LPMO activity assay, Asc is frequently used as the external electron dono[24, 26, 30]. However, as a reducing reagent, Asc interferes in the HRP colorimetric detection results. Thus, we use ascobate oxidase to remove residual Asc, which perfectly performed i.e., with the addition of small amount of ascobate oxidase (5 U/ml) and short reaction time (within 1 min), the interference of Asc on HRP reaction is completely eliminated (Fig. 8). In addition, efficient removal of Asc by ascorbic acid oxidase results in fast termination of the LPMO reaction. Therefore, ascobate oxidase has two functions in our SsGOOX based HRP colorimetric assay, elimination of the interference of Asc and fast termination of the LPMO reaction.

Based on sequence alignment analysis, we selected two AA9 LPMOs from *T. terrestris* for

enzyme activity assay. One is TtAA9F, which is determined as C1 type LPMO through MALDI-TOF analysis and HPAEC-PAD analysis of the cleavage products released from PASC; the other one is TtAA9G, which is determined as C4 type LPMO. It is noteworthy that GOOX can also play an important role in regioselectivity analysis of LPMOs. The oxidative cleavage products of LPMO reaction when further treated with SsGOOX, the peak profile of HPAEC-PAD analysis changed significantly, and the changes were different for C1 oxidized products and C4 oxidized products (Fig. 7). By detecting the concentration of reducing cello-oligosaccharides and derivatives, the SsGOOX based HRP colorimetric method can detect as low as 28.6 nmol/L of C1 type LPMO (for TtAA9F, 28.6 nmol/L equals to 0.929 mg/L) and 27.9 nmol of C4 type LPMO (for TtAA9G, 27.9 nmol/L equals to 0.726 mg/L). The standard curves of A_{515} versus LPMO concentration possess coefficients of determination greater than 0.994. Thus, we suggest that the SsGOOX based HRP colorimetric method for LPMO activity assay presented here has the following advantages: i) after LPMO reaction, the whole assay process can be conveniently operated conveniently on a microtiter plate and the assay results can be recorded with a microtiter plate reader, therefore it is a high-throughput assay method; ii) the assay targets directly at the main activity of LPMOs rather than the side activities; iii) activities of both the C1 and C4 type LPMOs can be assayed; iv) it is sensitive and accurate; v) except a microplate reader, no other expensive instrument is needed, which portrays it as a cheap and ready to use method.

In the assay process, three tool enzymes are used, HRP, ascorbate oxidase, and SsGOOX. HRP and ascorbate oxidase are both commercially available, while SsGOOX is prepared by ourselves. The HRP colorimetric system is a convenient and commonly used tool for the detection of H_2O_2 . Ascorbate oxidase is added right after the LPMOs reaction which removes Asc residue and

simultaneously terminates the LPMOs reaction, thus the step of Asc removal does not add any complexity to the assay process. SsGOOX is added along with the HRP colorimetric mixture, therefore the application of SsGOOX also does not add any complexity. If SsGOOX or other comparable GOOXs are commercially available, it will be convenient to use this GOOX based LPMO activity assay method.

For C1 type LPMO activity assay, there might exist an underestimation of their activity because the aldonic acids released cannot be detected by the SsGOOX based HRP colorimetric assay. Therefore, a ratio of the amount of total soluble lytic products to the amount of native cello-oligosaccharides (T/R ratio) is introduced to compensate this underestimation. Sampling the product of LPMO reaction for activity assay is carried out in the initial speed region, i.e., within 30 min of the reaction (Fig. 10). In the initial speed region of an enzymatic reaction, the reaction speed is generally assumed to be constant, i.e. the formation of total oligosaccharides and derivatives is linearly correlated with the reaction time. On the other hand, the formation of reducing oligosaccharides also has linear correlation with the reaction time as reflected in Fig. 10. Therefore, the ratio of the amount of total oligosaccharides and derivatives to the amount of reducing oligosaccharides (T/R ratio) should be constant in the initial speed region which was determined as 4.75 according to the HPAEC-PAD analysis results.

Conclusion

We have established a sensitive, robust, and direct gluco-oligosaccharide oxidase based HRP colorimetric assay for the detection of LPMO activity. This method can detect the activities of both the C1 and C4 type LPMOs at concentrations as low as 28.6 nmol/L and 27.9 nmol/L,

respectively, targeting their main activities with a standard curve of R^2 value greater than 0.994.

The assay process can be operated on microtiter plates ready to use for high-throughput screening,

and therefore has potential applications in large scale screening of LPMOs with high activity or in

screening procedures for directed evolution of LPMOs. Based on the assay method, we have

proposed a definition of LPMO activity unit that accurately reflects the main activities of LPMOs

and can be used for the comparison of LPMOs of different origins.

Methods

Strains, plasmids, and cultivation conditions

Escherichia coli DH5 α ' was used for plasmid construction and propagation, and was cultivated in

LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, supplemented with 100 μ g/mL ampicillin

if necessary). *Trichoderma reesei* QM9414 strain (ATCC 26921) was used as a host for the

heterologous expression of the recombinant proteins. The cultivation conditions of *T. reesei* were

followed as described previously[29]. *Thielavia terrestris* (ATCC 38088) was cultivated on PDA

agar at 45°C. Plasmid pUC19 was used for the construction of vectors or expression cassettes.

Plasmid pAN7-1 containing the hygromycin B resistant cassette was used as an assisting plasmid

for *T. reesei* transformation [31].

Enzymes and chemicals

Phosphoric acid swollen cellulose (PASC) was prepared with Avicel PH-101 (Sigma-Aldrich) as

raw material following the method of Cannella et al [32]. Horseradish peroxidase (HRP) was

purchased from Macklin (Shanghai), of which one unit of enzyme activity was defined as the

amount of enzyme that form 1.0 mg purpurogallin from pyrogallol in 20 sec at pH6.0, 20°C. Ascorbic acid oxidase was purchased from Solarbio (Beijing), of which one unit of enzyme activity is defined as the amount of enzyme that oxidizes 1.0 μmol of L-ascorbate to dehydroascorbate per min at 25°C. 4-amino-antipyrine (4-AAP) was purchased from Macklin (Shanghai). 3,5-dichloro-2-hydro-xybenzenesulfonic acid (DCHBS) was purchased from Solarbio (Beijing). Other chemicals were in analytical grade and purchased from BBI Life Sciences Corporation (Shanghai).

Construction of expression vector and Protoplast transformation

The genomic DNA of *T. reesei* and *T. terrestris* was extracted using the fungal genomic DNA extracting kit (Sangon Biotech, Shanghai). The promoter and the terminator sequences of the *pdg* gene were PCR amplified from the genomic DNA of *T. reesei* QM9414 as template. The coding sequence of GOOX from *Sarocladium strictum* was codon-optimized for expression in *T. reesei* (Table S2) and was commercially synthesized by IGEbio (Guangzhou, China), with addition of signal peptide sequence of *T. reesei cbh1* at N-terminal and a 6×histidine tag at C-terminal. Genes of TtAA9F and TtAA9G (GenBank sequence numbers: THITE-2142696 for TtAA9F and THITE-170174 for TtAA9G) including their native signal peptide sequences were PCR amplified from the genomic DNA of *T. terrestris*, and a 6×histidine tag was fused with the C-terminal of each gene during PCR. The expression cassettes were constructed by sequential ligation of the *pdg* promoter, the objective genes, and the *pdg* terminator into pUC19 with ClonExpress™ II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). For protoplast transformation of *T. reesei*, the

linearized constructions and pAN7-1 were co-transformed using the polyethylene glycol method as described in Punt et al[31].

Recombinant protein expression and purification

Recombinant protein production in *T. reesei* was performed as described in Li et al[29]. Total Protein concentration was determined with the standard Bradford reagent kit (Sangon Biotech, Shanghai, China). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10% polyacrylamide gel slabs. The protein band was visualized by staining with Coomassie Brilliant Blue G250 and the molecular weight of the observed proteins was estimated according to the pre-stained broad-range protein standard marker (Thermo Scientific PageRuler Prestained Protein Ladder). Expression of the target proteins was verified through Western blotting with anti-6×His tag mouse monoclonal antibody and HRP-conjugated rabbit anti-mouse IgG (BBI Life Sciences Corporation, Shanghai). Purification of the recombinant proteins was performed on an ÄKTA Purifier UPC100 FPLC-System (GE Healthcare) as principally described as follows. The supernatant was filtered with a 0.45 µm pore size polyvinylidene fluoride (PVDF) membrane to remove the residual insoluble substances. The filtrate was put into a dialysis bag with a 7000 molecular weight cutoff covered with PEG 20000 powder, and incubated at 4°C for 12 hours for concentration. The concentrated sample was dialyzed against the affinity chromatography binding buffer (20 mM sodium phosphate, pH 8.0, 500 mM NaCl) for 12 hours, during which the buffer was changed once. After dialysis, the sample was filtered with a 0.22 µm pore size PVDF membrane, and the recombinant proteins were purified using Ni Sepharose 6 Fast Flow on the FPLC-system. The flow rates of eluent A (20 mM sodium phosphate, pH 8.0, 500

mM NaCl) and eluent B (eluent A+500 mM imidazole) were adjusted to fulfill the gradient elution condition, i.e., in the loading stage, the imidazole concentration was maintained at 25mM; in the washing stage, the imidazole concentration was adjusted in a stepwise gradient (50-75 mM) to remove nonspecifically bound proteins; and then imidazole concentration in the elution buffer was maintained at 250 mM imidazole to elute the target proteins. Fractions containing the purified protein were concentrated by Amicon Ultra Centrifugal Filters (Millipore, Germany) and were further purified by gel filtration chromatography using a Superdex 200 Increase 10/300 GL column (GE Healthcare) eluted by an eluent containing 20 mM Tris (pH 7.5) and 150 mM NaCl at a flow rate of 0.5 ml/min. To recover the possible loss of Cu²⁺ during purification, the purified LPMO solutions were respectively mixed with equal volume of copper sulfate solution with a concentration of 3 times of the LPMOs, and incubated for 1 h at 4°C. The excessive copper was then removed by desalting the protein using Sephadex G-25 Medium (GE Healthcare).

Setting up the SsGOOX based HRP colorimetric method for cellobiose concentration assay

The amounts of 4-AAP and DCHBS used for the assay were first optimized. For optimization of 4-AAP dosage, 20 µl 4-AAP solutions with different concentrations were mixed with equal volume of 1 mM cellobiose, and loaded on a 96-well microtiter plate. Then the chromogenic reaction was started by addition of 20 µl of 100 mM DCHBS, 20 µl of 1 µM GOOX, 20 µl of 500 U/ml HRP and 100 µl of sodium phosphate buffer (100 mM, pH7.0). A₅₁₅ was detected after incubating at room temperature for 30 min using a Synergy MX microplate reader (BioTek, Vermont, USA). For optimization of DCHBS dosage, similar process was carried out, except that

the roles of 4-AAP and DCHBS were exchanged, and the concentration of 4-AAP in the reagent mixture was set at 0.1 mM.

The standard curve for cellobiose analysis was set up by using a series of cellobiose solutions with concentrations ranging from 0.01-0.35mM as the substrate. Twenty microliter cellobiose solutions were loaded on a 96-well microtiter plate, and 180 μ L reagent mixture which consists of 20 μ L of 1 mM 4-AAP, 20 μ L of 20 mM DCHBS, 20 μ L of 1 μ M GOOX, 20 μ L of 500 U/ml HRP and 100 μ L of sodium phosphate buffer (100 mM, pH7.0) was added to each well to start the chromogenic reaction. A_{515} was then continuously detected at room temperature on the Synergy MX microplate reader. An assay with 20 μ L pure water substituting for cellobiose solution was used as blank. The relation between A_{515} and cellobiose concentration was analyzed and the standard curve was plotted in proper cellobiose concentration range.

Characterization of the enzymatic properties of recombinant SsGOOX

The properties of SsGOOX were characterized by measuring its catalytic reaction velocity at different conditions. The HRP colorimetric assay on 96-well microtiter plates was used to measure hydrogen peroxide production and the reaction velocity[18]. The HRP reaction mixture contained 0.1 mM 4-AAP, 2 mM DCHBS, 50 U/ml HRP, 100 mM phosphate buffer (pH7.0). To determine the optimal reaction temperature, 100 nM recombinant SsGOOX was used to oxidize 1 mM cellobiose in 40 mM Britton-Robinson buffer (pH7.0) for 2 min at different temperatures [33]. Then 20 μ L reaction mixture was added to 180 μ L HRP colorimetric assay mixture, and A_{515} was measured when the color reaction proceeded to 3 min at room temperature. The optimal reaction pH was determined mainly following the above approach, except that the reaction pH was set at

different values in the range of pH 2 to 12 using 40 mM Britton-Robinson buffer and the reaction temperature was set at 50°C. The thermal stability of the enzyme was determined in triplicate by incubating 1 µM GOOX in 100 mM phosphate buffer (pH 7.0) for 0, 2, 4, 6, 8, 10, 15, 20, and 60 min at 50°C, 55°C, 60°C, respectively; and the residual enzyme activity was measured at pH 7.0 and 50°C.

For enzyme kinetic study, 10 nM of recombinant SsGOOX and a series of cellobiose concentrations ranging from 0.02-5 mM were used. The initial reaction velocities were obtained by measuring reaction product when the reaction proceeded to 30 s at room temperature, pH 7.0, and then data were fit using the modified Hill's equation to determine kinetic parameters using non-linear least squares regression in the curve fitting tool in Matlab©.

MALDI-TOF MS analysis

MALDI-TOF MS analysis of the oligosaccharide products released by LPMO reaction was carried out on a 5800 MALDI-TOF MS (AB SCIEX), using 5-chloro-2-mercapto-benzothiazole (CMBT) and 2, 5-dihydroxybenzoic acid (DHB) as the matrix as described previously [34]. MS data acquisition mass range was from m/z 500 to 2500.

HPAEC-PAD analysis

The supernatants of LPMO reactions were diluted 1000 times, and 25 µL diluted samples were injected for HPAEC analysis on a Dionex ICS5000+ system equipped with a pulsed amperometric detector (PAD detector) and a CarboPac PA200 column (3×30 mm guard column followed by a 3×250 mm analytical column). Products of LPMO reactions were separated on the PA200 column

678 with gradient elution at a flow rate of 0.4 ml/min at 30 °C. The flow rates of eluent A (0.1 M
679 NaOH) and eluent B (0.1 M NaOH+1 M sodium acetate) were automatically adjusted to fulfill the
680 gradient elution condition, i.e., the concentration of sodium acetate in the eluent increasing from 0
681 to 140 mM (14 min), 140 to 300 mM (8 min), 300 to 400 mM (4 min), and then held constant at
682 500 mM (3 min) before re-equilibration in 0.1 M NaOH (4 min). The oligosaccharides with the
683 degree of polymerization (DP) ranged from DP2 to DP5 (Elicityl, Crolles, France) and their
684 oxidation products by GOOX were used as standards.

685 Removing the residual Asc with ascorbic acid oxidase

686 4 µl of ascorbic acid oxidase solutions with different concentrations (0, 25, 50, 75, 125, 250, 500,
687 1000, 1500, 2000 U/ml), were respectively mixed with 96 µl 2 mM cellobiose solution and 100 µl
688 freshly prepared 2 mM Asc solution, and incubated at room temperature for 1 min. Then 20 µl of
689 the reaction mixtures were added into 180 µl SsGOOX based HRP colorimetric reagent containing
690 0.1 mM 4-AAP, 2 mM DCHBS, 100 mM phosphate buffer (pH 7.5), and 100 nM GOOX,
691 50 U/ml horseradish peroxidase pre-loaded on a 96-well microtiter plate. A₅₁₅ was recorded on the
692 microtiter plate reader. A control reaction without Asc but with ascorbic acid oxidase (20 U/ml in
693 the Asc oxidizing reaction mixture) was set to verify that ascorbic acid oxidase alone does not
694 affect the SsGOOX based HRP colorimetric assay.

695 LPMO activity assay

696 PASC was used as the substrate for LPMOs reaction. The 196 µl LPMO reaction mixture consisted
697 of 100 µl PASC suspension (90 g/L for C1 LPMO activity assay, 30 g/L for C4 LPMO), 10 µl

LPMO with a certain concentration, 76 μ l 100 mM phosphate buffer (pH7.5), and 10 μ l 20 mM freshly prepared Asc solution. The first three solutions were added into a 1.5 ml eppendorf tube and pre-incubated on a thermomixer (Eppendorf® Thermomixer Comfort) with shaking at 50°C for 3 min, the Asc solution was then added to start the reaction thereafter. The reaction was performed with shaking (1200 r/min) at 50°C for 30 minutes. After the LPMO reaction, the eppendorf tube was immediately placed on ice. With the addition of 4 μ l ascorbic acid oxidase (1000 U/ml), the tube was vortexed on the thermomixer for 5 min at room temperature to remove the residual Asc and to terminate the LPMO reaction. The reaction mixture was then centrifuged at 12,000 r/min in a mini centrifuge at 4°C for 5 min. Finally 20 μ l supernatant was added to 180 μ l SsGOOX based HRP colorimetric reagent consisting of 20 μ l of 1 mM 4-AAP, 20 μ l of 20 mM DCHBS, 20 μ l of 1 μ M GOOX, 20 μ l of 500 U/ml HRP and 100 μ l of sodium phosphate buffer (100 mM sodium phosphate, pH7.0) pre-loaded on the wells of a microtiter plate. A₅₁₅ was continuously detected for 25 min on a microtiter plate reader.

List of abbreviations

4-AAP: 4-amino-antipyrine; AA9: auxiliary activities family 9; Asc: ascorbic acid; CBM1: cellulose-binding domain 1; DCHBS: 3,5-dichloro-2-hydroxybenzenesulfonic acid; DP: degree of polymerization; FAD: flavin adenine dinucleotide; GOOX: gluco-oligosaccharide oxidase; HPAEC: high performance anion exchange chromatography; HRP: horseradish peroxidase; LoB: limit of blank; LoD: limits of detection; LPMO: lytic polysaccharide monooxygenases; MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PASC: phosphoric acid swollen cellulose.

719 **Declarations**

720 **Ethics approval and consent to participate**

721 Not applicable

722 **Consent for publication**

723 Not applicable

724 **Availability of data and materials**

725 All data generated or analysed during this study are included in this published article and its
726 supplementary information files.

727 **Competing interests**

728 The authors declare that they have no competing interests.

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733 **Authors' contributions**

734 GL, JW and NX designed the research; SW, JT and MA performed research and analyzed data;
735 GL, MA and JW wrote the paper. All authors read and approved the final manuscript.

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Figures

Figure 1

SDS-PAGE (A) and Western blot (B) analysis of the recombinant SsGOOX protein. M: molecular marker; S1 and W1: culture supernatant of the parent strain *T. reesei* QM9414; S2 and W2: culture supernatant of the recombinant strain; S3 and W3: purified recombinant SsGOOX.

Figure 2

Simulation of the experimental data using the normal substrate inhibition model (the dashed line) and the modified Hill's model (the solid line). Error bars show standard deviation ($n=3$; independent experiments).

Figure 3

Cellobiose exhaustion upon recombinant SsGOOX oxidation over time indicated by HRP reaction. $\bullet$: 0.01mM ; $\square$: 0.04mM ; $\triangle$: 0.08mM ; $\circ$: 0.32mM.

Figure 4

The relationship between A_{515} and cellobiose concentration in SsGOOX based HRP colorimetric method for cellobiose concentration assay. The standard curve of low cellobiose concentration range is shown in the insert.

Figure 5

SDS-PAGE and Western blot analysis of recombinant TtAA9F (A) and recombinant TtAA9G (B). M: molecular weight marker; S1: SDS-PAGE analysis of the culture supernatant of the parental strain; S2: SDS-PAGE analysis of the culture supernatant of the recombinant strain; S3: SDS-PAGE analysis of the purified recombinant protein; W1: Western blot analysis of the culture supernatant of the parental strain; W2: Western blot analysis of the culture supernatant of the recombinant strain; W3: Western blot analysis of the purified recombinant protein.

Figure 6

MALDI-TOF-MS analysis of the products generated by two recombinant LPMOs with ascorbic acid as an electron donor. A: Products generated by the recombinant TtAA9F; B: Products generated by the recombinant TtAA9G; C: magnified DP6 peaks of A; D: magnified DP6 peaks of B.

Figure 7

HPAEC-PAD analysis of the products generated by reactions of TtAA9F (A) and TtAA9G (B) towards PASC. Control (lower line): control reaction without Asc addition; Normal reaction (middle line): normal LPMO reaction with Asc as the external donor; Normal reaction+GOOX (upper line): after normal LPMO reaction, GOOX was added to oxidize the reducing sugars. Non-oxidized oligosaccharides from DP2 to DP5 and the corresponding aldonic acid oligosaccharides (oxidized at C1) were used as standard.

Figure 8

Elimination effect of ascorbate oxidase on residual Asc in SsGOOX based HRP colorimetric assay. The reaction time for ascorbic acid oxidation was 1 min, the cellobiose concentration for detection was set at 0.1 mM, and the absorbance without ascorbic acid addition was defined as 100%.

Figure 9

Detection of the LPMO activity with the SsGOOX based HRP colorimetric assay method. A) The A_{515} -concentration curve of TtAA9F, a linear relation was found in the range 0-3.6 μM ; B) The A_{515} -concentration curve of TtAA9G, the linear range was 0-6.4 μM ; C) Comparison of the raw assay readings of 12 blanks and 12 independently diluted samples containing 15 nM TtAA9F; D) Comparison of the raw assay readings of the blanks and the 15 nM TtAA9G samples; E) Comparison of the LoB and 60

independently assayed results of 28.6 nM TtAA9F samples; F Comparison of the LoB and 60 independently assayed results of 27.9 nM TtAA9G at samples.

Figure 10

Determination of the initial speed range for LPMO activity assay. A: TtAA9F; B: TtAA9G.

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