# Chapter 7

# Analyzing Activities of Lytic Polysaccharide Monooxygenases by Liquid Chromatography and Mass Spectrometry

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## Abstract

Lytic polysaccharide monooxygenases perform oxidative cleavage of glycosidic bonds in various polysaccharides. The majority of LMPOs studied so far possess activity on either cellulose or chitin and analysis of these activities is therefore the main focus of this review. Notably, however, the number of LPMOs that are active on other polysaccharides is increasing. The products generated by LPMOs from cellulose are either oxidized in the downstream end (at C1) or upstream end (at C4), or at both ends. These modifications only result in small structural changes, which makes both chromatographic separation and product identification by mass spectrometry challenging. The changes in physicochemical properties that are associated with oxidation need to be considered when choosing analytical approaches. C1 oxidation leads to a sugar that is no longer reducing but instead has an acidic functionality, whereas C4 oxidation leads to products that are inherently labile at high and low pH and that exist in a keto-gemdiol equilibrium that is strongly shifted toward the gemdiol in aqueous solutions. Partial degradation of C4-oxidized products leads to the formation of native products, which could explain why some authors claim to have observed glycoside hydrolase activity for LPMOs. Notably, apparent glycoside hydrolase activity may also be due to small amounts of contaminating glycoside hydrolases since these normally have much higher catalytic rates than LPMOs. The low catalytic turnover rates of LPMOs necessitate the use of sensitive product detection methods, which limits the analytical possibilities considerably. Modern liquid chromatography and mass spectrometry have become essential tools for evaluating LPMO activity, and this chapter provides an overview of available methods together with a few novel tools. The methods described constitute a suite of techniques for analyzing oxidized carbohydrate products, which can be applied to LPMOs as well as other carbohydrate-active redox enzymes.

**Key words** Lytic polysaccharide monooxygenase, High-performance anion-exchange chromatography, Porous graphitized carbon, Aldonic acid, Gemdiol, Hydrophilic interaction liquid chromatography

## 1 Introduction

### 1.1 Chitin

The fact that LPMOs (previously known as CBM33 and GH61) are enzymes was discovered in 2010 by Vaaje-Kolstad et al. [1]. The first described activity for a LPMO was CBP21 (or *Sm*LPMO10A),

D. Wade Abbott and Alicia Lammerts van Bueren (eds.), *Protein-Carbohydrate Interactions: Methods and Protocols*, Methods in Molecular Biology, vol. 1588, DOI 10.1007/978-1-4939-6899-2_7, © Springer Science+Business Media LLC 2017

a chitin-active C1-oxidizing bacterial LPMO. The formation of oxidized chito-oligosaccharides was analyzed at high resolution using hydrophilic interaction liquid chromatography (HILIC) (Fig.1a) and MALDI-ToF (using both ion doping and isotope labeling; Fig. 1b). The HILIC method was developed for separating native chito-oligosaccharides and chitoaldonic acids in the same analysis. Whereas native chito-oligosaccharides retain well in acetonitrile–water, proper retention of aldonic acids (i.e., charged carboxylic acids) requires increased ionic strength and pH of the elution buffer.

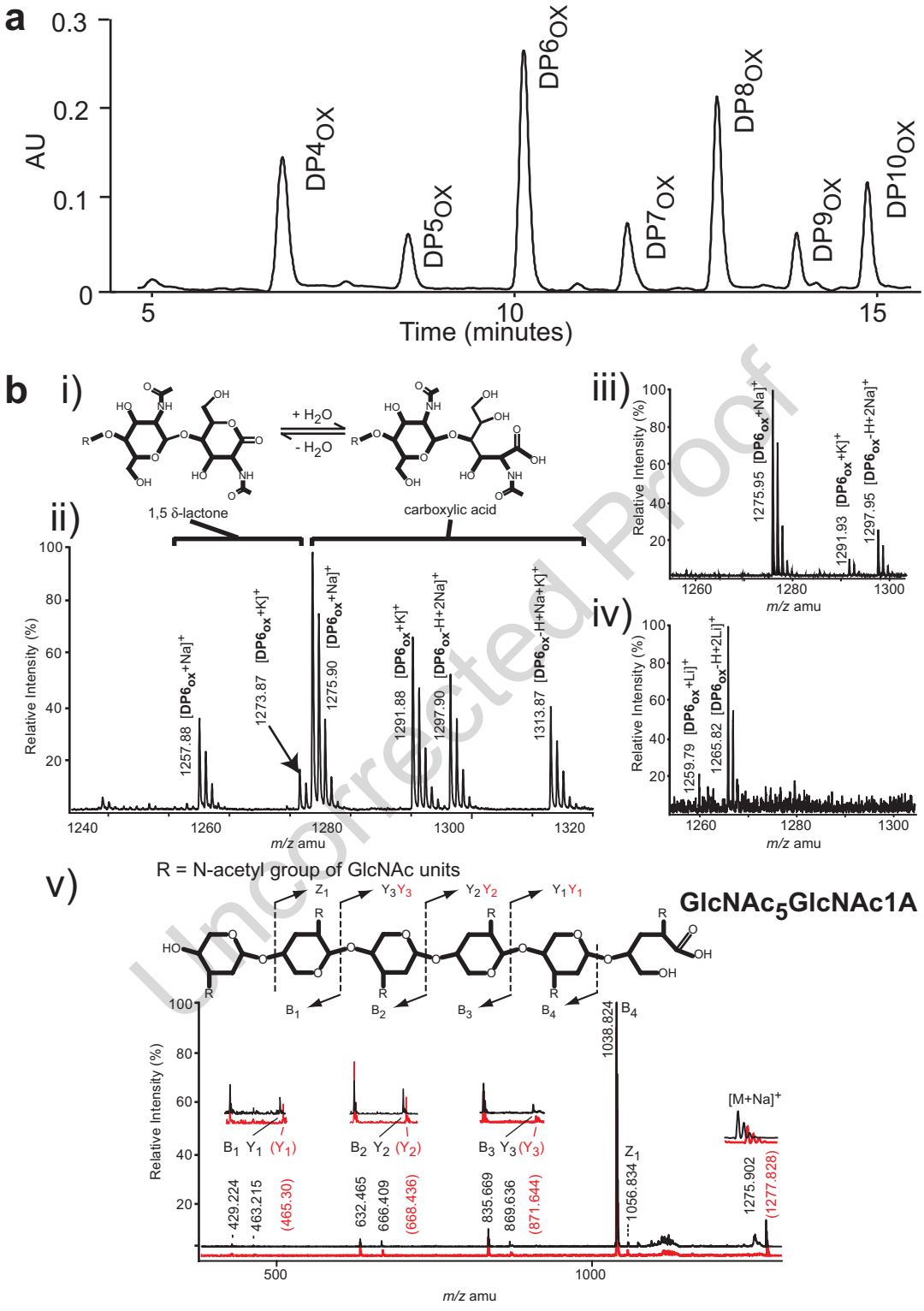
In this early work, detection of oxidized oligomeric products by mass spectrometry was shown to be very useful, but also challenging, due to the equilibrium between the aldonic acids and the corresponding lactones; and the overlapping masses of the sodium adducts of oxidation products and the commonly observed K-adducts of the corresponding native oligosaccharides. In this respect, the combination of MALDI-ToF MS with isotope labeling and/or metal doping is very useful [1] (Fig. 1b, c). Metal doping is simple and should probably be used routinely. The use of labeling techniques is more complicated, but also a powerful tool for the identification of oxidations, as shown in Fig. 1b.

### *1.2 Cellulose*

As predicted upon the discovery of LPMO activity on chitin in 2010, LPMOs acting on cellulose were described soon after, in 2011, by several groups [3–6]. Oxidized cello-oligosaccharides were analyzed by high-performance anion-exchange chromatography (HPAEC; Fig. 2) and MALDI-ToF MS. Analysis of cello-oligosaccharides requires different detection methods (e.g., pulsed amperometric detection, charged aerosol detection or ESI-MS) compared to chito-oligosaccharides because the former do not



**Fig. 1** Analysis of C1-oxidized chito-oligosaccharides. (**a**) UHPLC-HILIC analysis of oxidized chito-oligosaccharides. Some ionic strength (15 mM Tris–HCl, pH 8.0) was essential to obtain retention of aldonic acids. Note that α and β-anomers would be separated under these chromatographic conditions if the oligosaccharide would have a normal reducing end (as in **c**, below). The lack of such separation thus indicates that the reducing end is modified. (**b**) (*i*) Equilibrium between the lactone form and the aldonic acid form of oxidized chitobiose. (*ii*) MS analysis at lower pH (promoting the lactone form) without metal doping, showing both the lactone (1257 for the sodium adduct) and the aldonic acid form (1275 for the sodium adduct) of the hexameric C1-oxidized product, as well as the distribution of sodium and potassium adducts. Note that the mass difference between sodium (23) and potassium (39) is 16. Furthermore, note that aldonic acids form diagnostic sodium and potassium salts, meaning that one proton is replaced by $Na^+$ or $K^+$. (*iii*) Analysis of the sample of (*ii*) at higher pH (almost no lactone) and (*iv*) after lithium (7) doping gives a simpler spectrum representing the chitoaldonic acids: *m/z* 1259 for the lithium adduct and *m/z* 1265, for the lithium salt of the lithium adduct. (*v*) Fragmentation mass spectra of chitohexaaldonic acid (indicated above the spectrum) formed in reactions with $H_2{}^{16}O$ (*black*) or $H_2{}^{18}O$ (*red*). Only the Y ions show *m/z* +2 for reactions run in $H_2{}^{18}O$, showing that the oxidation is in the down-stream end. (**c**) Analysis of $GlcNAc_2$ in its native and oxidized (aldonic acid) form. The oxidized disaccharide was generated by reaction of $GlcNAc_2$ with a chito-oligosaccharide oxidase called ChitO (*blue* chromatogram; *see* [2] for details). Note that native $GlcNAc_2$ (*green* chromatogram) elutes earlier, and with anomer separation. (**a**) and (**b**) were reproduced from [1] with permission from AAAS; (**c**) was reproduced from [2]



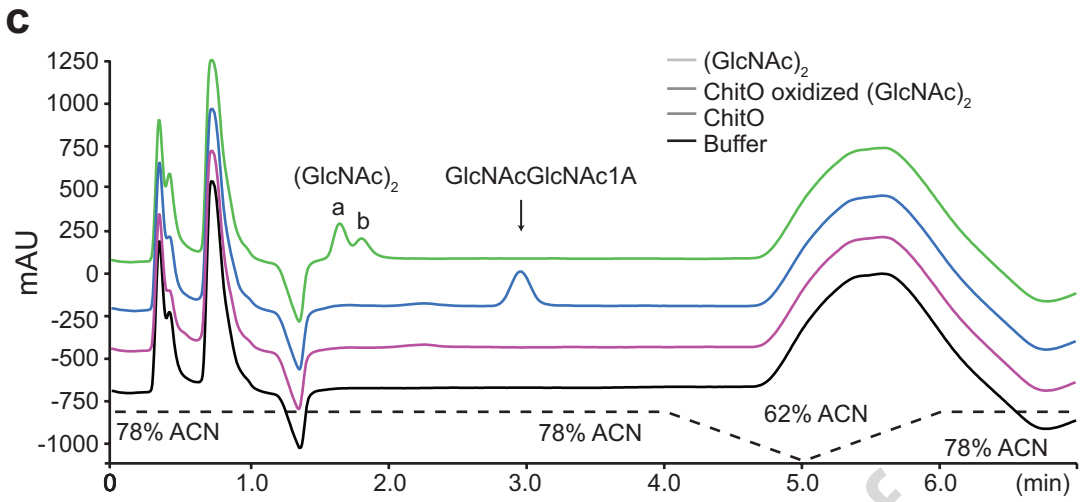



**Fig. 1** (continued)

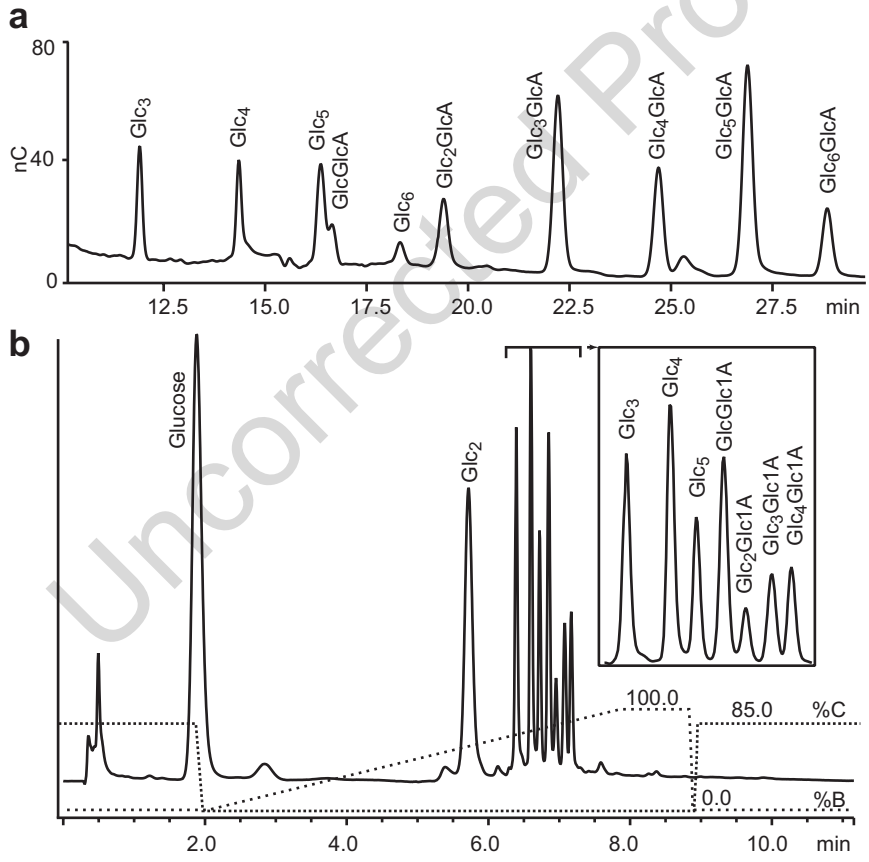





**Fig. 2** HPAEC analysis of C1 oxidized cello-oligosaccharides. (**a**) Standard procedure (*see* Subheading 3.2); native cello-oligosaccharides elute first followed by the aldonic acids. There is a slight overlap between the two product clusters, which implies that the C1-oxidized monomer and dimer elute among the late eluting native oligosaccharides. (**b**) the faster procedure (*see* Subheading 4.2); by shortening the column length to a guard column, run times of approximately 10 min are sufficient, allowing high throughput HPAEC, if reduced resolution is acceptable. (**a**) is reproduced from [3] with permission from John Wiley and Sons; (**b**) is reproduced from ref. [7] with permission from Elsevier

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absorb UV light. Aldonic acids are stable at high pH and HPAEC is therefore a suitable method for analyzing C1-oxidized cellodextrins. In later work, it was shown that the normally time-consuming HPAEC procedure could be made much faster by only using a guard column that provides sufficient separation of aldonic acids and native species with a total run time of only 10 min (Fig. 2b; [7]). At the alkaline pH during the HPAEC analysis, the equilibrium between the lactone and acid is strongly shifted toward the aldonic acid, and this makes HPAEC ideal for analysis of C1-oxidized products (the p$K_a$ of cellobionic acid is 3.5 [8]).

In contrast to the chemically stable aldonic acids, oxidation in the non-reducing end (C4-oxidation) results in products that are much more prone to decomposition at extreme pH. It was recently shown that the gemdiols undergo on-column decomposition during HPAEC [8] (Fig. 3), leading to products with additional oxidations and, most importantly, native cello-oligosaccharides that have one less glucose that the original C4-oxidized product.

Due to this undesirable effect during HPAEC an alternative method based on porous graphitized carbon (PGC) chromatography has been developed to enable simultaneous screening of C1 and C4 oxidized cellodextrins (Fig. 4; [8]). PGC chromatography may be combined with charged aerosol detection (CAD), where sufficiently high sensitivity can be obtained by employing low ion-strength eluents (low nanomole range; [8]). While CAD



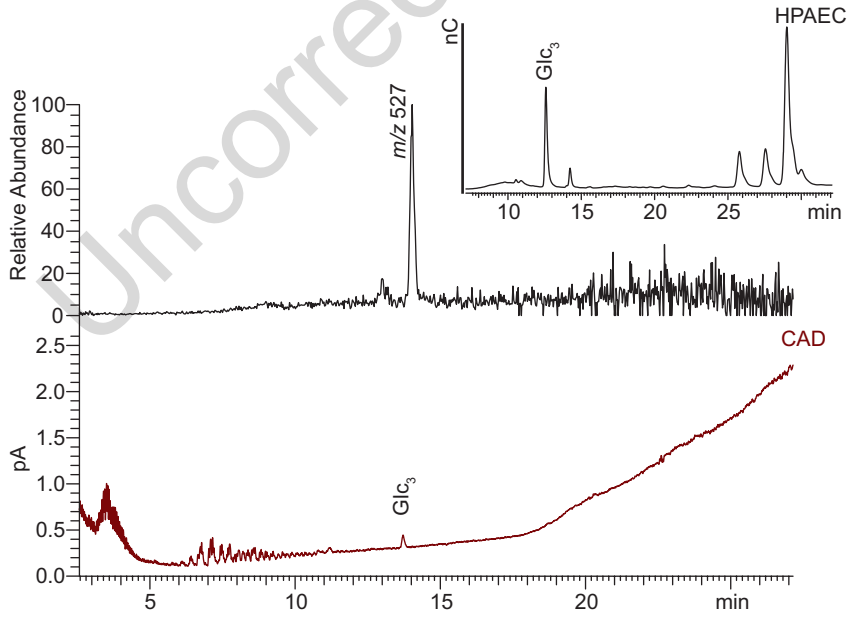


**Fig. 3** Decomposition of C4-oxidized cellodextrins during HPAEC. A purified C4 oxidized tetramer was subjected to standard HPAEC (*upper right chromatogram*) and the peak eluting at 12.8 min (labeled $Glc_3$) was collected and reinjected on a PGC column where it coelutes with cellotriose (*lower choromatogram*; 13.8 min). Mass spectrometry analysis of the compound confirms that it is a native trimer (*m/z* = 527; sodium adduct). This figure was reproduced from [8] with permission from Elsevier

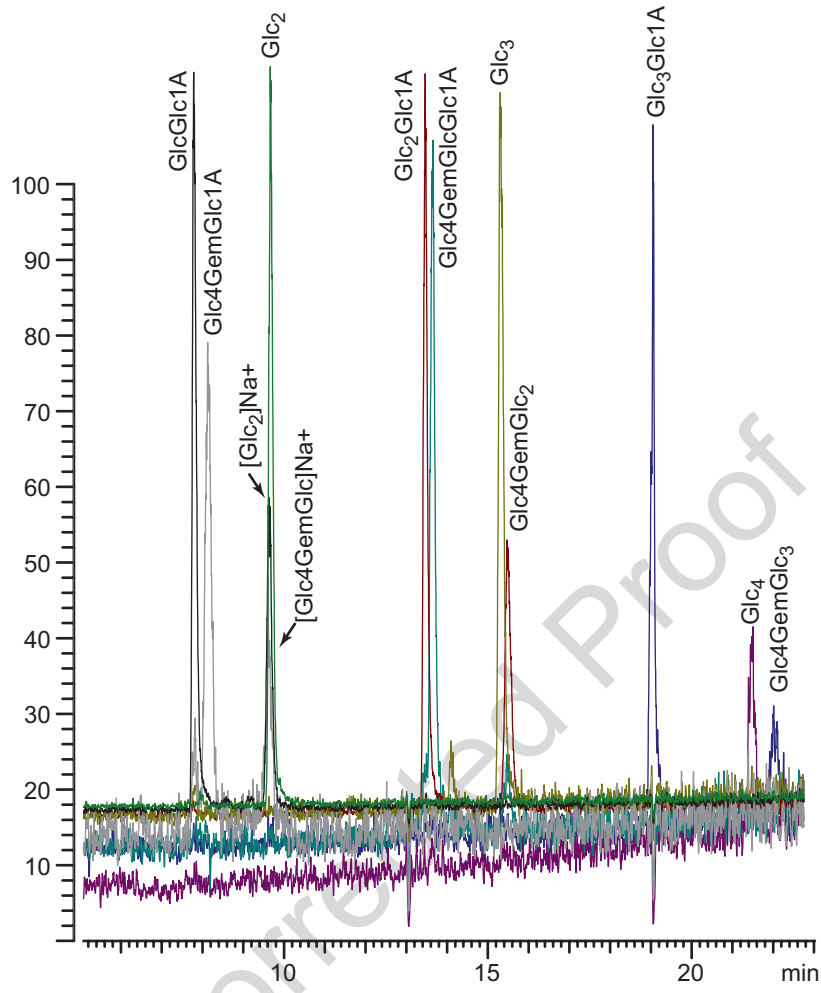




**Fig. 4** Porous graphitized carbon chromatography of a mixture of C1-oxidized, C4-oxidized, double oxidized, and native cello-oligosaccharides. The chromatogram comprises 12 overlayed Extracted Ion Chromatogram (EIC) traces (lithium adducts if not otherwise indicated). The traces show that the PGC column offers superb separation between C1 and C4 oxidized cello-oligosaccharides, whereas native and C4-oxidized as well as C1-oxidized and double-oxidized species partially co-elute. This method may easily be combined with a universal detector like charged aerosol detection (CAD) for quantification purposes. The figure was reproduced from [8] with permission from Elsevier

detection provides sensitivity sufficient to enable kinetic analysis, limitations in product separation are such that the method is only suitable for oligosaccharides up to DP5, which needs to be taken into consideration when working with LPMOs that release higher DP products. Since the oxidative modifications in the non-reducing end render products with a high degree of similarity to native cello-oligosaccharides several products tend to co-elute. The big advantage here is that the PGC-CAD method, in contrast to HPAEC, can easily be combined with MS detection which allows discrimination between co-eluting species. Furthermore, native products may be removed by treatment with a beta-glucosidase

(which acts from the non-reducing end and will only work on the native compounds). Obviously, because of the partial co-elution of C4-oxidized products and their native counterparts, it is of utmost importance to ensure that there is no background formation of native oligosaccharides by contaminating cellulases.

### 1.3 Do LPMOs Have Glycoside Hydrolase Side Activities?

The question whether or not LPMOs possess additional glycosyl hydrolase activity has been discussed repeatedly, due to the inherent appearance of native oligosaccharides during product analysis, for both C1 and C4 oxidizing LPMOs. Native oligosaccharides are formed from the substrate when an oxidation event occurs close to the reducing end (in the case of C1 oxidation) or non-reducing end (in the case of C4 oxidation; Fig. 5). In order to investigate

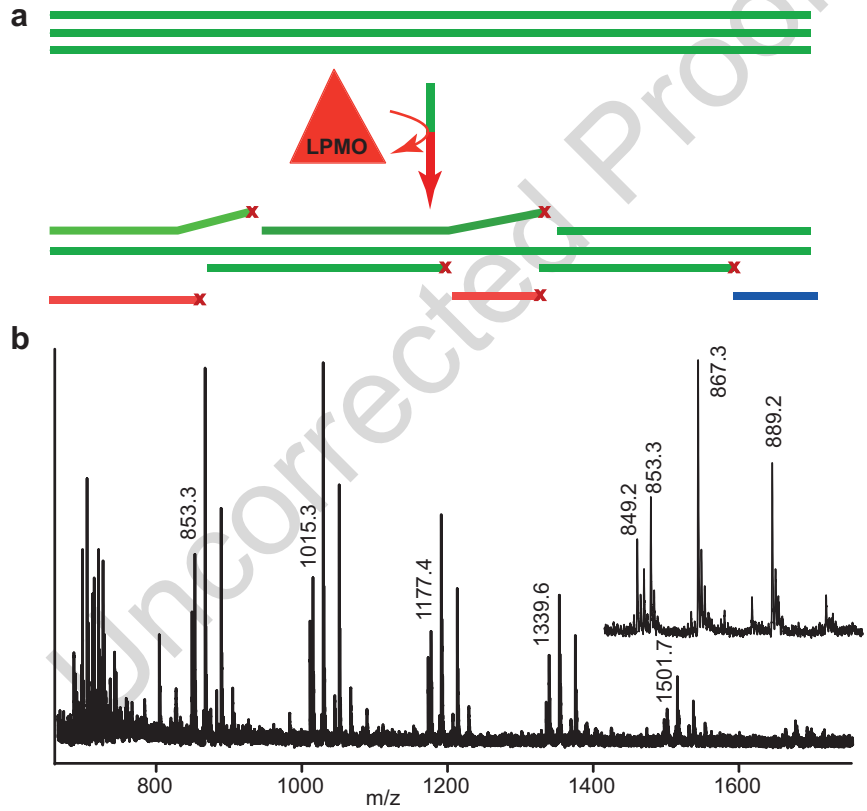


**Fig. 5** LPMO activity on reduced cellulose. (**a**) Schematic presentation of LPMO activity (C1 oxidation) on a normal cellulose fiber (*green*). Oxidation events are marked by *red crosses*. Oxidation events may result in soluble shorter products (*red* and *blue*) and longer insoluble products (*green*). Soluble oxidized oligosaccharides are marked as *red lines* with *red crosses*. The *blue line* indicates the release of a native product, which may happen when the oxidation event occurs close to the reducing end of the substrate. (**b**) MALDI-ToF spectra of reduced PASC treated with a C1-oxidizing LPMO (*Pc*GH61D). The spectra show that the vast majority of the released native oligosaccharides are reduced (*m/z* values corresponding to reduced celloligosaccharides are 853.3, 1015.3, 1177.4, 1339.6, and 1501.7). The *inset* shows details for the pentamer (sodium adducts labeled): 849, DP5-lactone; 853, DP5, reduced; 867, DP5ox, alodonic acid; 889, DP5ox, sodium salt of the aldonic acid. (**a**) is reproduced from [7] with permission from Elsevier; (**b**) is reproduced from [4])

the true occurrence of glycosyl hydrolase activity, the substrate may be reduced to its corresponding glucitol in the downstream end. Upon reaction with LPMO, any glycoside hydrolase activity would reveal itself by high release of native oligosaccharides compared to the amount of reduced oligosaccharides. This approach was applied to C1-oxidizing *Pc*GH61D (or *Pc*LPMO9D) which resulted in increasing amounts of oxidized oligosaccharides compared to glucitol oligosaccharides over time (3:1 after 4 h; 13:1 after 20 h). Only minor amounts of native oligosaccharides were released, indicating that there is no significant glycoside hydrolase activity (Fig. 5; [4]).

C4-oxidizing LPMOs seem to produce larger amounts of native products, but this is due to chemcial modfications during the analytical process, as discussed to above. A nice overview over the (apparent) production of native cellodextrins by varying types of LPMOs may be found in Fig. 1 of ref. [7].

### 1.4 Analyzing MS Data and Differentiating Between C1 and C4 Oxidized Products

When analyzing C1 and C4 oxidized products using MALDI, a general feature for aldonic acids is that they form salts of their adducts, and this formation of double adducts is typical for carboxylic acids ([9, 10]). Since the aldonic acid to lactone equilibrium is favored toward the aldonic acid under MALDI conditions, signals corresponding to the lactone form tend to be weak. For C4 oxidized products, the 4-keto to gemdiol equilibrium is less skewed, and, due to efficient dehydration during spotting of MALDI sample plates, the keto signal (with $m/z$ −2 compared to the native), is much more pronounced than the lactone signal (also $m/z$ −2 compared to the native) for C1 oxidized products. Thus, despite similar masses of the products (note that the aldonic acid and the gemdiol have identical masses too), mass spectra will show characteristic differences that relate to C1 vs C4 oxidation. MS-MS approaches will yield different fragmentation patterns for C1 and C4 oxidized species [11]. In short, C4 oxidized species tend to show double water loss and dominant ring fragmentation, while C1 oxidized species do not exhibit these features during fragmentation but instead show diagnostic decarboxylation. For details on fragmentation, readers are directed to Isaksen et al. [11]. Extensive fragmentation data on xyloglucan is presented in Agger et al. [12].

In mass spectrometry, analyzing products from LPMO reactions is a major challenge because of the overlapping masses of common species. The mass difference of oxidized and native sugars is $m/z$ 16 while the mass difference between sodium and potassium adducts is also $m/z$ 16. In most experimental conditions both sodium and potassium adducts may be present, meaning that the native-potassium $[M+K]^+$ and the oxidized-sodium $[M+Na]^+$ species will have overlapping $m/z$ values. This poses considerable problems in interpreting MS data and ion doping (*see* Subheading 3) is regularly used to reveal the true nature of the products.

**Table 1**
**Theoretical and observed masses of native and oxidized cello-oligomers harboring potassium and sodium adducts, respectively**

| | Native $[M+K]^+$ | | | Oxidized $[M+Na]^+$ | | | |
|---|---|---|---|---|---|---|---|
| | Theoretical ($m/z$) | Observed ($m/z$) | Error (ppm) | Theoretical ($m/z$) | Observed ($m/z$) | Error (ppm) | Required resolution |
| $Glc_2$ | 381.0794 | 381.0787 | 1.84 | 381.1003 | 381.1000 | 0.79 | 18,234 |
| $Glc_3$ | 543.1322 | 543.1315 | 1.29 | 543.1532 | 543.1533 | −0.18 | 25,864 |
| $Glc_4$ | 705.1850 | 705.1835 | 2.13 | 705.2060 | 705.2058 | 0.28 | 33,581 |
| $Glc_5$ | 867.2378 | 867.2362 | 1.84 | 867.2588 | 867.2585 | 0.35 | 41,298 |
| $Glc_6$ | 1029.2907 | 1029.2892 | 1.46 | 1029.3116 | 1029.3118 | −0.19 | 49,249 |

The observed data were achieved using a high-resolution Q-Exactive mass spectrometer with the resolution set to 140,000. The required resolution was calculated as $R = M/\Delta M$, where $\Delta M$ is the difference between the two masses that one wants to separate



Importantly, modern mass spectrometers can achieve resolutions up to several hundred thousand using the orbitrap principle and Fourier transformation, while time-of-flight mass spectrometers typically achieve resolutions of up to around 40,000. By employing the newest high resolution methods it is actually possible to discriminate between species with "overlapping" $m/z$ values. Table 1 shows relevant adduct pairs with overlapping $m/z$ values and the resolution required for being able to unambiguously discriminate between those. Figure 6 illustrates the separation of these ion pairs using a Q-Exactive mass spectrometer employing the orbitrap principle and demonstrates that differentiation between native potassium adducts and oxidized sodium adducts can be achieved.

### 1.5 Verification of Product Identity and Product Stability

Product identities have been addressed in various manners, as discussed above. Notably, the identities of both C1-oxidized cello-oligosaccharides [7] and C4-oxidized products have also been verified by NMR [11]. Initially, it was proposed that some LPMOs could oxidize C6 [6], but there is little proof to support this oxidation mode, which, notably, would likely not lead to cleavage. This being said, it is not unlikely that other oxidations may occur, either directly by the LPMO, possibly as a side reaction, or indirectly, via tautomerization.

### 1.6 Recent Developments

Continuous developments in research on LPMOs and other carbohydrate-active redox enzymes will require an expanded repertoire of screening methods capturing a wider range of products. Today, LPMOs have been shown to be active on hemicelluloses (glucomannan, mixed linked beta-glucan and xyloglucan) [12, 13],

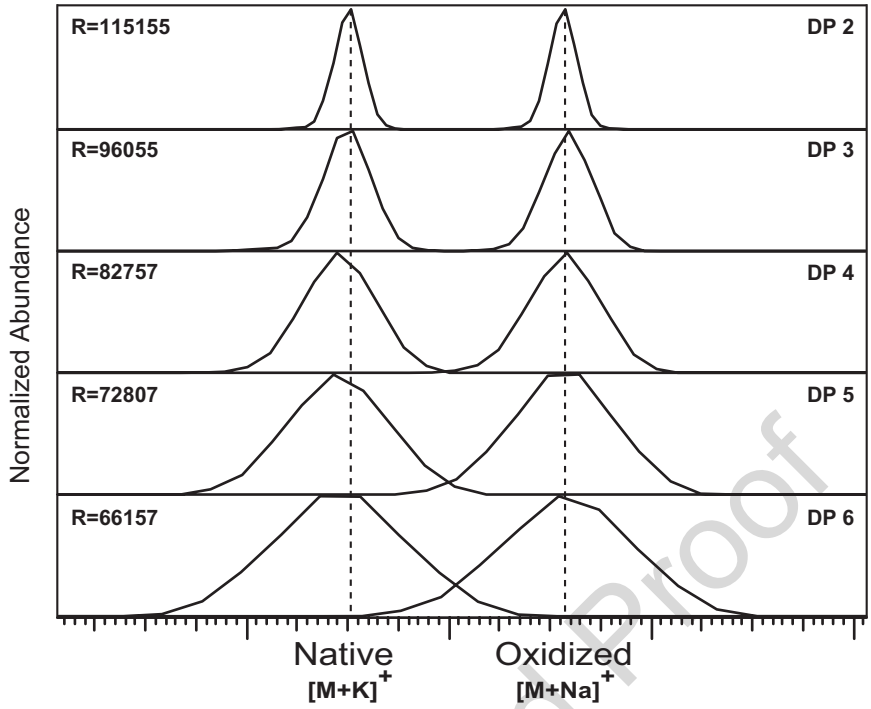


**Fig. 6** The power of high-resolution mass spectrometry. The figure shows native (K adduct) and oxidized cello-oligosaccharides (Na adduct) in the range of DP 2-6. MS-acquisitions were done using a Q-Exactive mass spectrometer in profile mode with 140,000 set resolution on two separate samples: (I) 0.005 g/L $Glc_{2-6}$ standard in 1.5 mM KCl and (II) products of an LPMO reaction. Mass spectra in a fixed relevant range (average *m/z* of native and oxidized ± 0.04 Da) of samples I and II were overlaid and the intensities normalized. The *X*-axis represents this *m/z* window of 0.08 Da in total. *R* indicates the peak width resolution as reported by the Thermo Xcalibur software



starch [14, 15], and xylan [16]. Some examples of LPMO action on more complex, natural samples have been shown in studies on xyloglucan and mannan by Agger et al. [12] and studies on xylan by Frommhagen et al. [16]. Another factor potentially contributing to sample complexity is the wide range of reductants that can activate LPMOs ([17, 18]). Certain reductants can cause challenges in analytics since sample compounds may give interfering signals. Since the effect of sample background varies depending on the choice of method (e.g., ascorbic acid has interfering peaks with C4 oxidized products in HPAEC, but not with C1 oxidized products) the effect of sample background must be evaluated for each individual analytical case. Furthermore, some reductants and the use of extreme reaction conditions may cause side reactions [19] which unavoidably results in even more complex product mixtures. Below, we will outline several of the techniques currently used to detect the activity of LMPOs.

## 2 Materials

### 2.1 HILIC Chromatography

1. Equipment: UHPLC system (Agilent 1290) with a diode array UV detector.
2. Columns: BEH amide column (2.1 × 150mm) and a BEH Amide VanGuard pre column (2.1 × 5 mm) both having a column material particle size of 1.7 μm.
3. Acetonitrile (ACN) HPLC grade.
4. Tris–HCl (15 mM, pH 8). Dissolve 2.36 g of Tris–HCl in approx 950 mL of Milli-Q water. Adjust to pH 8 with HCl, fill up to a final volume of 1 L with Milli-Q water.

### 2.2 HPAEC

1. Equipment: Ion exchange chromatography system with pulsed amperometric detection (PAD) (ICS3000, Dionex).
2. Columns: CarboPac PA1 (2 × 250 mm) and a CarboPac PA1 guard (2 × 50 mm) columns (Dionex, Thermo).
3. MilliQ water. Measure the desired volume of Milli-Q water (Type I, 18.2 MΩ·cm) directly in a dedicated HPAEC mobile phase bottle. Sonicate for 20 min to remove dissolved carbon dioxide and transfer immediately hereafter to the HPAEC system and store under $N_2$-saturated headspace.
4. Sodium Hydroxide (0.1 M). Measure exactly 2 L of Milli-Q water (Type I, 18.2 MΩ·cm) directly in a dedicated HPAEC mobile phase bottle. Sonicate for 20 min to remove dissolved carbon dioxide and transfer immediately hereafter to the HPAEC system and store under $N_2$-saturated headspace. Add 10.4 mL of NaOH from a 50% liquid solution. Do not use NaOH pellets. Close the mobile phase bottle and swirl gently to ensure proper mixing. Maintain $N_2$-saturated headspace until the mobile phase is discarded.
5. Sodium acetate (1 M in 0.1 M NaOH). Dissolve 82.03 g of anhydrous sodium acetate (≥99% purity) in 1 L of Milli-Q water (Type I, 18.2 MΩ·cm). Filter the solution through no less than a 0.45 μm filter directly into a dedicated HPAEC mobile phase bottle. Sonicate for 20 min to remove dissolved carbon dioxide and transfer immediately hereafter to the HPAEC system and store under $N_2$-saturated headspace. Add 5.2 mL of NaOH from a 50% liquid solution. Do not use NaOH pellets. Close the mobile phase bottle and swirl gently to ensure proper mixing. Maintain $N_2$-saturated headspace until the mobile phase is discarded.

### 2.3 PGC Chromatography

1. Equipment: UHPLC system (Ultimate3000RS, Dionex) set up with charged aerosol detection (Corona ultra) and an ESI-MS detector (Velos pro).

2. Columns: Porous graphitized carbon columns; Hypercarb (2.1 × 150mm; 3 μm) and a Hypercarb guard (2.1 × 10 mm; 3 μm) from Thermo Electron Corporation, San José, USA.
3. Ammonium acetate (10 mM, pH 8). Dissolve 0.771 g of ammonium acetate in approx 950 mL of Milli-Q water. Adjust pH with ammonia until pH 8 and fill up to a final volume of 1 L with Milli-Q water.
4. Acetonitrile (ACN) HPLC grade.
5. Sodium chloride (1 μM NaCl, no buffer). Dissolve 0.058 g of NaCl in 1 L Milli-Q water (1 mM). From this 1 mM solution, mix 1 mL with Milli-Q water to a final volume of 1 L.

### 2.4 MALDI-ToF Analysis and Lithium Doping

1. Equipment: Bruker Ultraflex MALDI-TOF/TOF instrumentwith a Nitrogen 337 nm laser beam (Bruker Daltonics GmbH, Bremen, Germany).
2. Lithium chloride solution (the LiCl concentration should be approximately twice the concentration of the buffer used in the LPMO reaction). Dissolve the desired amount of LiCl in Milli-Q water.
3. 2,5-dihydroxybenzoic acid (DHB) solution: dissolve 4.5 mg DHB (Bruker Daltonics) in 150 μL acetonitrile and 350 μL water.
4. MTP 384 target plate ground steel TF from Bruker Daltonics (or equivalent).

### 2.5 High Resolution MS to Discriminate Between Potassium and Sodium Adducts by Direct Infusion Q-Exactive MS

1. Q-Exactive hybrid quadrupole orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany).
2. MilliQ water.
3. Potassium chloride (KCl, 1.5 mM). Dissolve 111.8 mg KCl in MilliQ water and adjust to a final volume of 1 L.

### 2.6 Isotope Labeling

1. Tris–HCl (*see* Subheading 2.1).
2. Ascorbic acid (AA, 1 mM), prepare a fresh 100 mM stock solution of reduced ascorbic acid by dissolving 17.6 mg AA in 1 mL MilliQ water.
3. $N_2(g)$.
4. $H_2{}^{18}O$ and $^{18}O_2$ from Cambridge Isotope laboratories (catalogue numbers OLM-240-97-1 and OLM-212-PK, respectively).

### 2.7 Reduction of Cellulose

1. Phosphorous acid swollen cellulose prepared from Avicel PH-101 cellulose from Fluka analytical (Sigma-Aldrich, St. Louis, USA).
2. Sodium hydroxide (NaOH, 12.5 mM).
3. Sodium borohydride ($NaBH_4$) anhydrous.
4. Glacial acetic acid.

## 3 Methods

The protocols provided below cover most available methods for characterizing LPMOs and analyzing oxidized products by HPLC and mass spectrometry. For more details readers are directed to the original publications related to the method in question. Where applicable, notes are appended in Subheading 4.

### 3.1 HILIC Chromatography for the Analysis of C1 Oxidized Chitin Oligosaccharides

1. Use an instrumental setup as mentioned in Subheading 2.1 or similar.
2. Dissolve sample in 72% (v/v) acetonitrile (ACN). Inject 5 μL sample (*see* **Note 1**).
3. Operate the system at 30 °C (column temperature) and a flowrate of 0.4 mL/min.
4. Keep starting conditions 72% ACN (A):28% 15 mM Tris–HCl pH 8.0 (B) for 4 min, then use an 11 min linear gradient to 62% A: 38% B, which is held for 3 min.
5. Recondition column by applying a 2 min gradient to initial conditions and subsequent operate at initial conditions for 5 min.
6. Monitor eluted oligosaccharides by recording UV absorption at 205 nm (*see* **Note 1**).

### 3.2 HPAEC-PAD for the Analysis of Various Cello-Oligosaccharides

1. Use an instrumental setup as mentioned in Subheading 2.2 or similar.
2. Centrifuge samples for 3 min in an Eppendorf centrifuge at maximum speed and transfer supernatants to HPLC vials without any further adjustments.
3. Set column temperature 30 °C and use 0.25 mL/min flow rate.
4. Use mobile phases containing 0.1 M NaOH (A) and 0.1 M NaOH, 1 M sodium acetate (B) (*see* **Note 2A**).
5. Use the following gradient: a 10 min linear gradient from 100% A (starting condition) to 10% B, a 15 min linear gradient to 30% B, a 5 min exponential gradient (Dionex curve 6) to 100% B.
6. Recondition column by running initial conditions (100%A) for 9 min [3] (*see* **Note 2A**).
7. For other applications and mass spectrometry adaptations (*see* **Note 2B** and **C**).

### 3.3 Simultaneous Analysis of Aldonic Acids and C4-Oxidized Cello-Oligosaccharides by Porous graphitized Carbon (PGC) Chromatography

1. Use an instrumental setup as written in Subheading 2.3 or similar.
2. Centrifuge samples for 3 min in an Eppendorf centrifuge at maximum speed and transfer supernatant to HPLC vials without any further adjustments.
3. Operate the column at 0.4 mL/min and 70 °C.

4. Use the following gradient: 0–1 min, 100% eluent A (10 mM $NH_4$-acetate, pH 8.0); 1–15 min, linear gradient to 27.5% eluent B (acetonitrile); 15–28 min, linear gradient to 60% B; 28–35 min, isocratic at 60% B [7] (*see* **Note 3**).
5. Recondition the column by applying 100% eluent A for 9 min.
6. The chromatography system was composed as follows: Dionex UltiMate3000 RSLC set up with detection by electrospray ionization-MS (VelosPro LTQ linear iontrap, Thermo Scientific) or optionally with parallel use of MS with a Charged Aerosol Detector (CAD) (ESA inc., Dionex, Sunnyvale, USA). ESI-MS detection is used for qualitative detection, whereas the CAD is used for quantitation. The CAD detector is a universal detector, where the response is independent of the analyte, making it possible to prepare calibration curves with easily accessible standards that are structurally similar, but not identical, to the analyte. Such standards need to have the same elution time as the analyte to prevent gradient effects (*see* **Note 3**).

### 3.4 MALDI-ToF Analysis and Lithium Doping

1. To prepare samples for MALDI-ToF analysis reactions should be run at low buffer concentrations (as a rule of thumb, less than 50 mM, but lower is better), and no MS-incompatible ions like phosphate/nitrate should be used.
2. Centrifuge samples in an Eppendorf centrifuge at maximum speed for 2 min at room temperature.
3. Apply 2 μL saturated DHB solution to a MALDI plate.
4. Apply 1 μL sample, and mix with 3).
5. Dry the spot under a stream of warm air.
6. Analyze the sample on a MALDI-ToF instrument (*see* **Note 4A**).
7. Mix 1 μL sample with 9 μL LiCl solution and vortex for 5 s.
8. Apply 2 μL saturated DHB solution to a MALDI plate. DHB is the standard matrix used for all MALDI experiments, but other matrices may work equally well.
9. Add 1 μL of the lithium-doped sample from (1) to (2) and mix.
10. Dry the spot under a stream of warm air.
11. Analyze the sample on a MALDI-ToF instrument (*see* **Note 4B**).

### 3.5 High Resolution MS to Discriminate Between Potassium and Sodium Adducts by Direct Infusion Q-Exactive MS

The following procedure describes the manual analysis of oligosaccharides using direct injections on a Q-Exactive hybrid quadrupole orbitrap mass spectrometer. Other high-resolution mass spectrometers equipped with a nano-flow ion source can also be used with minor adaptations of this protocol.

1. Mount the Nanospray Flex ion source to the Q-Exactive, and change the nano head to the Offline nano ES head to allow manual use of tapered capillary emitters.

2. Prepare the sample by diluting with water or potassium chloride (1.5 mM) when conducting potassium doping.
3. Cut the emitter to a suitable length and load 3 μL sample directly into the emitter using a gel-loader tip. Avoid bubbles. Use a new emitter for every sample.
4. Assemble the emitter in the ion source and position the tip in front of the skimmer and apply positive pressure using a syringe to start the liquid flow. If no flow is observed, a gentle "crash" into the skimmer to scratch the emitter tip may be necessary. The optimal flow lays between 50 and 300 nL/min and, although this is difficult to control, the flow rate can be estimated by the time it takes for the complete sample to be injected (i.e., 3 μL fully injected in 10 min gives a flow of 300 nL/min).
5. Apply the settings from Table 2 and switch on the Q-Exactive. Adjust the emitter position and the spray voltage, if needed, to achieve a stable spray.
6. Acquire MS full scan data for 20 s. Optionally, the Q-Exactive can be set to cycle between MS full scans and MS/MS fragment scans by providing an inclusion list of selected precursor ions. Fragmentation can be achieved using stepped normalized collision energy from 25 to 48. In order to achieve high quality MS/MS spectra, the number of microscans should be at least three and the maximum injection time set to 800 ms. (*see* **Note 5**).

**Table 2**
**Q-Exactive parameters for direct injections**

| | |
|---|---|
| Sheet gas flow rate | 0 |
| Aux gas flow rate | 0 |
| Sweep gas flow rate | 0 |
| Spray voltage (kV) | 0.9–1.5[a] |
| Capillary temperature | 250 °C |
| S-lens RF level | 50 |
| Scan range | 150–2000 *m/z* |
| Resolution | 140,000 |
| Polarity | Positive |
| AGC target | 5e6 |
| Maximum inject time | 100 ms |
| Spectrum scan mode | Profile |
| Micro scans | 1 |

[a]The spray voltage normally needs to be adjusted to the needle distance and the sample concentration

### 3.6 Isotope Labeling

Stable isotope reagents such as $H_2{}^{18}O$ and $^{18}O_2$ can be used in LPMO reactions to demonstrate the incorporation of molecular oxygen and water in the products formed by these enzymes. Identification of products containing the $^{18}O$ isotope is achieved by mass spectrometry where products have *m/z* +2 compared to products formed in $^{16}O$ conditions. Such experiments and product analysis have been described in detail in Vaaje-Kolstad et al. [1] and the protocols used by these authors are outlined below. The reaction volume, substrate concentrations, etc. described are optimal for demonstrating activity of a chitin-active LPMO toward chitin, but may need optimization if the methods are used for other substrates and enzymes.

#### 3.6.1 Reactions in Buffered $H_2{}^{18}O$

1. Suspend 2.0 mg of dry substrate in 1.0 mL pure $H_2{}^{18}O$ in a 2.0 mL glass vial. This leaves a headspace of approximately 1 mL when the vial is sealed. Seal the vial airtight and mix thoroughly. Let the substrate suspension hydrate overnight at room temperature.
2. Dissolve a sufficient amount of reducing agent (e.g., ascorbic acid) in an appropriate volume of pure $H_2{}^{18}O$ to yield a final concentration of 1.0 M. Keep the solution in an aluminum foil wrapped test tube (to shield from light) on ice.
3. In order to achieve the correct pH in the $H_2{}^{18}O$ reaction solution, transfer 10 µL of a 1.0 M nonvolatile buffer (e.g., Tris–HCl pH 8.0) to a 2.0 mL glass vial and evaporate off the liquid by heating with dry air (approximately 60 °C).
4. Transfer 498 µL of the substrate suspension to the glass vial containing the dried buffer and mix thoroughly to dissolve the buffer components. Subsequently, add 0.5 µL of the reducing agent solution (dissolved in $H_2{}^{18}O$) and 0.75 µL of a 660 µM solution of the LPMO (dissolved in $H_2{}^{16}O$) to the buffered substrate suspension to yield final concentrations of 1 mM reducing agent and 1 µM enzyme. A high concentration of the enzyme stock solution is desirable in order to keep $H_2{}^{16}O$ contamination at a minimum (replacing the $H_2{}^{16}O$ in the enzyme stock solution to $H_2{}^{18}O$ is possible, but is not considered as an option due to the high cost of pure $H_2{}^{18}O$).
5. Seal the glass vial airtight and incubate the reaction for an appropriate time (usually 1 h or more) at an appropriate temperature (enzyme dependent) and with vigorous mixing (e.g., 1000 rpm in an Eppendorf Thermomixer).analyze products by MS.

#### 3.6.2 Reactions in a Solution Saturated with $^{18}O_2$

1. Prepare a buffered LPMO reaction solution (e.g., 20 mM Tris–HCL pH 8.0) containing 2.0 mg/mL substrate and 1.0 mM reducing agent (e.g., ascorbic acid) in a glass vial that can be closed airtight with a screw cap containing a Teflon coated rubber septum. Make sure that the reaction volume only represents approximately 50% of the vial volume.

2. Close the vial tightly with the screw cap and connect to a Schlenk line (for details, *see* procedure described under the heading "molecular oxygen free reaction" in Vaaje-Kolstad et al. [1] to remove oxygen from the headspace and dissolved oxygen from the solution. This is achieved by performing five cycles of degassing and $N_2$ filling. It is important to have a slight $N_2$ over pressure after the final $N_2$ filling in order to avoid contamination of the head space with air when removing the vial from the Schlenk line.
3. Remove the vial from the Schlenk line and perforate the septum with the needle of a Hamilton syringe preloaded with a concentrated LPMO solution. Add the LPMO to the reaction mixture by injecting an appropriate volume (as low as possible in order to minimize addition of dissolved $^{16}O_2$) to a final concentration of 1.0 μM. Withdraw the Hamilton syringe from the vial.
4. Connect a gas cylinder containing compressed $^{18}O_2$ gas to the vial by pushing a needle fitted to the outlet of the gas cylinder through the vial septum.
5. Using the Schlenk line, place the vial under vacuum in order to remove atmospheric gas residing in the tubing connected to the $^{18}O_2$ gas container and the headspace of the vial.
6. Disconnect the vial from the Schlenk line needle and fill the head space of the vial with $^{18}O_2$ gas by slowly opening the gas cylinder regulator.
7. After 30 s, close the gas cylinder regulator and carefully remove the needle from the vial.
8. Incubate the vial containing the LPMO reaction mixture for an appropriate number of hours (usually 1–24) at the desired temperature with vigorous mixing (e.g., 1000 rpm in an Eppendorf Thermomixer) and analyze products by MS.
9. The lactone—aldonic acid equilibrium will lead to exchange of oxygen atoms (*see* **Notes 4–6**).

AU2

### 3.7 Reduction of Cellulose

Reduced phosphoric acid swollen cellulose (PASC) can be prepared with the following procedure:

1. Use a 2 mL 2% (w/v) PASC suspension in water and centrifuge for 3 minutes at 21,000 × *g*. Remove the supernatant and resuspend the pellet in 1 mL MilliQ $H_2O$. Centrifuge again for 3 minutes at 21,000 × *g* and remove the supernatant.
2. Resuspend the pellet in 4 mL 12.5 mM NaOH.
3. Add 25 mg $NaBH_4$ and leave the tubes at ambient temperature overnight with occasional stirring.
4. Quench the reaction by neutralizing with 100 μL glacial acetic acid, followed by centrifugation as described above.

5. Wash the pellet four times with MilliQ $H_2O$ and finally resuspend in MilliQ $H_2O$ to obtain a 2% (w/v) solution of reduced PASC.

## 4 Notes

1. Samples must have the same proportion of organic solvent as in the chromatographic starting conditions, if not, this is likely to compromise resolution. Some ionic strength (provided by the added Tris–HCl) is needed in order to obtain retention of the aldonic acids. Furthermore, an adapted version of this method that is more suitable for the shortest products appears in [2]. This study also describes a method for enzymatically generating chitoaldonic acid standards using a chito-oligosaccharide oxidase [2].
2. (A) When eluents are prepared note that when approx half of the 50% NaOH solution has been used, discard the remaining for the purpose of mobile phase preparation due to risk of carbonate contamination. It is critical to follow this procedure for mobile phase preparation or to follow equivalent recommendations by the instrument vendor, in order to achieve satisfactory quality of analysis. The most important things to pay attention to are (a) water and chemical quality, (b) sufficient degassing for removal of dissolved carbon dioxide, (c) storage in atmospheres with reduced content of carbon dioxide ($N_2$ or He-saturated headspace), (d) regular change of mobile phases (2–3 days shelf life), and (e) to avoid all kinds of detergents in mobile phases, hence no detergent washing of mobile phase bottles between eluent preparations. Restrict cleaning to rinsing with Milli-Q water (Type I, 18.2 MΩ cm). Extensive exchange of mobile phases on the column and regeneration after each eluent changes is also important in order to remove accumulation of carbonate contaminations on the column which compromise resolution. (B) This method is used for native and oxidized cello-oligosaccharides, and may be adapted to be used for xyloglucan fragments as described in [12]. If there is a need for higher throughput, a 10 min method for separation and detection of aldonic acids may be used [7]. (C) For validation purposes HPAEC may be coupled to ESI-MS as explained in [8], but this requires anion suppressor, additional pumps and flow splitting. If you do not have access to online MS detection with your HPAEC a simpler approach that does not require a complex instrument setup and is based on offline MS is described in [8]. In short the latter procedure implies manual fractionation, desalting and MS analysis by MALDI-ToF, direct infusion ESI-MS, or injection onto another, simpler LC-MS system.

3. The elution of products may vary slightly depending on which UHPLC system you are using. This is due to for example varying dead volumes/gradient mixing in the hardware that is used. Gradient needed may PGC allows simultaneous detection of C1 and C4 oxidized products only in the range from DP2-5. *See* details in Westereng et al. [8]. Native cello-oligosaccharides co-elute with C4-oxidized cello-oligosaccharides and double oxidized compounds co-elute with C1-oxidized oligosaccharides.

   Due to incompatibility between an alkaline mobile phase and the detection principle of CAD, it is beneficial to lower the pH of eluent A to 6.5 in cases where CAD is applied [8]. Sensitivity in the low nanomole range is usually needed and improved sensitivity with CAD may be achieved by lowering the ionic strength. Analysis of uncharged compounds may be achieved at ionic strengths down to 1 μM NaCl and this enables product quantification with a sensitivity that is suitable for characterizing LPMO activities [8].

4. (A) Under standard conditions, more than one type of adduct is commonly observed during MALDI analysis. The most dominant adduct is sodium, but also potassium, hydrogen, and $NH_4$ adducts can occasionally be observed. One simple way of overcoming this multiplicity of signals, which hampers product identification, is ion doping to force the adduct composition to a defined adduct type. An example of lithium doping is given in Fig. 1, which shows complete lithium adduct formation. (B) Normally the mixing of the sample and the LiCl solution (or other doping reagent) as indicated is sufficient to achieve complete doping. Testing two to three different concentrations of doping reagent may be necessary to find conditions that provide sufficient doping. Note that adding too much of doping reagent can give problems due to ion suppression. It is important to run LPMO reactions with low buffer concentrations in order to avoid ion suppression and extensive background signals. Doping with other ion salts, such as NaCl and KCl, may be performed in a similar manner as outlined above, but note that adduct formation efficiency varies between ions. Details on adduct formation may be found in [20].

5. The actual flow rate in the emitter is difficult to control and is dependent on several factors such as sample viscosity, emitter opening size, applied positive pressure, distance from needle to skimmer, and spray voltage. Some trial and error must be expected to optimize these parameters. Typically, without spray voltage applied, the positive pressure alone should be able to form small droplets at the emitter tip. If no liquid appears, a small "crash" into the skimmer may scratch the emitter open. If too much liquid comes out, the emitter opening is too large,

and a new emitter needs to be installed. This method is not applicable for high-throughput analysis or quantitative measurements as a new emitter, including manual optimization, is needed for each sample.

6. Aldonic acids dissolved in aqueous buffers are in a pH-dependent equilibrium with the corresponding δ-lactone. The δ-lactone is formed by dehydration of the aldonic acid. The aldonic acids is re-formed by hydrolysis of the δ-lactone. Thus, when performing $^{18}O$–isotope labeling experiments, care must be taken to avoid (a) exchange of the incorporated $^{18}O$ atom with $^{16}O$ $^{18}O_2$ experiments and (b) incorporation of a second $^{18}O$ atom in $H_2{}^{18}O$ experiments. Since the aldonic acid-δ-lactone equilibrium is strongly dominated by the aldonic acid at alkaline pH, it is preferable to conduct isotope labeling experiments at pH>7. The isotope labeling experiments performed by Vaaje-Kolstad et al. [1] were all conducted at pH 8.0. At this pH the δ-lactone forms of the C1 oxidized products are not observed using MALDI-ToF MS and the equilibrium is such that there is enough time to carry out product analyses before the exchange of oxygen atoms becomes noticeable.

### 4.1 Future Perspectives

In this chapter we outline several crucial aspects of carbohydrate analysis that can be applied to analyze soluble products generated by LPMOs. More work is needed for developing effective methods for monitoring the insoluble products, i.e., oxidations on the insoluble material. Insight into oxidations on insoluble products may in some case be obtained by completely solubilizing LPMO-treated material with hydrolases and then analyze soluble oxidized products. Less quantitative methods based on labeling oxidized chain ends and microscopy are also available [21]. In addition to this, the field of size exclusion chromatography (SEC) in both the aqueous and nonaqueous mode has seen large improvements in the past years. In particular, several column producers today design SEC columns for UHPLC conditions and this enables higher throughput, higher resolution, and smaller injection volumes than what we have seen so far. Using SEC in ionic liquid mode for analyzing molecular distributions in cellulose [22] as well as for studying the molecular distribution of product mixtures after enzymatic treatments has a large potential to broaden our understanding of the effects of LPMO treatments.

Research on LPMOs has only just begun and, despite major achievements [23–25], there is much exciting research ahead. The analytical tools described above will be invaluable for further unravelling of LPMO function in nature and in the biorefinery.

## Acknowledgments

This work was supported by the Norwegian Research Council through grants and 193817, 214138, 214613, 216162, 243663, and 244259.

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