Solubilization of crystalline chitin with a single LPMO: generating chito-oligosaccharides with unprecedented bioactivity

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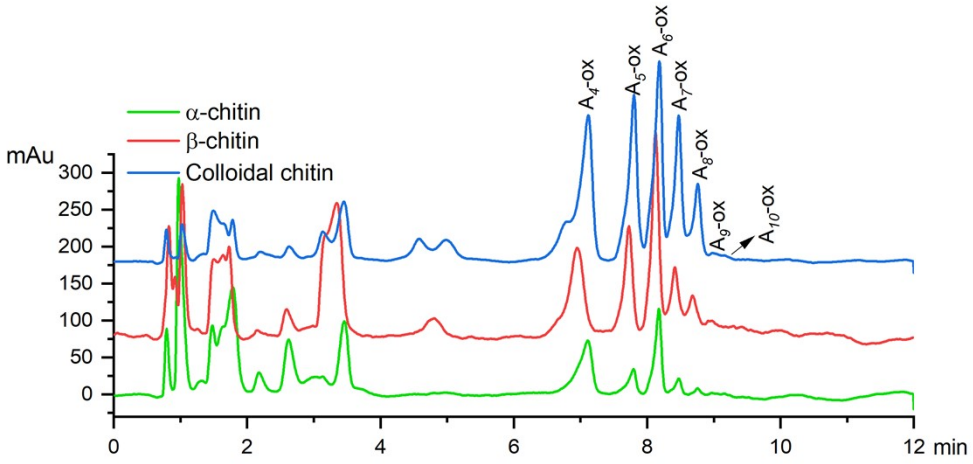


Figure S1. HPLC analysis of oxidized chito-oligosaccharides produced from α-chitin, β-chitin and colloidal chitin in 48 h LPMO standard reactions. The chromatograms belong to the experiments shown and described in Fig. 1 of the main manuscript. The chromatograms show soluble products generated in reactions with α-chitin (green), β-chitin (red) or colloidal chitin (blue). Similar reactions without the addition of AscA did not yield any oxidized products. The larger peaks between 0 and 4 min represent background compounds that were also observed in the control reactions without added AscA, whereas some of the minor peaks between 0 and 4 min are short oxidized and non-oxidized chitin fragments.

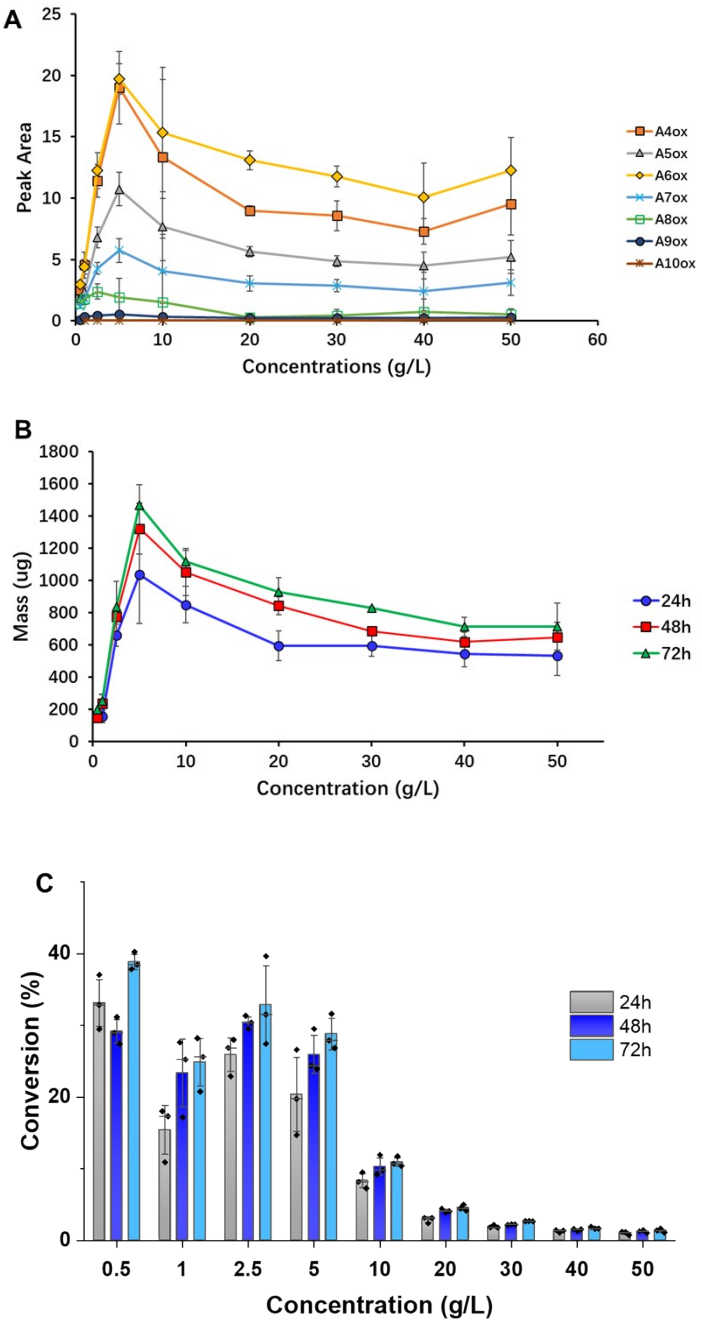


Figure S2. Qualitative and quantitative analysis of soluble products generated in reactions with different chitin concentrations. The reactions contained 1µM CBP21, 1mM AscA, and different concentrations of β-chitin in 20 mM Tris-HCl, pH 8.0. Panel (**A**) shows the formation of each individual oxidized chito-oligosaccharide after 72 h. Panels **B** and **C** show quantitative analysis of soluble products, which was done after hydrolysis of the products with *Sm*CHB. The values shown are the average of three independent experiments with standard deviations shown as error bars

Panels **B** and **C** show that the amount of soluble products increases slowly between 24 h and 72 h despite the expected lack of ascorbic acid. This is commonly observed and relates to two processes: (i) already existing chitin fragments that had remained associated with the insoluble substrate slowly dissociate & (ii) oxidised ascorbic acid, dehydroascorbic acid (DHA) is unstable and converts slowly into multiple derivatives,

some of which are redox active and can fuel the LPMO reaction[1] .

Panels **A** and **B** show that the amount of soluble products decreases slightly with increasing substrate concentration. This is due to the fact that lower substrate concentrations lead to a higher probability of the same chitin chain being cleaved twice at locations close to each other, which is a prerequiste for the formation of soluble products. The fraction of soluble products decreases as more substrate is available in the reaction, as discussed extensively in a previous report[2].

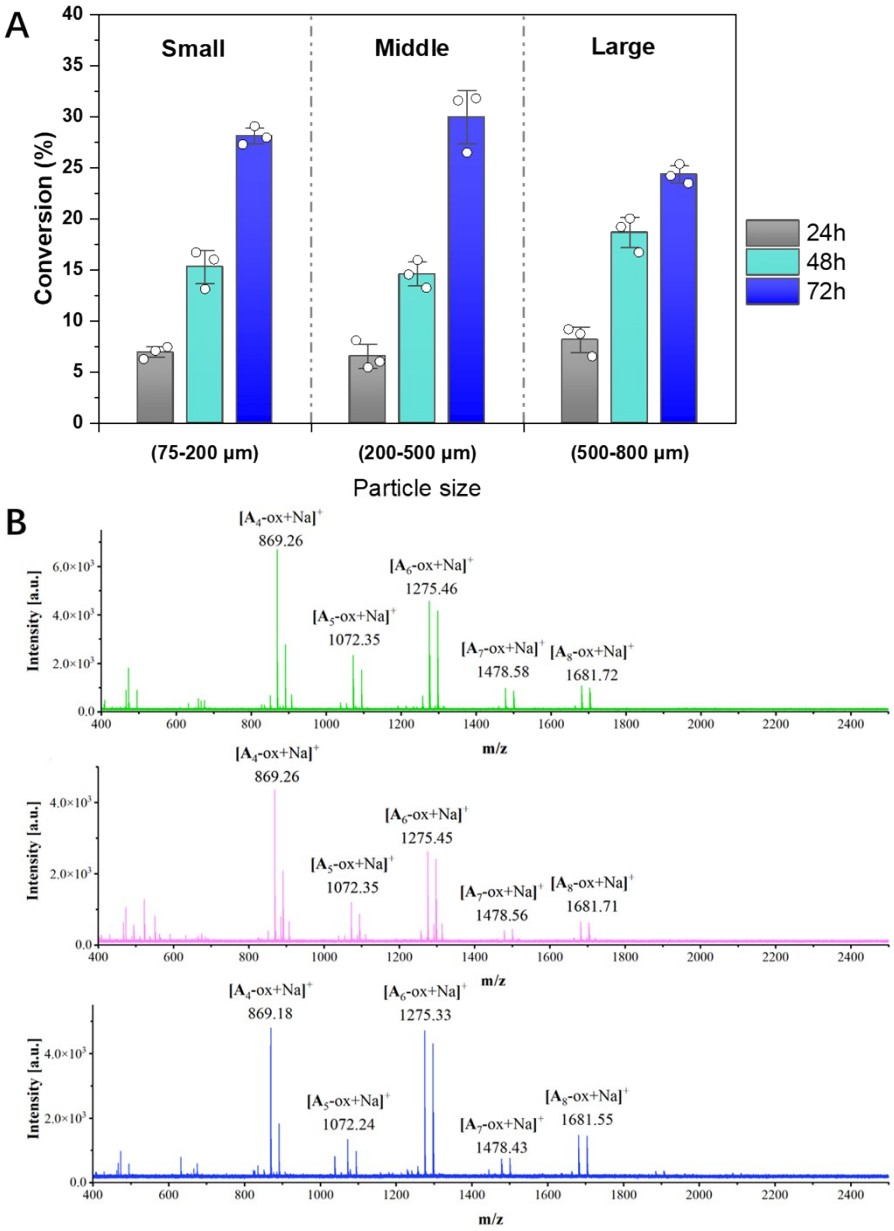


Figure S3. Impact of different particle sizes on chitin Solubilization by CBP21. Reactions contained 1 µM CBP21, 1 mM AscA and 10 g/L β-chitin in 20 mM Tris-HCl, pH 8.0, and were incubated at 37 °C, with shaking at 850 rpm. Fresh AscA, to a final concentration of 1 mM was added at 24 h and 48 h. Regarding the chitin materials with different particle sizes, the flaked β-chitin was ball-milled in-house and fractionated to different particle sizes of 75-200 µm, 200-500 µm or 500-800 µm. Panel **(A)** shows the degree of conversion over time. The values shown are the average of three independent experiments with standard deviations shown as error bars. (**B**) MALDI-TOF MS analysis of products generated in reactions with β-chitin with different particle sizes. The spectra are for the samples taken after 72 h of reaction, for the small (green), medium (pink) and large (blue) particle sizes, from top to bottom,

respectively. The indicated *m*/*z* values are the sodium adducts of the aldonic acid form of the oxidized tetramer (869) to octamer (1681). The other, major peak seen for each of these products is the sodium salt of the sodium adduct of the aldonic acid (*m*/*z* + 22).

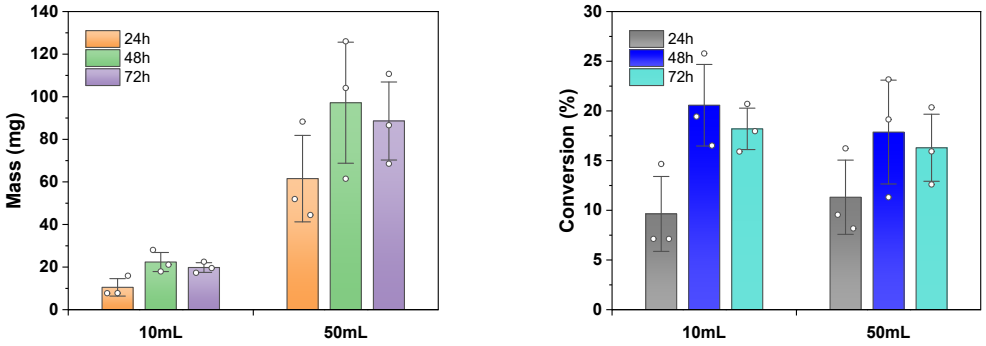


Figure S4. Large-scale preparation of oxCHOS in 100 mL sealed glass bottles. The reaction volumes were 10 or 50 mL and the reactions contained 100 or 500 mg of substrate, respectively. The reaction mixtures contained 1 μM CBP21, 1 mM AscA, and 10 g/L of β-chitin in 20 mM Tris-HCl, pH 8.0, and were incubated at 37 °C, with shaking at 225 rpm. Fresh AscA to a final concentration of 1 mM was added at 24 h and 48 h. Quantitative analysis of solubilized products was carried out after each 24 h period, after hydrolysis of these products with *Sm*CHB. The conversion of chitin for each of the 24 h steps was calculated by summing up the molar amount of GlcNAc and twice the molar amount of the oxidized dimer, giving the total amount of solubilized GlcNAc, which was compared to the total molar amount of GlcNAc added to the reaction in the form of chitin. The values shown are the average of three independent experiments with standard deviations shown as error bars.

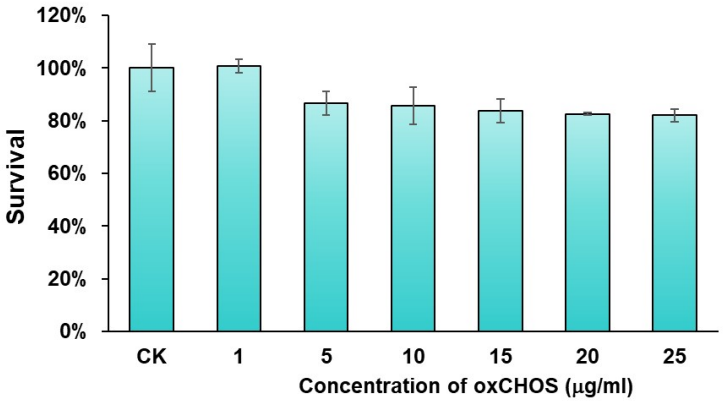


Figure S5. Effects of different concentrations of LPMO-generated oxCHOS on the viability of RAW264.7 macrophages. RAW264.7 cells at a density of 1 $\times 10^6$ cells/ mL were incubated with 1-25 μg/mL oxCHOS for 24 h, after which viability was assessed using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. The data are presented as means ± SD of three independent biological replicates. CK, control sample with no added CHOS. The graph shows that, at the concentrations used in this study, cytotoxic effects were minimal (>80% survival after 24 h).

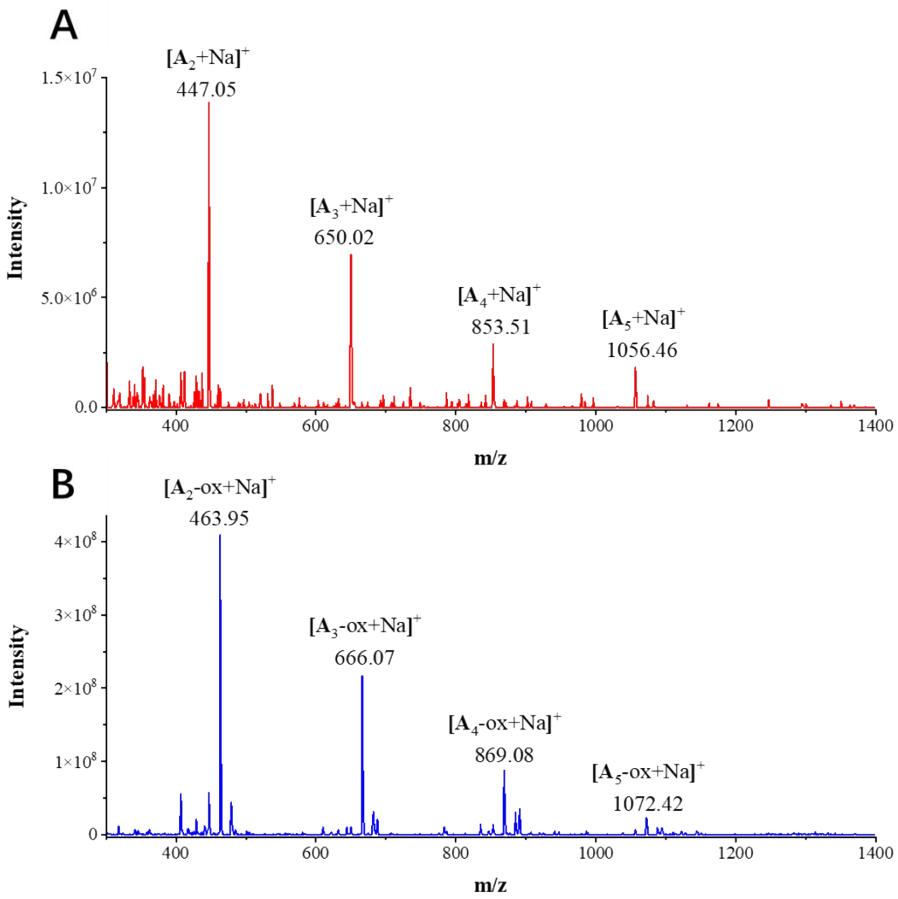
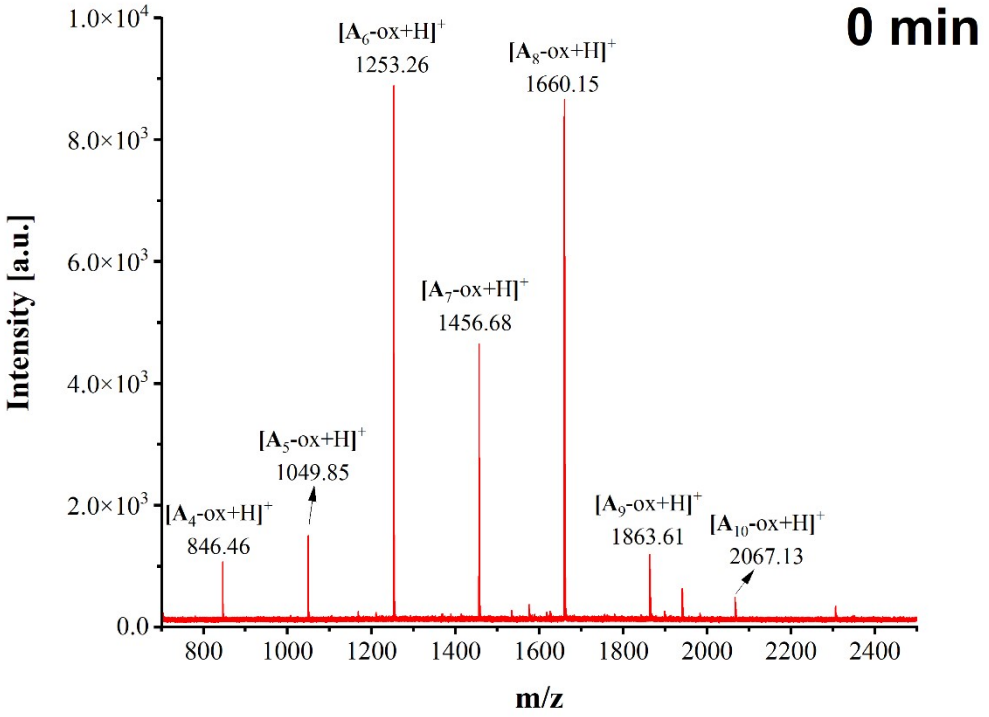
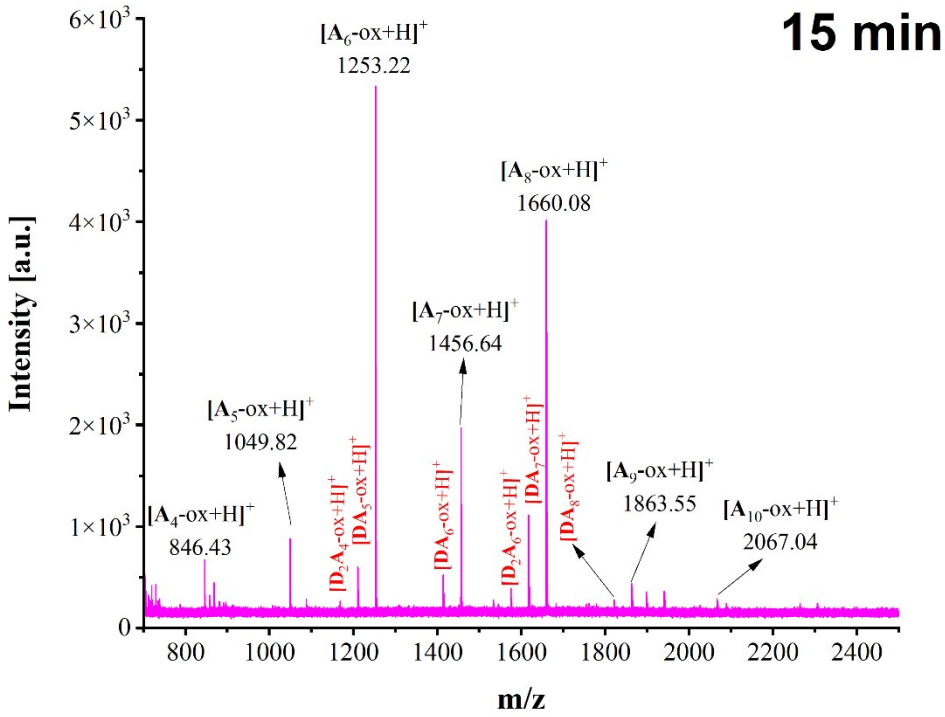


Figure S6. MALDI-TOF MS spectra of CHOS (A) and oxCHOS (B) with low degree of polymerization (DP2-5). Low DP oxCHOS were prepared with chito-oligosaccharide oxidase using native chito-oligosaccharides (CHOS) as the starting material. Chito-oligosaccharide oxidase was removed using an Amicon Ultra-15 centrifugal filter with 10 kDa MWCO (Merck, Darmstadt, Germany), after which the filtrate containing low DP oxCHOS was desalted using a dialysis membrane with 100 Da MWCO (Spectrum Labs, USA). The *m/z* values shown in the graphs are for the sodium adducts of the native oligomers (**A**) and the sodium adducts of the oxidized oligomers in the aldonic acid form (**B**; see also Fig. S3).


0 min
Intensity [a.u.]
m/z
[A6-ox+H]+
1253.26
[A8-ox+H]+
1660.15
[A7-ox+H]+
1456.68
[A5-ox+H]+
1049.85
[A4-ox+H]+
846.46
[A9-ox+H]+
1863.61
[A10-ox+H]+
2067.13


15 min
Intensity [a.u.]
m/z
[A6-ox+H]+
1253.22
[A8-ox+H]+
1660.08
[A7-ox+H]+
1456.64
[A5-ox+H]+
1049.82
[A4-ox+H]+
846.43
[D2A4-ox+H]+
[DA5-ox+H]+
[DA6-ox+H]+
[D2A6-ox+H]+
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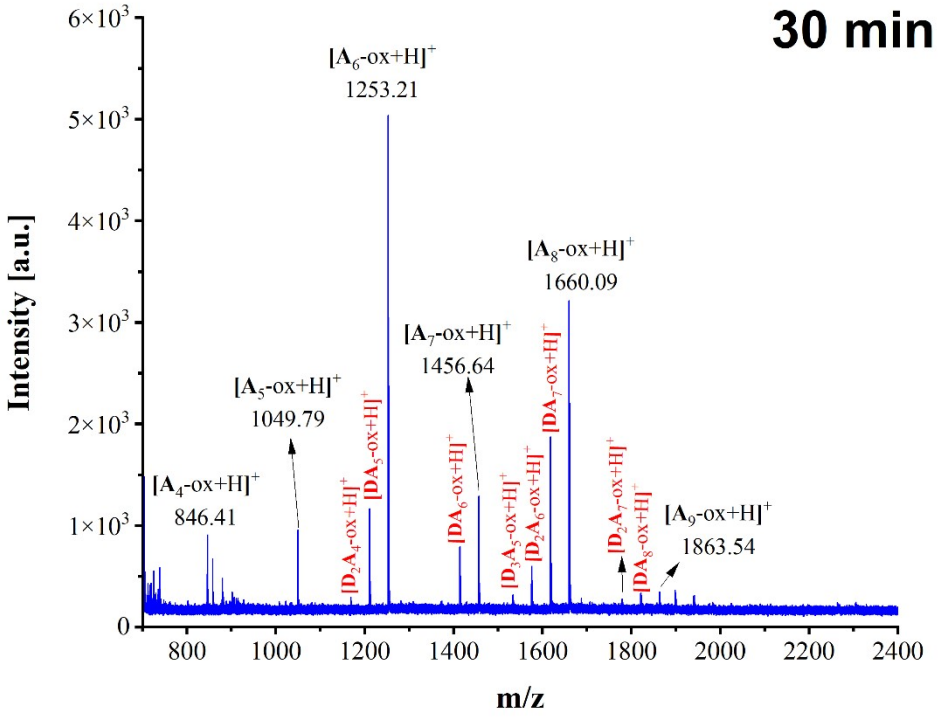
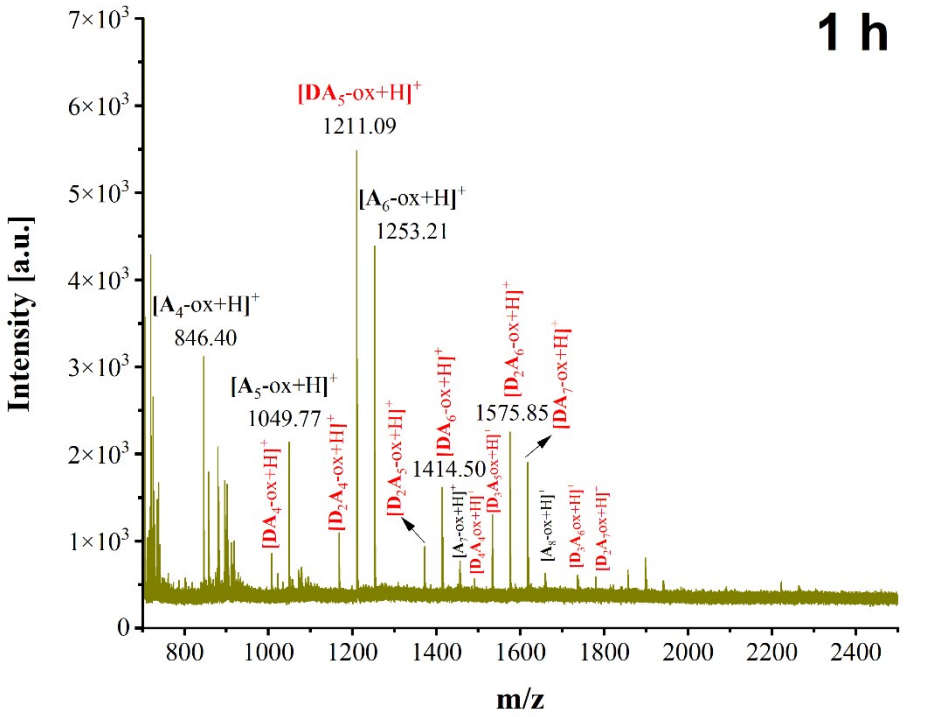
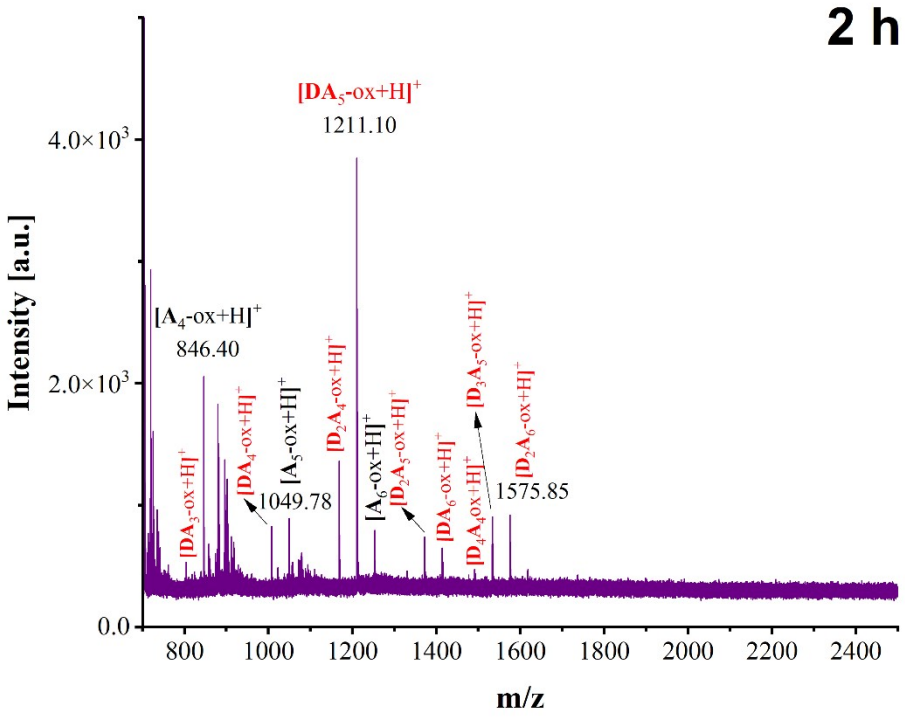
[DA8-ox+H]+
[A9-ox+H]+
1863.55
[A10-ox+H]+
2067.04


30 min
Intensity [a.u.]
m/z
[A6-ox+H]+
1253.21
[A8-ox+H]+
1660.09
[A7-ox+H]+
1456.64
[A5-ox+H]+
1049.79
[A4-ox+H]+
846.41
[A9-ox+H]+
1863.54


1 h
Intensity [a.u.]
m/z
[DA5-ox+H]+
1211.09
[A6-ox+H]+
1253.21
[A4-ox+H]+
846.40
[A5-ox+H]+
1049.77
1575.85
1414.50


2 h
[DA5-ox+H]+
1211.10
4.0×10^3
2.0×10^3
0.0
Intensity [a.u.]
[A4-ox+H]+
846.40
[DA3-ox+H]+
[DA4-ox+H]+
1049.78
[A5-ox+H]+
[D2A4-ox+H]+
[A6-ox+H]+
[D2A5-ox+H]+
[DA6-ox+H]+
[D4A4ox+H]+
1575.85
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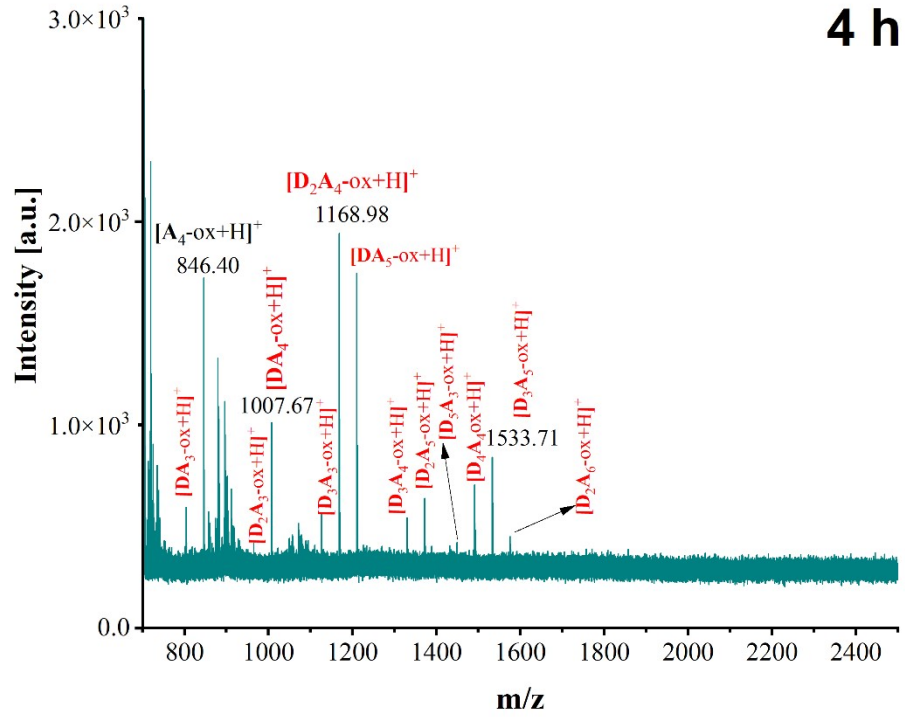
1000
1200
1400
1600
1800
2000
2200
2400
m/z


4 h
3.0×10^3
2.0×10^3
1.0×10^3
0.0
Intensity [a.u.]
[D2A4-ox+H]+
1168.98
[A4-ox+H]+
846.40
[DA5-ox+H]+
[DA3-ox+H]+
[DA4-ox+H]+
1007.67
[D2A3-ox+H]+
[D3A3-ox+H]+
[D3A4-ox+H]+
[D2A5-ox+H]+
[D5A3-ox+H]+
[D4A4ox+H]+
1533.71
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1200
1400
1600
1800
2000
2200
2400
m/z

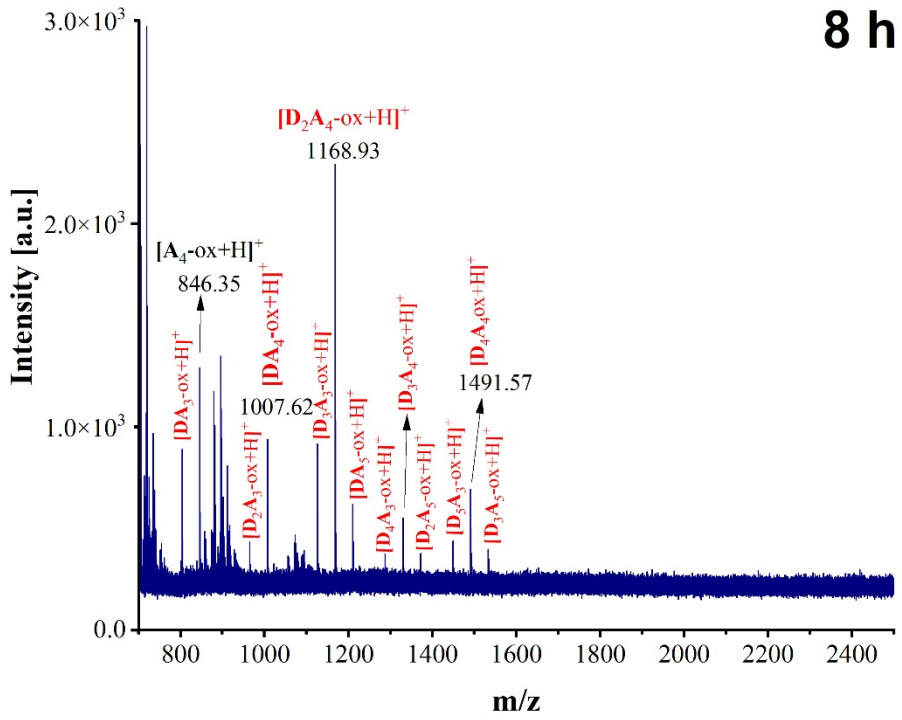

Figure S7. MALDI-TOF MS spectra of LPMO-generated oxidized chito-oligosaccharides after treatment with *Ar*CE4A. The reaction contained 1.0 mg/ml substrate (oxCHOS), 10 µM $CoCl_2$ and 25 nM *Ar*CE4A in 10 mM Tris-HCl, pH 8.0, and was incubated at 37°C, in a shaker at 225 rpm for 0 min, 15 min, 30 min, 1 h, 2 h, 4 h, or 8 h. Reactions were quenched by adding acetonitrile to a final concentration of 50% (v/v); due to a slightly changed analytical procedure the spectra are dominated by hydrogen adducts, as opposed to other mass spectra shown in this paper, which are dominated by sodium adducts. **A** represents a GlcNAc unit; **D** represents a GlcN unit. For clarity, only a few *m/z* values are shown. The shown *m/z* values correspond to the hydrogen adducts of the aldonic acid form of the fully acetylated oxidized tetramer to decamer and selected deacetylated products. Each deacetylation leads to a reduction of the *m/z* value by 42. Similar analyses for reactions run for less than one hour are shown in Fig. 6D of the main manuscript. It is worth noting that as deacetylation increased product detection became harder, as shown by the increasing noise levels in the mass spectra.

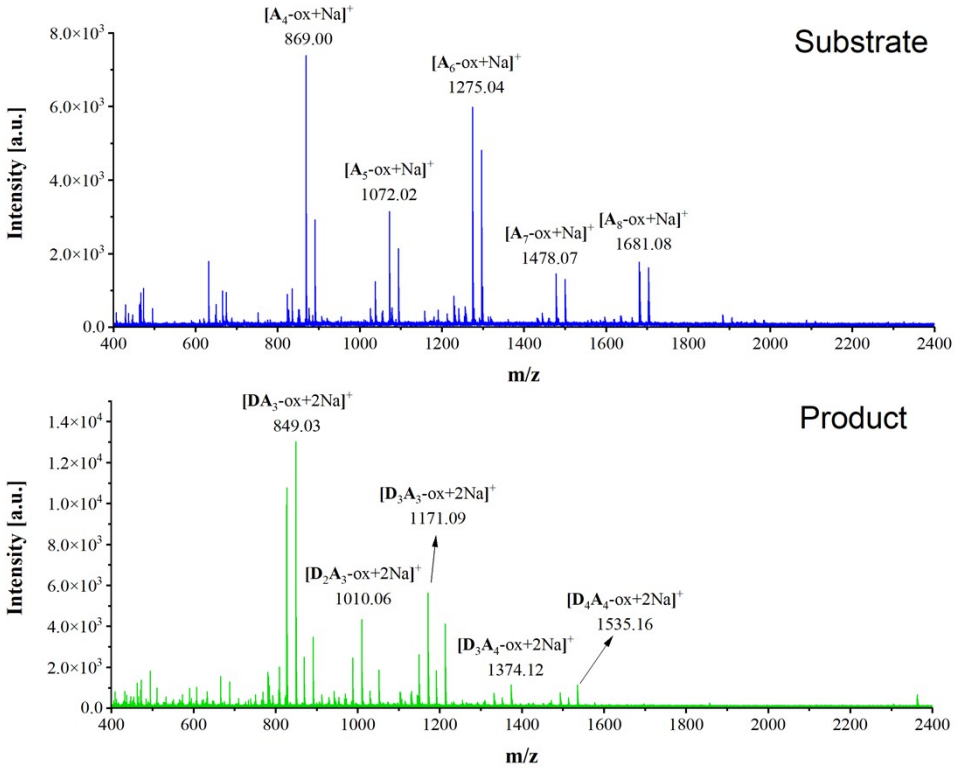

Figure S8. MALDI-TOF MS spectra of LPMO-generated oxidized chito-oligosaccharides before and after deacetylation with *An*CDA from *Aspergillus nidulans* FGSC A4. The reaction contained 1 µM *An*CDA, 10 µM $CoCl_2$, and 1 mg/mL oxCHOS in 50 mM Tris-HCl, pH 8.0, and was incubated at 37°C for 24h, with shaking at 850 rpm. MS analysis was carried out directly after the 24 h incubation, without quenching the reaction. **A** represents a GlcNAc unit; **D** represents a GlcN unit. For clarity, not all *m/z* values are shown. The indicated *m*/*z* values in the upper panel are the sodium adducts of the aldonic acid form of the oxidized tetramer (869) to octamer (1681). In the lower panel, labels refer to the sodium salt of the sodium adduct of partially deacetylated oxidized oligomers in the aldonic acid form; regular sodium adducts are also visible at *m/z* -22 relative to the labeled peaks. Each deacetylation reduces the *m*/*z* value by 42. For example, the peak with *m*/*z* 869 (upper panel) is the sodium adduct of the aldonic acid form of the oxidized chitin tetramer, while the peak with *m*/*z* 849 in the lower panel represents the sodium salt of the sodium adduct of the singly deacetylated oxidized tetramer in its aldonic acid form (**DA**$_3$-ox; 869 - 42 + 22).

References

1. A. A. Stepnov, I. A. Christensen, Z. Forsberg, F. L. Aachmann, G. Courtade and V. G. H. Eijsink, *FEBS Lett.*, 2022, **596**, 53-70.
2. G. Courtade, Z. Forsberg, E. B. Heggset, V. G. H. Eijsink and F. L. Aachmann, *J. Biol. Chem.*, 2018, **293**, 13006-13015.