Supplementary Material

Complementary sample preparation strategies for analysis of cereal β-glucan oxidation products by UPLC-MS/MS

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Figure S1 Thin layer chromatography (TLC) of a control experiment to establish behavior of polymeric β-glucan (BG) and oligosaccharides on graphitized carbon solid phase extraction (SPE). Preconditioned SPE cartridge (see experimental section for details) was loaded with two portions (each 2.5 mL) of 0.6% barley BG spiked with maltooligosaccharides Glc\(_n\) (\(n = 1\) (1 mM); \(n = 2, 3\) (each 0.4 mM); \(n = 4–6\) (each 0.2 mM)), the SPE then washed with H\(_2\)O (3 mL), and eluted with 1:3 ACN/H\(_2\)O (2 × 2.5 mL; fraction 1 & 2). Solutions collected during loading (two spots), washing (1 spot), and elution (2 spots) were applied on the TLC plate (~3 µL each), and the spots made visible after development (BuOH/ACOH/H\(_2\)O 2:1:1) by dipping in an ethanolic solution of 5% (v/v) H\(_2\)SO\(_4\) + 0.7% (w/v) 4-cumylphenol and heating for 15 min at 120°C. The TLC plate clearly shows how polymeric BG is not retained and breaks through during loading and washing (intense spot at baseline). The same is the case for glucose (\(n = 1\)). The rest of the spiked oligosaccharides with \(n = 2–6\) are retained (small losses during washing for \(n = 2\)) and need aqueous ACN to fully elute in fraction 1 (no spots in fraction 2). BuOH, n-butanol; ACOH, acetic acid.
**Figure S2** UPLC-MS/MS of released oligomers observed from oxidized BBG (100 mM H₂O₂, 50 µM FeSO₄) after direct SPE (strategy I). (A) Base peak ion chromatogram (BPI) using negative mode and an ACN/H₂O gradient up to 50% H₂O (0.1% NH₃ additive). (B) MS/MS of released Glc, and (C) MS/MS of oxo-Glc species (plus the cross-ring cleavage product Glc₃Ara), with their proposed main oligosaccharide structures in symbolic representation, based on retention time behavior and MS/MS patterns. ****, disaccharide signal from catalase material.
Figure S3 UPLC-MS/MS of released oxo-Glcₙ species with C=O located somewhere else than the non-reducing end as observed from oxidized BBG (100 mM \( \text{H}_2\text{O}_2 \), 50 µM \( \text{FeSO}_4 \)) after enzymatic treatment/SPE (strategy II; 10x concentrated by evaporation under a stream of \( \text{N}_2 \)). (A) Base peak ion chromatogram (BPI) using negative ion mode, a slower aqueous ACN gradient (0.17 mL/min) with 15 cm BEH amide column (doubling the retention time compared to Figure 5a in the manuscript), and basic eluent (0.1% \( \text{NH}_3 \) additive). (B) MS/MS of oxo-Glcₙ species. (For MS/MS of oxo-Glcₙ (C=O at non-reducing end) and of the (5a*) species (= oxo-Glc₅), see Figure 5a/b in the manuscript; for XICs of each oxo-Glcₙ, see Figure S4).
Comparison: Harsh vs. mild oxidation of BBG after enzyme digestion/SPE (strategy II)

(A) Harsh oxidation: BPI (top) and XICs of oxo-Glcₙ

(B) Mild oxidation: BPI (top) and XICs of oxo-Glcₙ

Figure S4 Comparison of UPLC-MS base peak ion (BPI) and extracted ion chromatograms (XIC) from (A) harsh (100 mM H₂O₂) and (B) mild (250 µM AH₂) oxidation of BBG after lichenase+β-glucosidase treatment/SPE (strategy II; 10x concentrated by evaporation under a stream of N₂). Negative ion mode, a slower aqueous ACN gradient (0.17 mL/min) with 15 cm BEH amide column, and basic eluent (0.1% NH₃ additive) were used. The peaks are labeled with their base peak m/z. Note that the mild oxidation conditions lead to a different product profile of oxo-Glcₙ species with oxoGlcGlcₙ₋₁ not being the predominant product as is the case for n = 3,4 under the harsh conditions. This phenomenon is subject to further investigation. The BPI of the mild oxidation (B) shows native Glcₙ peaks (m/z 341, 503, 665, 827), all of which also occur in the non-oxidized control in the same proportions (whereas no oxo-Glcₙ signals in control), and might be residues of DP3–5 (from lichenase treatment) that were not fully hydrolyzed by β-glucosidase. The sharp m/z 341 peak (13 min) originates from the catalase material (disaccharide). *, oxo-Glcₙ isomers with the carbonyl not at the non-reducing end. **, in-source fragmentation peak of the respective n+1 species.
Figure S5 Comparison of (A) negative and (B) positive ion mode in the UPLC-MS analysis of BBG oxidation products after lichenase & β-glucosidase/SPE treatment (strategy II) with the respective base peak ion chromatograms (BPI) and extracted ion chromatograms (XIC) of oxo-Glc₄ as example. Note that in the positive mode, isomeric oxo-Glc₄ products have different preferences regarding ionization, with the main oxoGlcGlc₃ product ionizing preferably as [M+H]⁺ or [M+H₂O+NH₄/Na]⁺, in agreement with the carbonyl-hydrate (geminal diol) equilibrium of the oxo-group (R₂C=O + H₂O = R₂C(OH)₂). Other oxo-Glc₄ isomers with mid-chain oxo-groups (labeled with * in the BPIs) preferably ionize as ammonium or sodium adducts in the positive ion mode.
Figure S6 (A) Collision induced dissociation (CID) of Glc-2AB from reductive amination strategy III: Proposed structures for the observed most prominent MS/MS fragments. MS/MS spectra of (B) 2-AB labeled standards, and (C) of 2-AB labeled reducing termini from harsh BBG oxidation after enzyme treatment/SPE (fraction 2; see Figure 7b for BPI). Fragments are labeled for (C), with labels in purple containing the 2-AB moiety, while labels in blue do not. Differences in relative fragment intensities of Ery- and Ara-2AB between standards and BBG oxidation products might originate from Ara and Ery being different isobaric pentoses or tetroses, respectively, e.g. formed through epimerization. Oxidation product GlcGlc-2AB could be identified to be the β-(1→3)-linked isomer due to the observed MS/MS fragment m/z 191 and matching retention time (R_t) of ~3.3 min (β-(1→4)-isomer elutes earlier). Due to lack of standards, the linkage type of GlcAra-2AB could not be unambiguously confirmed by MS/MS, but is assumed to be β-(1→2)-linked originating from a β-(1→3)-Glc unit, analogous to the β-glucosidase resistant, confirmed Glcβ(1→3)Glc-2AB.
Figure S7 Detection of lytic C5-oxidation products with C=O labeling strategy III (reductive amination).\(^1\) Proposed mechanisms to explain the observed epimeric mixture of Glc\(^{5\text{oxo}}\)Glc-2AB\(_0\) (2 peaks), but not for Glc\(^{5\text{oxo}}\)Glc-2AB\(_0\) (predominantly 1 peak), on the basis of observations made by Baxter and Reitz (1994) in their aza-sugar synthesis from 5-oxo-hexoses (see Figure S8a for chromatogram, and Figure S8 for the full mechanism). Under the assumption that the hydride attack (NaBH\(_3\)CN) on the intermediate iminium ion takes place axially from the side that avoids formation of a boat-conformation transition state, a (A) \(\beta\)-(1→3)-linked unit has disfavoring steric obstacles for both possible intermediates, namely 1,2-allylic strain \((A_{1,2}\); left) pushing the equilibrium to the right side, and a blocked top side from \(R = \text{Glc}_m\) (right). Consequently, products from both intermediates are formed. (B) A \(\beta\)-(1→4)-linked unit has two factors favoring the conformation on the right-hand side: higher 1,2-allylic strain due to \(R = \text{Glc}_m\) (left), and an accessible top that additionally might have a directing effect of the free hydroxyl group at C3 by anchimeric assistance (right). This would explain why predominantly one product was formed for \(5\text{oxo}\)Glc-2AB\(_0\) \((\beta\)-(1→4)-linked before \(\beta\)-glucosidase), while both epimers were detected in comparable amounts for \(\beta\)-(1→3)-linked Glc\(^{5\text{oxo}}\)Glc-2AB\(_0\). The high dependence on selectivity regarding substitution pattern and configuration was also observed by Baxter and Reitz (1994), as for instance unsubstituted 5-oxo-glucose gave high selectivity (>95%) for one epimer after reductive amination, while mannose (C2 epimer of glucose) and per-O-acetylated 5-oxo-Glc had low selectivity (67:33 and ~50:50, respectively).

\(^1\) Glc\(^{5\text{oxo}}\) is the only primary oxidation product with 6 carbons expected to result in such a cyclization: For instance, a \(\gamma\)-keto-aldehyde (C4-oxidation) could also lead to a cyclisation by reductive amination resulting in a 5-membered pyrrolidine derivative with the same \(m/z\). However, the C4-oxidation would have to occur on a reducing end for C1 to be a free aldehyde (in equilibrium with its hemiacetal form), and reducing ends are in low amounts compared to the total sugar units, most of which (>99%) are mid-chain units. Lytic C5-oxidation is the only process that directly leads to a suitable substrate for the observed cyclisation without the need for two oxidation processes happening on the same glucose unit. Misidentification of dehydration side reactions can also be excluded: A loss of 18 Da corresponds to \(-\text{H}_2\text{O}\), or a dehydration, but cannot be a side product of Glc reducing end labeling, as test reactions with glucose and oligomer standards under identical reductive amination conditions showed no such products. It also cannot be a result of lactone formation with the carboxyl of the label, as this would lead to the correct \(m/z\) for 2-\(\text{AA} (-\text{H}_2\text{O})\), but not for 2-\(\text{AB} (-\text{NH}_3;\) would give the same \(m/z\) of 282.10 as \(5\text{oxo}\)Glc-2AA, which was not observed).
Figure S8 UPLC-MS/MS of oxo-products from BBG oxidation (harsh conditions) detected as 2-AB labeled species in SPE fraction 1 after reductive amination, enzyme treatment & SPE (strategy III; negative ion mode, basic eluent). (A) Overlaid extracted ion chromatogram (XIC) and (B) MS/MS spectra of C=O labeled 5-oxo-reducing ends (stereocenter * of epimers set arbitrarily). The inset labeled with “T” is the structure of 2-AB labeled L-threo-tetrodialdose (oxo-Tet-2AB$_2$), which is also a C5-oxidation product that was also observed by Schuchmann & von Sonntag in their Glc irradiation study (Schuchmann and Sonntag, 1977). (C) XICs of labeled oxo-Glc$_n$ products and (D) their respective MS/MS spectra. For each $n$, the average MS/MS is shown, as surprisingly little differences were found between the isobaric individual peaks of 2AB-(oxo-Glc$_n$) resolved by UPLC-MS. The fragments are labeled assuming the labeled oxo-group being at the non-reducing end as in the depicted structures (since they are the main isomers as detected in strategy II; see Figure 5a). Fragments labels in red contain the oxidized unit (incl. 2-AB), while labels in blue do not.

2 It is noteworthy that in the MS/MS of these labeled reducing ends, some fragments in the spectra bear $m/z$ that are easily mistaken to be purely glucose-derived fragments, but can be differentiated thanks to the qToF detection with high enough resolution (e.g. $m/z$ 161.07 = [2AB-C$_2$H$_3$-H] $\text{vs. } m/z$ 161.04 = [Glc - H$_2$O - H]). In some cases, this can be relevant as some cross-ring fragments (e.g. $m/z$ 263.08, 281.09) which are indicative of the glycosidic linkage type are near-isobaric to 2-AB-labeled glucose fragments (e.g. $m/z$ 263.10, 281.11). This also applies to MS/MS fragments in Figure S6.

3 However, Y-ions are not typically encountered if the reducing end is still intact, as fragmentation in the negative mode predominantly occurs from the reducing to the non-reducing end link a zipper (A, B, & C ions). Hence, these ions, including the cross-ring fragments labeled with **, originate most likely from oxo-Glc$_n$ isomers with labeled mid-chain oxo-groups and are actually their C-type ions.
Figure S9 Comparison of (A) extracted ion chromatograms (XIC) from negative ion UPLC-MS (0.1% NH$_3$ eluent) and MS/MS of (B) Glc$_n$ standards (mixed-linkage & cello-oligomers) with (C) isobaric oligosaccharides formed under the reductive amination conditions during C=O labeling (strategy III, SPE fraction 1) of oxidized BBG (100 mM H$_2$O$_2$). The latter oligomers are presumably the result of direct C=O-reduction (instead of imin-reduction) of oxo-Glc$_n$ species with NaBH$_3$CN to epimeric mixtures of HexGlc$_{(n-1)}$ (Hex = any hexose, e.g. glucose & its epimers (epimeric center in parenthesis) mannose (C2), allose (C3), galactose (C4); not necessarily at the non-reducing end). Note that retention times differ clearly from the standards in (A), and that all HexGlc$_{(n-1)}$ have a β-(1→3)-linked reducing end unit as evident in their MS/MS (C) (no A$_n$ ions).
MS/MS of CO$_2$H labeled standards and acidic oxidation products from BBG oxidation (strategy IV)

Figure S10 MS/MS spectra of CO$_2$H labeled standards and oxidation products from EDC-mediated amidation of carboxylic acids with PhNH$_2$ (sample preparation strategy IV). Note that the observed cross-ring fragments (A-ions) of labeled GlcAGlc ($m/z$ 430) indicate a β-(1→4)-linkage, which is unexpected and might be a peeling product of GlcAGlc$_2$ (bottom spectrum).
Figure S11 Symbols, structures and abbreviations: Lytic & non-lytic products initiated by HO•-attack on indicated carbons of β-glucan repeating unit (β-(1→3)-linked as example), as well as the resulting UPLC-MS detected products after enzyme treatment/SPE (strategy II) and with prior C=O/CO₂H labeling (strategy III & IV). Attack on a β-(1→4)-linked unit gives the analogous products (attack on C4 being lytic, on C3 non-lytic), but different sizes of detected products after sample preparation strategies II–IV (e.g. monosaccharides instead of Glcβ(1→3)[oxidized/labeled unit]). Cross-ring cleavages C1-C2, C2-C3 & C4-C5 according to Schuchmann and Sonntag (1977).
**Table S1** Sample preparation strategies of oxidized BG solutions and the resulting lost and preserved information about the oxidation products for each case, as well as the corresponding species detected by UPLC-MS and confirmed by MS/MS.\(^a\)

| Sample preparation\(^b\) | Lost information\(^c\) | Preserved information | Detected as\(^d\) |
|--------------------------|------------------------|-----------------------|-------------------|
| **I: SPE**               | Polymeric oxidation products | Released oligosaccharides (neutral & acidic) with \(n = 2–8\) | (oxo-)Glc\(_n\), Glc\(_{n-1}\)Ara, Glc\(_{n-1}\)Ery, Glc\(_{n-1}\)GlcA, Glc\(_{n-1}\)Glc1A, GlcAGlc\(_{n-2}\)Glc1A GlcAGlc\(_{n-2}\)Ara\(^h\) |
| **II: Lichenase + \(\beta\)-glucosidase, SPE** | Neutral (new) reducing ends, lytic C1-oxidation\(^g\) | (lytic) oxo-products, C6-oxidation to CO\(_2\)H, \(\beta\)-(1→3)-linked C1-oxidation products | oxoGlcGlc\(_{n-1}\), GlcAGlc\(_{n-1}\), GcGlc1A, oxo-Glc\(_n\)\(^i\) |
| **III: Carbonyl (C=O) labeling, precipitation, enzymes, SPE\(^c\)** | (lytic) oxo-products (e.g. from lytic C3/C4-oxidation),\(^1\) acidic products | Neutral (new) reducing ends (incl. cross-ring cleavage products & lytic C5-oxidation) | (Glc)Glc-2AB, (Glc)Ara-2AB, (Glc)Ery-2AB, (Glc)\(^{5\text{oxo}}\)Glc-2AB |
| **IV: Carboxylic acid (CO\(_2\)H) labeling, precipitation, enzymes, SPE\(^d\)** | Neutral (new) reducing ends, (lytic) oxo-products | C1- and C6-oxidation to CO\(_2\)H, acidic products from cross-ring cleavage | (Glc)Glc1A-NHPh, (Glc)GlcA-NHPh, PhNH-GlcAGlc\(_{n-1}\), Ara1A-NHPh, GlcEry1A-NHPh |

\(^{a}\) Refers to harsh oxidation conditions used for method development (0.6% BG, 50 \(\mu\)M FeSO\(_4\), 100 mM H\(_2\)O\(_2\)). Lost or preserved information as well as the detected products in the case of the two labeling procedures (III & IV) refer to only the respective labeled products. oxo-Glc\(_n\), gluco-oligomer with an oxidized hydroxyl group (\(\rightarrow\text{C}=\text{O}\)) on any of the units; oxoGlcGlc\(_{n-1}\), gluco-oligomer with carbonyl specifically at the non-reducing end unit; -2AB, reducing end unit labeled with 2-AB by reductive amination (\(\rightarrow\)aminodeoxyxylitol); -NHPh or PhNH\(_2\), anilide of acid unit. For other abbreviations and structures, see **Figure S11**.

\(^{b}\) Oxidized BG solution treated with phosphate buffer and catalase first (except for strategy I: SPE).

\(^{c}\) Monosaccharide and other small products (\(\prec\text{C}_4\)) are lost in all cases due to SPE purification/fractionation.

\(^{d}\) (Glc) and (oxo-) in parenthesis refer to products both with and without the indicated additional structural feature.

\(^{e}\) Labeling by reductive amination with 2-AB (as example, 2-AA also possible) and NaBH\(_4\)CN. Enzymes: lichenase + \(\beta\)-glucosidase treatment.

\(^{f}\) Labeling by amidation with aniline (PhNH\(_2\)) and EDC. Enzymes: lichenase + \(\beta\)-glucosidase treatment.

\(^{g}\) Information only partially lost, namely \(\beta\)-(1→4)-linked C1-oxidation products.

\(^{h}\) Mixed-linkage mixtures with random position of both \(\beta\)-(1→3)-linkage and oxidized sugar unit (e.g. GlcA, C=O group).

\(^{i}\) The predominant products exhibit clearly defined position of the \(\beta\)-(1→3)-linkage (reducing end unit) and the oxidized monomer unit (as the non-reducing end; \(n = 2–5\)). Prominent exceptions are Glc\(_B\)(1→3)Glc1A & Glc\(_B\)(1→3)GlcA, as well as some oxo-Glc\(_n\) products (\(n = 2, 4, 5\)).

\(^{i}\) Partially lost through direct C=O reduction to epimeric mixtures HexGlc\(_{n-1}\) as side-reaction of reductive amination.
References

Baxter, E.W., and Reitz, A.B. (1994). Expeditious Synthesis of Aza sugars by the Double Reductive Amination of Dicarbonyl Sugars. *The Journal of Organic Chemistry* 59(11), 3175-3185. doi: 10.1021/jo00090a040.

Schuchmann, M.N., and Sonntag, C.V. (1977). Radiation-Chemistry of Carbohydrates .14. Hydroxyl Radical Induced Oxidation of D-Glucose in Oxygenated Aqueous-Solution. *J. Chem. Soc. Perk. T. 2* (14), 1958-1963. doi: 10.1039/P29770001958.