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Reaction pathways during oxidation of cereal β -glucans

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ABSTRACT

Oxidation of cereal β -glucans may affect their stability in food products. Generally, polysaccharides oxidise via different pathways leading to chain cleavage or formation of oxidised groups within the polymer chain. In this study, oxidation pathways of oat and barley β -glucans were assessed with different concentrations of hydrogen peroxide (H_2O_2) or ascorbic acid (Asc) with ferrous iron (Fe^{2+}) as a catalyst. Degradation of β -glucans was evaluated using high performance size exclusion chromatography and formation of carbonyl groups using carbazole-9-carbonyloxyamine labelling. Furthermore, oxidative degradation of glucosyl residues was studied. Based on the results, the oxidation with Asc mainly resulted in glycosidic bond cleavage. With H_2O_2 , both glycosidic bond cleavage and formation of carbonyl groups within the β -glucan chain was found. Moreover, H_2O_2 oxidation led to production of formic acid, which was proposed to result from Ruff degradation where oxidised glucose (gluconic acid) is decarboxylated to form arabinose.

KEYWORDS

β -glucan, oxidation, hydrogen peroxide, ascorbic acid, formic acid

ABBREVIATIONS

H_2O_2 , hydrogen peroxide; Asc, ascorbic acid; HPSEC, high performance size exclusion chromatography; CCOA, carbazole-9-carbonyloxyamine; BBG, barley β -glucan; OBG, oat β -glucan

1 INTRODUCTION

Interest towards cereal (1→3)(1→4)-β-D-glucans (β-glucans) has increased since their ability to lower blood cholesterol and sugar levels were recognised and health claims concerning these health effects approved by European Food Safety Authority (EFSA, 2010, 2011a, 2011b). Oat and barley are the most significant sources of cereal β-glucans with contents varying from 3% to 7% and from 3% to 11%, respectively (Cui & Wood, 2000). Cereal β-glucans are composed of cellulose-like blocks of mainly three (DP3) or four (DP4) glucosyl units linked by (1→4)-β-D-linkages. These blocks are attached with (1→3)-β-D-linkages, which gives flexibility and enhances the water-solubility of cereal β-glucans. β-Glucan can form viscous solutions with viscosity depending on the concentration of the solution and the molar mass of the β-glucan. The health effects of β-glucan have been linked to the solubility of the molecule and its viscosity in solutions (Lazaridou & Biliaderis, 2007; Wood, 2010). The molar ratio of DP3 to DP4 may affect aggregation and rheological properties of β-glucans, and thus, the higher DP3/DP4 in barley (1.8–3.5) compared to oat (1.5–2.3) is giving rise to the differences in the functionality of these β-glucans.

Recent studies have shown that degradation of β-glucans can occur due to oxidation reactions, which can affect their technological and physiological functionality (Faure, Andersen, & Nyström, 2012; Kivelä, Gates, & Sontag-Strohm, 2009; Kivelä, Nyström, Salovaara, & Sontag-Strohm, 2009). The oxidation of β-glucan can be initiated by the hydroxyl radicals ($\cdot\text{OH}$), which are formed in the presence of hydrogen peroxide (H_2O_2) and metal catalyst (e.g. ferrous ion Fe^{2+} or Cu^{2+}) via Fenton-type reactions (Haber & Weiss, 1934). Ascorbic acid (Asc) can act as a reducing agent facilitating the formation of H_2O_2 from oxygen, and it therefore can also initiate oxidation reactions (Guo, Yuan, Wu, Xie, & Yao, 2002). Oxidative degradation has been shown to be significant when oat and barley β-glucans are oxidised with Fenton's reagent, as indicated by the decrease in molar mass and

viscosity (Kivelä, Henniges, Sontag-Strohm, & Potthast, 2012; Mäkelä, Sontag-Strohm, & Maina, 2015). The radical-mediated oxidation is non-selective and depending on the carbon atom that is attacked it can result in glycosidic bond cleavage, formation of carbonyl groups along the polysaccharide chain and glucose ring opening and fragmentation (Schuchmann & von Sonntag, 1977; von Sonntag, 1980). Faure, Sánchez-Ferrer, Zabara, Andersen and Nyström, (2014) studied the oxidation of barley β -glucan at elevated temperature (85°C) and showed fast degradation (molar mass non-detectable with light scattering after 2 h of oxidation) and formation of new carbonyl-based functional groups. In addition, their results suggested β -(1 $\rightarrow$ 3) glycosidic linkage to be more prone to cleavage than β -(1 $\rightarrow$ 4) linkage.

Our previous study showed that in addition to depolymerisation, some aggregates were formed during oxidation, as shown in asymmetric flow field-flow fractionation (AsFFFF) analysis (Mäkelä et al., 2015). The aggregates were few (3–6%) and were hypothesised to possibly result from inter-chain cross-linking due to the reaction of β -glucan hydroxyl groups with carbonyl groups that were formed during oxidation. Bamford and Collins (1950) studied the oxidation of glucose under alkaline conditions and showed that glucose decomposed to formic acid and arabonic acid. Formic acid has also been shown to form due to oxidation in acidic conditions (Jin et al., 2005; Robert, Barbati, Ricq, & Ambrosio, 2002). In this study it was therefore hypothesised that the main reaction pathway during oxidation of cereals β -glucans is glycosidic bond cleavage and the subsequent formation of new reducing ends. The reducing ends formed are further susceptible to oxidation, which could lead to sequential degradation of the reducing end glucosyl units to form formic acid and new monosaccharide units at the reducing end. The aim of this study was therefore firstly to evaluate depolymerisation and formation of carbonyl groups during the oxidation of β -glucan with either H₂O₂ or Asc at different concentrations. Secondly, the

formation of formic acid and the subsequent changes in the reducing end glucosyl units were determined.

2 MATERIALS AND METHODS

2.1 Preparation and oxidation of sample solutions

Barley β -glucan (high viscosity, purity >94%, the weight average molar mass (M_w) 495 000 g/mol) and oat β -glucan (high viscosity, purity >94%, M_w 361 000 g/mol) were purchased from Megazyme (Ireland). 0.7% (w/v) Barley and oat β -glucan solutions (BBG and OBG, respectively) were prepared by wetting the sample with 99.5% ethanol (Altia, Finland) prior to dissolution with MilliQ water (Millipore system, Merck Millipore, Germany). Consequently, the samples were kept at 85°C for 2 h with continuous stirring. After 2 h, the samples were kept stirring for an hour at room temperature.

Oxidation reactions were initiated with 10 mM, 40 mM or 70 mM H_2O_2 (30 % hydrogen peroxide, Merck, Germany) or Asc (L(+)-ascorbic acid, AnalaR NORMAPUR®, VWR Chemicals, Belgium). For all solutions, 1 mM iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) (Merck, Germany) was also added, and the final β -glucan concentration in the solutions was adjusted to 5.6 mg/ml with MilliQ water. A non-oxidised control sample diluted to the same concentration with MilliQ water was used for comparison. All reactions were carried out at room temperature.

2.2 Carbonyl content and molar mass analyses

The molar mass of non-labelled samples were analysed using high performance size exclusion chromatography (HPSEC) in 0.01 M LiBr/DMSO. The analysis was done as specified by Mäkelä et al. (2015) and the data analysed using OmniSEC software as described by Fishman, Doner, Chau and Hoagland (2000). Carbonyl content analysis with simultaneous

molar mass analysis of the labelled molecules was done according to Röhring et al. (2002a, b) with some modifications. The 4 day oxidised samples were precipitated with ethanol to remove the oxidation reagents. After centrifugation, the samples were washed twice with ethanol and finally dried at room temperature. For carbonyl analysis, 10 mg samples and 20 mg standards (six cellulose samples with known carbonyl content (Potthast et al., 2015)) were labelled with CCOA (carbazole-9-carboxyloxyamine). The samples were activated by wetting them with MilliQ and rinsed with 96% ethanol on a filter paper. The activated samples were suspended into 1 ml of 0.9% LiCl in DMAc and the suspensions were shaken overnight at room temperature. 2 ml of 1.25 mg/ml CCOA in acetate buffer (pH 4) was added to each sample and the samples were shaken at 40°C for 7 days. After labelling the samples were centrifuged and the precipitates were dissolved in 1 ml of 0.9% LiCl in DMAc. The samples were filtered (0.45 µm) prior to analysis.

The HPSEC system included a fluorescence detector (FL3000, Thermo Scientific, USA) for monitoring the CCOA label ($\lambda_{\text{ex}} = 290$ nm and $\lambda_{\text{em}} = 340$ nm) a MALLS detector (Wyatt Dawn DSP, Wyatt Technology, USA) with an argon ion laser ($\lambda_0 = 488$ nm), and a refractive index detector (Shodex RI-71, Japan). Four serial PLgel-mixed ALS (7.5 mm x 300 mm) columns (Agilent, Germany) were used. In addition, the system consisted of a degasser (Dionex DG-2410, Thermo Scientific, USA), an autosampler (1100, Agilent, Germany), a pulse damper pump, and a column oven (STH 585, GynkoteK, Germany) at 25°C. The operating conditions of the HPSEC were as follows: the flow rate was 1.00 ml/min, the injection volume 100 µl, the run time 45 min. DMAc/LiCl (0.9%, w/v) after filtering through a 0.02 µm filter was used as eluent. The dn/dc value of 0.136 was used. The MALLS data were evaluated with Astra software (Wyatt Technology, USA) using the first-order Zimm fit. The carbonyl group content was analysed using Chromeleon and GRAMS/32 software (Thermo Scientific, USA).

The total carbonyl group amounts, $c(\text{C=O})_{\text{tot}}$, are reported in $\mu\text{mol/g}$. The equivalent amount of reducing end groups, $c(\text{C=O})_{\text{reg}}$, was determined from the number average molar mass (M_n) using Equation 1.

$$c(\text{C=O})_{\text{reg}} = \frac{1}{M_n} \times 10^6 \mu\text{mol/g} \quad (\text{Equation 1})$$

2.3 Formic acid analysis

2.3.1 NMR spectroscopy analysis

Formation of formic acid was detected with nuclear magnetic resonance (NMR) spectroscopy analysis using barley β -glucan oxidised with 70 mM H_2O_2 /Asc and 1 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. NMR analysis was carried out on a 600 MHz Bruker Avance III NMR spectrometer (Bruker BioSpin, Germany) equipped with a QCI cryoprobe. The measurements were performed at 22°C. For the analysis, 7 mg/ml of sample was dissolved in D_2O and the oxidation reaction was initiated before NMR analysis. 1D ^1H experiments were carried out at several time points using Bruker 1D NOESY with pre-saturation and spoil gradients pulse program (noesygppr1d) where the residual water signal is suppressed by 4 s volume selective pre-saturation.

2.3.2 Formic acid content analysis

The formic acid (FA) content of non-oxidised and oxidised samples was measured at two time points of oxidation (24 hours and 4 days) using the Megazyme Formic acid assay (Ireland). In the analysis formate dehydrogenase (FDH) and NAD^+ are added to the sample and the formation of NADH in Reaction 1 is followed spectrophotometrically.

The samples were prepared and analysed according to the assay instructions. The pH of the oxidised samples was neutralised by adding an appropriate amount of 0.1 M NaOH to the sample. For the non-oxidised samples, the same volume of MilliQ was added to equalise the concentration of the samples.

Since the formic acid analysis is based on the spectrophotometric measurement of NADH, which is formed in Reaction 1, Asc was suspected to affect the reaction since it may act as a reducing agent. Thus, the effect of ascorbic acid (10 mM, 40 mM and 70 mM Asc including 1 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was tested with reagent blank samples. The results of these reagent blanks were subtracted from the results of the samples oxidised with ascorbic acid.

2.4 Arabinose and glucose content

2.4.1 Preparation of samples

For monosaccharide analyses the non-oxidised and oxidised samples (5.6 mg/ml) were enzymatically hydrolysed. 60 μl of 50 U/ml lichenase (endo-(1-3)(1-4)- β -D-glucan 4-glucanohydrolase, Megazyme, Ireland) and 40 μl of 2 U/ml β -glucosidase (Megazyme, Ireland) were added to 200 μl of sample and the sample was simultaneously diluted to 2.8 mg/ml with MilliQ. The samples were incubated for 24 h at 40°C before inactivating the enzymes by boiling the samples for 10 min. The samples were stored at -20°C until glucose and arabinose were analysed.

The samples were diluted appropriately for glucose and arabinose analysis using glucose (0.005–0.200 mg/ml) and arabinose (0.002–0.100 mg/ml) standards. For both standards and samples, deoxygalactose (0.05 mg/ml) was added as an internal standard. The glucose and arabinose contents of the samples were calculated as percentages of the theoretical monosaccharide (glucose) amount in the native sample.

2.4.2 Monosaccharide analysis in HPAEC-PAD

Samples were filtered (0.45 μ m) prior to monosaccharide analysis, which was done with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) according to Johansson et al. (2006) with some modifications. The instrument consisted of three HPLC pumps (Waters 515 HPLC pumps, USA), an auto-sampler (Waters 2707, USA), two SSI pulse equalisers (model LP 21, Scientific systems Inc, USA), guard column CarboPac PA-1 (50 $\times$ 4 mm, Dionex Corporation, USA), analytical column CarboPac PA-1 (250 $\times$ 4 mm, Dionex Corporation, USA) and a pulsed amperometric detector (Waters 2465, USA). The temperature of the column was maintained at 30 °C. The flow rate of the mobile phase was 1 ml/min and 200 mM NaOH (A) and MilliQ water (B) were used as eluents, the ratio of A:B ranging from 1:99 to 99:1 with elution time. A post-column addition of 300 mM NaOH (Eluent C) with 0.3 ml/min flow rate was used. The temperature of the detector was 30 °C and the pulse potentials and durations of the detector were: E1=0.05 V, t1=400 ms, E2=0.75 V, t2=120 ms, E3=-0.80 V, t3=130 ms, ts=20 ms. The data handling was done using Empower 3 software (Waters, USA).

2.5 Statistical analysis

The molar masses of the non-oxidised and oxidised samples were calculated as average of the two replicates. The content of formic acid, glucose and arabinose and the oligosaccharide composition were analysed from three replicate samples and the results are reported as averages $\pm$ standard error of mean (SEM).

Statistical analyses were accomplished with Statistical Package for the Social Science (SPSS Statistics version 23, IBM, USA), using one-way analysis of variance (ANOVA) with post-hoc Tukey test. For arabinose results, the data were transformed into logarithmic mode

prior to statistical analysis because of the 10-fold differences in the values. Differences were considered as significant at $P < 0.05$.

3 RESULTS

3.1 Carbonyl content of the oxidised beta-glucans

To ensure that the molar mass was not changing due to precipitation or the CCOA labelling processes, the molar mass of freshly prepared samples was also analysed. As shown in table 1, the M_w was similar for both the fresh sample and labelled samples indicating CCOA labelling did not cause further degradation of the samples. The M_w of both BBG and OBG decreased due to oxidation even though there were differences in the extent of degradation. The M_w decrease of non-labelled BBG was 86% (from 500 000 g/mol to 69 000 g/mol) and 50% (from 500 000 g/mol to 250 000 g/mol) when oxidised with 70 mM H_2O_2 and 70 mM Asc, respectively. The corresponding percentages for non-labelled OBG were 52% (from 420 000 g/mol to 200 000 g/mol) for both oxidants.

The CCOA method applied covers all aldehyde and keto groups present. If the oxidation is accompanied by degradation also the number of reducing end groups increases. The combination with SEC-MALLS allows to calculate those newly formed reducing ends from the number average molar mass (M_n). The difference to the total amount of carbonyl groups hence corresponds to the groups introduced by oxidative processes (*cf.* Table 1). The carbonyl content was shown to increase due to oxidation and the increase was more significant in samples oxidised with H_2O_2 than in those oxidised with Asc (Table 1). In both BBG and OBG samples the carbonyl content increased only 2–4-fold when oxidised with Asc but with different concentrations of H_2O_2 the increase was 17–28-fold and 9–14-fold in BBG and OBG, respectively (Table 1, Figure 1 a-f). In BBG and OBG samples oxidised with

70 mM H₂O₂ (Figure 1 c, d) a shoulder was seen in LS signal in the area where larger molecules elute indicating some aggregation in the samples.

Table 1. Molar masses (number average molar mass = M_n , weight average molar mass = M_w , Z-average molar mass = M_z), total carbonyl content ($c(\text{C=O})_{\text{tot}}$) and equivalent concentration of reducing end groups ($c(\text{C=O})_{\text{reg}}$) of non-oxidised and oxidised BBG and OBG analysed after 4 days of oxidation at room temperature. The oxidation was initiated with different concentrations (10/40/70 mM) of H₂O₂ or ascorbic acid (Asc) and 1 mM FeSO₄·7H₂O as a catalyst.

Sample	Treatment	Fresh samples ^a			Labelled samples ^b				
		M_n (x10 ³ g/mol)	M_w (x10 ³ g/mol)	M_z (x10 ³ g/mol)	M_n (x10 ³ g/mol)	M_w (x10 ³ g/mol)	M_z (x10 ³ g/mol)	$c(\text{C=O})_{\text{tot}}$ (μmol/g) ^c	$c(\text{C=O})_{\text{reg}}$ (μmol/g) ^d
BBG	Non-oxidised	440	500	560	460	560	660	2.4	2.2
	10 mM H ₂ O ₂	100	150	310	97	160	270	40	10
	40 mM H ₂ O ₂	65	98	190	67	120	320	57	15
	70 mM H ₂ O ₂	45	69	130	42	62	89	66	24
	10 mM Asc	230	320	560	210	310	420	7.0	4.8
	40 mM Asc	220	290	600	210	310	40	8.8	4.8
	70 mM Asc	170	250	450	170	260	380	9.4	5.8
OBG	Non-oxidised	300	420	600	280	400	540	3.3	3.6
	10 mM H ₂ O ₂	140	190	340	150	200	260	31	6.6
	40 mM H ₂ O ₂	150	210	420	150	230	320	40	6.6
	70 mM H ₂ O ₂	150	200	420	100	160	230	46	9.5
	10 mM Asc	130	230	430	170	250	350	7.1	5.8
	40 mM Asc	110	200	380	160	220	290	8.0	6.4
	70 mM Asc	110	200	390	150	240	370	6.9	6.7

^a Samples were analysed with HPSEC in 0.01 M LiBr/DMSO after preparation.

^b Samples were analysed after precipitation and CCOA labelling with HPSEC in 0.9 % LiCl/DMAc.

^c The total carbonyl content is the value determined from the CCOA labelling.

^d The calculated concentration of reducing end groups based on the M_n values of the labelled samples.

209

210 **Figure 1.** HPSEC chromatograms of barley (a, c, e) and oat (b, d, f) β -glucan before
 211 oxidation (a, b) and after 4 days of oxidation with 70 mM H_2O_2 (c, d) and 70 mM ascorbic
 212 acid (e, f). 1 mM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was used as a catalyst in oxidised samples. Refractive index
 213 (RI) peak is shown with black line, light scattering (LS) peak with blue line, logarithmic

weight average molar mass ($\log M_w$) with green line and carbonyl group substitution ($DS_{C=O}$) with red line.

3.2 Formation of formic acid in oxidation of β -glucan

The 1D 1H spectrum of non-oxidised BBG is shown in Figure 2a. Figure 2b and 2c show BBG oxidised with 70 mM H_2O_2 and 1 mM $FeSO_4 \cdot 7H_2O$ as a catalyst for 2 and 3 hours, respectively. In the spectra, the β -(1 $\rightarrow$ 4) and β -(1 $\rightarrow$ 3) anomeric signals occur at δ 4.53 ppm and δ 4.78 ppm, respectively. The β -(1 $\rightarrow$ 3) signal is partially suppressed due to its close proximity to the suppressed water signal. According to analysis, a peak at δ 8.23 ppm, which was assigned to formic acid, was observed in the H_2O_2 oxidised sample (Figure 2). Neither the control non-oxidised sample (Figure 2a) nor sample oxidised with Asc (data not shown) showed the presence of formic acid. Formic acid accumulated with oxidation time as shown in Figure 2b and 2c, which were evaluated after 2 and 3 hours of oxidation respectively.

Figure 2. The 1D 1H spectrum of barley β -glucan showing the formation of formic acid during oxidation a) control sample with 1 mM $FeSO_4 \cdot 7H_2O$, b) sample oxidised with 70 mM H_2O_2 and 1 mM $FeSO_4 \cdot 7H_2O$ as a catalyst after 2 hours c) sample oxidised with 70 mM

H₂O₂ and 1 mM FeSO₄·7H₂O as a catalyst after 3 hours. The spectra were recorded at 600 MHz in D₂O at 22°C. Peaks are referenced to internal acetone (¹H = 2.225 ppm). * In the experiment, the anomeric proton of the β-(1→3)-linked glucosyl units is suppressed due to close proximity to the suppressed residual water signal.

The formic acid content of the samples, measured at two time points (day 1 and day 4), indicated that formic acid accumulated with oxidation time in samples oxidised with H₂O₂ (Figure 3). In non-oxidised samples and in the OBG samples oxidised with Asc, the formic acid content was negligible at both time points. The formic acid concentration was significantly higher in BBG samples than in OBG samples after 4 days of oxidation, the difference being about 4-fold in samples oxidised with 70 mM H₂O₂ for 4 days (13.6 mg/g of β-glucan in BBG compared to 3.3 mg/g of β-glucan in OBG). In OBG samples oxidised with Asc there was no formic acid formed (concentrations <0.7 mg/g of β-glucan) and in BBG samples the amount was minimal (varying from 0.87 to 1.50 mg/g of β-glucan).

246

247 **Figure 3.** Formic acid contents of non-oxidised and oxidised BBG and OBG samples. The
248 oxidation was initiated with different concentrations (10/40/70 mM) of H₂O₂ or ascorbic acid
249 (Asc) and 1 mM FeSO₄·7H₂O as a catalyst.

250

251 **3.3 Monosaccharide analysis**

252 Enzymatic hydrolysis of the samples indicated the presence of mainly glucose in both
253 oxidised and non-oxidised samples. However, in samples oxidised with H₂O₂, a small peak
254 eluting at about 12.5 min was also observed and it was identified as arabinose, based on
255 retention time. Arabinose and glucose in samples were therefore quantified and their content
256 calculated as percentage of theoretical amount of monosaccharide units (glucose) in β-glucan
257 in each sample.

258 In the samples oxidised with H₂O₂ the arabinose content increased with increasing
259 oxidant concentration at both time points (day 1 and day 4) (Figure 4). For example at day 4
260 the arabinose contents of the samples oxidised with 10 mM H₂O₂, 40 mM H₂O₂ and 70 mM
261 H₂O₂ were 0.14%, 0.55% and 0.85% in BBG and 0.25%, 0.82% and 1.30% in OBG,
262 respectively. Interestingly, the arabinose content was significantly higher in OBG samples
263 than in BBG samples. In addition, the amount of arabinose seemed to slightly decrease from
264 day 1 to day 4 in samples oxidised with 70 mM H₂O₂ (from 0.99% to 0.85% in BBG samples
265 and from 1.54% to 1.30% in OBG samples).

Figure 4. Arabinose contents of non-oxidised and oxidised BBG and OBG samples studied with HPAEC-PAD. The oxidation was initiated with different concentrations (10/40/70 mM) of H₂O₂ or ascorbic acid (Asc) and 1 mM FeSO₄·7H₂O as a catalyst.

Glucose contents of non-oxidised BBG and OBG were 98% and 93%, respectively (Figure 5). When oxidised with 70 mM H₂O₂ for 4 days, the glucose content of BBG decreased significantly by 10% (from 98% to 88%) and in OBG samples the corresponding decrease was 7.5% (from 93% to 86%), although the decrease was not statistically significant. When the samples were oxidised with ascorbic acid, there were no significant changes in the glucose content of either BBG or OBG.

Figure 5. Glucose contents of non-oxidised and oxidised BBG and OBG samples studied at day 4. The oxidation was initiated with different concentrations (10/40/70 mM) of H₂O₂ or ascorbic acid (Asc) and 1 mM FeSO₄·7H₂O as a catalyst.

4 DISCUSSION

4.1 Formation of carbonyls groups in the oxidation reactions of β -glucan

Our previous study indicated that oxidation of barley β -glucan led to significant decrease in M_w and these degraded β -glucan molecules consisted of more than 90% of the samples according to AsFIFFF analysis where single oxidised molecules and aggregates were separated (Mäkelä et al., 2015). A small portion of the samples oxidised with H₂O₂ was observed to form large aggregates, which was hypothesised to be caused by the formation of oxidised groups within the chain. In this study, we evaluated the presence of oxidised groups by analysing the carbonyl content with CCOA labelling and further studied the oxidation products.

Sample treatments (precipitation and CCOA labelling) were shown not to affect the M_w of the samples. When compared to non-labelled samples, which were analysed freshly in DMSO, the M_w of the labelled β -glucans did not differ considerably. Therefore, the labelling

process did not cause additional formation of reducing end carbonyl groups. The M_w results of BBG were similar with the ones obtained in our previous study (Mäkelä et al., 2015) and both non-oxidised BBG and OBG gave comparable M_w results to those reported by the manufacturer (500 000 g/mol compared to 495 000 g/mol for BBG and 420 000 g/mol compared to 361 000 g/mol for OBG). In OBG the oxidative degradation was shown to be significant but still less extensive than in BBG.

The carbonyl content was higher in BBG oxidised with both H_2O_2 and ascorbic acid compared to OBG. In OBG oxidised with Asc in the presence of Fe^{2+} , the carbonyl content was at lower level than shown by Kivelä et al. (2012). Furthermore, Kivelä et al. (2012) reported a 77% decrease in M_w when medium viscosity OBG was oxidised with 10 mM Asc and 0.1 mM $FeSO_4$ for 4 days compared to the 52% decrease obtained in this study for high viscosity OBG oxidised with 10 mM Asc and 1 mM $FeSO_4 \cdot 7H_2O$. Faure, Werder and Nyström (2013) have shown that increasing iron concentration will increase the formation of hydroxyl radicals in oxidation reactions of β -glucan. In the study by Kivelä et al. (2012), however, the M_w decrease was higher with lower iron concentration compared to our results. The purity of the OBG used in these studies was different (99% in medium viscosity OBG used by Kivelä et al. (2012) compared to >94% in high viscosity OBG used in this study), and thus, the impurities may have affected the oxidation reactions.

A clear difference between the oxidants H_2O_2 and Asc was seen when the amount of carbonyls in the oxidised samples were compared. Oxidation with Asc was not extensive and did not result in a significant amount of internal carbonyl groups ($c(C=O)_{tot} - c(C=O)_{reg} < 4 \mu\text{mol/g}$). On the contrary, oxidation with H_2O_2 resulted in a substantial increase in the number of reducing end and internal carbonyl groups. As shown in Table 1, with 70 mM H_2O_2 the total amount of carbonyls was higher in BBG compared to OBG (66 $\mu\text{mol/g}$ and 46 $\mu\text{mol/g}$ in BBG and OBG, respectively) but the amount of internal carbonyl groups

($c(\text{C=O})_{\text{tot}} - c(\text{C=O})_{\text{reg}}$) were relatively similar (42 $\mu\text{mol/g}$ and 37 $\mu\text{mol/g}$ in BBG and OBG, respectively). This indicated that more glycosidic bond cleavage occurred in BBG and this corresponds well with the M_w decrease. The difference between the two oxidants was in accordance to our earlier results (Mäkelä et al., 2015), which showed the formation of large aggregates in the samples oxidised with H_2O_2 but not in Asc treated samples. The aggregates may form from intermolecular interaction of β -glucan chains due to the presence of internal carbonyl groups. It has to be also noted that in our previous article (Mäkelä et al., 2015) we showed that pH of the samples was not the reason for the differences that were seen between the oxidants as the pH of the samples oxidised with H_2O_2 and Asc did not differ significantly (pH was 3.0–3.2 in all oxidised samples).

The hydroxyl radicals attack the carbohydrate chains non-selectively, and for example in the studies of oxidation of D-glucose altogether six different radical forms of glucose were detected with electron spin resonance (ESR) spectroscopy (Schuchmann & von Sonntag, 1977; von Sonntag, 1980). With polysaccharides, the oxidation can lead to elimination reactions and therefore formation of keto-groups on the anhydroglucose ring or the radical can cause degradation of the chain, depending on the position of the peroxy radical in the glucosyl unit. Our results support previous studies, which suggest that hydroxyl radicals attack different carbons on glucosyl units of the β -glucan resulting in both degradation and formation of oxidised groups within the β -glucan chain. Iurlaro et al. (2014) used tritium labelling to evaluate BBG oxidised with 1 μM CuSO_4 /10 mM H_2O_2 /10 mM Asc. Based on their study, they suggested that more mid-chain carbonyl groups were formed compared to new reducing end carbonyl groups (ratio of 3:1) during oxidation. With BBG the ratio of internal carbonyls to reducing end carbonyls were at similar level. Interestingly, for OBG the ratio was even higher, when oxidised with H_2O_2 .

4.2 Formation of formic acid and arabinose

In addition to depolymerisation and formation of carbonyl groups, the formation of formic acid and subsequent degradation of glucose molecules was also evaluated. According to the results, formic acid was formed in samples oxidised with H_2O_2 but not in samples treated with Asc. NMR analysis showed that formic acid already began to accumulate within the first few hours of oxidation with 70 mM H_2O_2 . Further evaluation of enzyme hydrolysed samples indicated that accumulation of formic acid was accompanied by the formation of arabinose in samples oxidised with H_2O_2 . The identification of arabinose was based on retention time in HPAEC-PAD analysis. Additionally, preliminary studies with gas chromatography analysis of alditol acetates of the enzyme hydrolysed samples also indicated the presence of arabinose based on retention time (data not shown).

The change in the content of formic acid and arabinose during oxidation of BBG and OBG was followed at two time points (day 1 and day 4). In addition, the glucose contents of non-oxidised and oxidised samples were compared after 4 days of oxidation. In non-oxidised samples the glucose content results were not significantly different from those reported by the manufacturer (98% compared to 94% in BBG and 93% compared to 96% in OBG). For BBG and OBG samples oxidised with H_2O_2 , the glucose contents were shown to decrease (10% and 7.5%, respectively), the decrease being more significant in BBG. This indicates that part of the glucose molecules are degraded or transformed during oxidation. However, with Asc there was no significant change in the glucose content, which further confirmed that the main oxidation pathway with Asc resulted in glycosidic cleavage. Consequently, no significant formic acid or arabinose formation was expected in the samples oxidised with Asc, which was also shown by the obtained data. In samples oxidised with H_2O_2 a significant amount of formic acid was produced and the content was dependent on the concentration of H_2O_2 and

the oxidation time. The formation of formic acid was significantly lower in OBG samples than in BBG, a trend that also correlated with the observed M_w decrease.

Jin et al. (2005) proposed an oxidation pathway where formic acid is cleaved from reducing end glucosyl units leaving an aldonic acid with one less carbon than the original aldose. The cleavage of formic acid from glucose was suggested to be sequential until six molecules of formic acid are formed from one glucose unit. In our study, however, arabinose rather than arabonic acid was observed. It has been shown that aldonic acids are decarboxylated by the action of Fenton's reagent (Larsen & Smidsrød, 1967; Stapley & BeMiller, 2007). The reaction (Ruff degradation) occurs when a complex between the aldonic acid, H_2O_2 and ferric ions (Fe^{3+}) is formed depending on the reaction conditions. Our study therefore suggests that oxidation of the β -glucan reducing end unit C1 results in formation of gluconic acid which further undergoes Ruff degradation to form arabinose and formic acid. Notably, in Asc induced oxidation, the Fe^{3+} formed during H_2O_2 decomposition is recycled back to Fe^{2+} (Guo et al., 2002) and therefore not available for Ruff degradation. Hence, this may explain why arabinose was not observed in samples oxidised with Asc.

According to the results obtained here, arabinose content increased in BBG and OBG, and the increase was dependent on the H_2O_2 concentration. Interestingly, the arabinose content was higher in OBG compared to BBG, although for the other markers of oxidation (M_w decrease, glucose decrease and formic acid content) the behaviour was contrary since the BBG seemed to oxidise more efficiently. This suggests that the formed arabinose undergoes oxidation and therefore further degrades to form lower aldoses and formic acid. The formed arabinose is most likely located in the reducing end of the oxidised β -glucan.

According to the results, OBG seemed to be less susceptible to oxidation than BBG. Our current studies show that the mineral content is higher in OBG than in BBG, and furthermore, OBG contains a 30-fold higher amount of phytic acid compared to BBG (data

not shown). This may explain the differences in the oxidation susceptibility of these β -glucans, since the phytic acid can act as an antioxidant because of its ability to bind iron (Graf & Eaton, 1990). Additionally, the results show that Asc oxidised β -glucan less extensively than H_2O_2 . In Asc initiated oxidation, the concentration of H_2O_2 (and thus also radicals) is dependent on the amount of molecular oxygen that is used for production of H_2O_2 . Therefore, the availability and solubility of oxygen may act as a rate limiting factor in Asc mediated oxidation. Additionally, this difference between Asc and H_2O_2 may be caused by the formation of hemiacetal linkages between hydroxyl groups of Asc and carbonyl groups of oxidised β -glucan. These carbonyl groups would still be measured in CCOA labelling method as described by Potthast, Rosenau, Kosma, Saariaho and Vuorinen (2005) but the interaction might decrease the amount of Asc that is free for oxidation reactions. Furthermore, Asc has been shown to oxidise in the presence of H_2O_2 (Deutsch, 1998; Grinstead, 1960), and thus, it is possible that part of the hydroxyl radicals formed in the presence of Asc are actually consumed to oxidise Asc instead of β -glucan. More studies should be done to clarify the differences in the oxidation pathways of these two oxidants and the underlying factors.

5 CONCLUSIONS

The current study shows that depending on the oxidation conditions, several oxidation pathways occur in oxidation of cereal β -glucan. When compared to H_2O_2 , the oxidation with ascorbic acid mainly resulted in glycosidic bond cleavage. However, with H_2O_2 , the formation of carbonyl groups within the chain was significantly more than the formation of reducing end carbonyl groups. Nonetheless, with H_2O_2 this study for the first time evidences the formation of formic acid during β -glucan oxidation with the consequential formation of arabinose, most-likely at the reducing ends of the oxidised β -glucan chains. The more

significant oxidation of β -glucan with H_2O_2 compared to oxidation with ascorbic acid possibly results from a higher initial amount of available hydroxyl radicals during H_2O_2 oxidation. The occurrence of these random non-specific oxidation reactions during food processing and storage affect both the technological and physiological functionality of cereal β -glucans.

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