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

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 $(1\rightarrow3)(1\rightarrow4)$-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 $(1\rightarrow4)$-β-D-linkages. These blocks are attached with $(1\rightarrow3)$-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 (·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

26   viscosity (Kivelä, Henniges, Sontag-Strohm, & Potthast, 2012; Mäkelä, Sontag-Strohm, &

27   Maina, 2015). The radical-mediated oxidation is non-selective and depending on the carbon

28   atom that is attacked it can result in glycosidic bond cleavage, formation of carbonyl groups

29   along the polysaccharide chain and glucose ring opening and fragmentation (Schuchmann &

30   von Sonntag, 1977; von Sonntag, 1980). Faure, Sánchez-Ferrer, Zabara, Andersen and

31   Nyström, (2014) studied the oxidation of barley β-glucan at elevated temperature (85˚C) and

32   showed fast degradation (molar mass non-detectable with light scattering after 2 h of

33   oxidation) and formation of new carbonyl-based functional groups. In addition, their results

34   suggested β-(1→3) glycosidic linkage to be more prone to cleavage than β-(1→4) linkage.

35   Our previous study showed that in addition to depolymerisation, some aggregates

36   were formed during oxidation, as shown in asymmetric flow field-flow fractionation

37   (AsFlFFF) analysis (Mäkelä et al., 2015). The aggregates were few (3–6%) and were

38   hypothesised to possibly result from inter-chain cross-linking due the reaction of β-glucan

39   hydroxyl groups with carbonyl groups that were formed during oxidation. Bamford and

40   Collins (1950) studied the oxidation of glucose under alkaline conditions and showed that

41   glucose decomposed to formic acid and arabonic acid. Formic acid has also been shown to

42   form due to oxidation in acidic conditions (Jin et al., 2005; Robert, Barbati, Ricq, &

43   Ambrosio, 2002). In this study it was therefore hypothesised that the main reaction pathway

44   during oxidation of cereals β-glucans is glycosidic bond cleavage and the subsequent

45   formation of new reducing ends. The reducing ends formed are further susceptible to

46   oxidation, which could lead to sequential degradation of the reducing end glucosyl units to

47   form formic acid and new monosaccharide units at the reducing end. The aim of this study

48   was therefore firstly to evaluate depolymerisation and formation of carbonyl groups during

49   the oxidation of β-glucan with either H_2O_2 or Asc at different concentrations. Secondly, the

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

51   determined.

52

53   **2 MATERIALS AND METHODS**

54   **2.1 Preparation and oxidation of sample solutions**

55   Barley β-glucan (high viscosity, purity >94%, the weight average molar mass (M_w) 495 000

56   g/mol) and oat β-glucan (high viscosity, purity >94%, M_w 361 000 g/mol) were purchased

57   from Megazyme (Ireland). 0.7% (w/v) Barley and oat β-glucan solutions (BBG and OBG,

58   respectively) were prepared by wetting the sample with 99.5% ethanol (Altia, Finland) prior

59   to dissolution with MilliQ water (Millipore system, Merck Millipore, Germany).

60   Consequently, the samples were kept at 85°C for 2 h with continuous stirring. After 2 h, the

61   samples were kept stirring for an hour at room temperature.

62   Oxidation reactions were initiated with 10 mM, 40 mM or 70 mM H_2O_2 (30 %

63   hydrogen peroxide, Merck, Germany) or Asc (L(+)-ascorbic acid, AnalaR NORMAPUR®,

64   VWR Chemicals, Belgium). For all solutions, 1 mM iron (II) sulphate heptahydrate

65   ($FeSO_4·7H_2O$) (Merck, Germany) was also added, and the final β-glucan concentration in the

66   solutions was adjusted to 5.6 mg/ml with MilliQ water. A non-oxidised control sample

67   diluted to the same concentration with MilliQ water was used for comparison. All reactions

68   were carried out at room temperature.

69

70   **2.2 Carbonyl content and molar mass analyses**

71   The molar mass of non-labelled samples were analysed using high performance size

72   exclusion chromatography (HPSEC) in 0.01 M LiBr/DMSO. The analysis was done as

73   specified by Mäkelä et al. (2015) and the data analysed using OmniSEC software as described

74   by Fishman, Doner, Chau and Hoagland (2000). Carbonyl content analysis with simultaneous

75   molar mass analysis of the labelled molecules was done according to Röhrling et al. (2002a,

76   b) with some modifications. The 4 day oxidised samples were precipitated with ethanol to

77   remove the oxidation reagents. After centrifugation, the samples were washed twice with

78   ethanol and finally dried at room temperature. For carbonyl analysis, 10 mg samples and 20

79   mg standards (six cellulose samples with known carbonyl content (Potthast et al., 2015)) were

80   labelled with CCOA (carbazole-9-carbonyloxyamine). The samples were activated by

81   wetting them with MilliQ and rinsed with 96% ethanol on a filter paper. The activated

82   samples were suspended into 1 ml of 0.9% LiCl in DMAc and the suspensions were shaken

83   overnight at room temperature. 2 ml of 1.25 mg/ml CCOA in acetate buffer (pH 4) was added

84   to each sample and the samples were shaken at 40˚C for 7 days. After labelling the samples

85   were centrifuged and the precipitates were dissolved in 1 ml of 0.9% LiCl in DMAc. The

86   samples were filtered (0.45 μm) prior to analysis.

87   The HPSEC system included a fluorescence detector (FL3000, Thermo Scientific,

88   USA) for monitoring the CCOA label (λ_{ex} = 290 nm and λ_{em} = 340 nm) a MALLS detector

89   (Wyatt Dawn DSP, Wyatt Technology, USA) with an argon ion laser ($\lambda 0$ = 488 nm), and a

90   refractive index detector (Shodex RI-71, Japan). Four serial PLgel-mixed ALS (7.5 mm x 300

91   mm) columns (Agilent, Germany) were used. In addition, the system consisted of a degasser

92   (Dionex DG-2410, Thermo Scientific, USA), an autosampler (1100, Agilent, Germany), a

93   pulse damper pump, and a column oven (STH 585, Gynkotek, Germany) at 25°C. The

94   operating conditions of the HPSEC were as follows: the flow rate was 1.00 ml/min, the

95   injection volume 100 μl, the run time 45 min. DMAc/LiCl (0.9%, w/v) after filtering through

96   a 0.02 μm filter was used as eluent. The *dn/dc* value of 0.136 was used. The MALLS data

97   were evaluated with Astra software (Wyatt Technology, USA) using the first-order Zimm fit.

98   The carbonyl group content was analysed using Chromeleon and GRAMS/32 software

99   (Thermo Scientific, USA).

100

101   The total carbonyl group amounts, $c(C=O)_{tot}$, are reported in µmol/g. The equivalent

102   amount of reducing end groups, $c(C=O)_{reg}$, was determined from the number average molar

103   mass (M_n) using Equation 1.

104    $c(C=O)_{reg} = \dfrac{1}{M_n} \times 10^6$ µmol/g   (Equation 1)

105

106   **2.3 Formic acid analysis**

107   *2.3.1 NMR spectroscopy analysis*

108   Formation of formic acid was detected with nuclear magnetic resonance (NMR) spectroscopy

109   analysis using barley β-glucan oxidised with 70 mM H_2O_2/Asc and 1 mM $FeSO_4 \cdot 7H_2O$.

110   NMR analysis was carried out on a 600 MHz Bruker Avance III NMR spectrometer (Bruker

111   BioSpin, Germany) equipped with a QCI cryoprobe. The measurements were performed at

112   22°C. For the analysis, 7 mg/ml of sample was dissolved in D_2O and the oxidation reaction

113   was initiated before NMR analysis. 1D ^1H experiments were carried out at several time points

114   using Bruker 1D NOESY with pre-saturation and spoil gradients pulse program

115   (noesygppr1d) where the residual water signal is suppressed by 4 s volume selective pre-

116   saturation.

117

118   *2.3.2 Formic acid content analysis*

119   The formic acid (FA) content of non-oxidised and oxidised samples was measured at two

120   time points of oxidation (24 hours and 4 days) using the Megazyme Formic acid assay

121   (Ireland). In the analysis formate dehydrogenase (FDH) and NAD^+ are added to the sample

122   and the formation of NADH in Reaction 1 is followed spectrophotometrically.

123

124    $FA + NAD^+ \xrightarrow{FDH} CO_2 + NADH + H^+$ (Reaction 1)
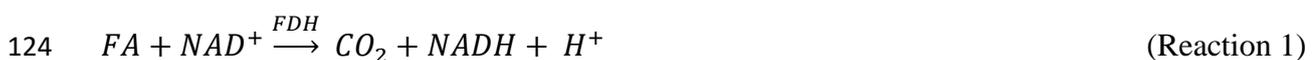
125

126   The samples were prepared and analysed according to the assay instructions. The pH

127 of the oxidised samples was neutralised by adding an appropriate amount of 0.1 M NaOH to

128 the sample. For the non-oxidised samples, the same volume of MilliQ was added to equalise

129 the concentration of the samples.

130   Since the formic acid analysis is based on the spectrophotometric measurement of

131 NADH, which is formed in Reaction 1, Asc was suspected to affect the reaction since it may

132 act as a reducing agent. Thus, the effect of ascorbic acid (10 mM, 40 mM and 70 mM Asc

133 including 1 mM $FeSO_4 \cdot 7H_2O$) was tested with reagent blank samples. The results of these

134 reagent blanks were subtracted from the results of the samples oxidised with ascorbic acid.

135

136 **2.4 Arabinose and glucose content**

137 *2.4.1 Preparation of samples*

138 For monosaccharide analyses the non-oxidised and oxidised samples (5.6 mg/ml) were

139 enzymatically hydrolysed. 60 μl of 50 U/ml lichenase (endo-(1-3)(1-4)-β-D-glucan 4-

140 glucanohydrolase, Megazyme, Ireland) and 40 μl of 2 U/ml β-glucosidase (Megazyme,

141 Ireland) were added to 200 μl of sample and the sample was simultaneously diluted to 2.8

142 mg/ml with MilliQ. The samples were incubated for 24 h at 40˚C before inactivating the

143 enzymes by boiling the samples for 10 min. The samples were stored at -20˚C until glucose

144 and arabinose were analysed.

145   The samples were diluted appropriately for glucose and arabinose analysis using

146 glucose (0.005–0.200 mg/ml) and arabinose (0.002–0.100 mg/ml) standards. For both

147 standards and samples, deoxygalactose (0.05 mg/ml) was added as an internal standard. The

148 glucose and arabinose contents of the samples were calculated as percentages of the

149 theoretical monosaccharide (glucose) amount in the native sample.

150

2.4.2 Monosaccharide analysis in HPAEC-PAD

Samples were filtered (0.45 µm) 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 (50×4 mm, Dionex Corporation, USA), analytical column CarboPac

PA-1 (250×4 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 ± 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

175   prior to statistical analysis because of the 10-fold differences in the values. Differences were

176   considered as significant at P<0.05.

177

178   **3 RESULTS**

179   **3.1 Carbonyl content of the oxidised beta-glucans**

180   To ensure that the molar mass was not changing due to precipitation or the CCOA labelling

181   processes, the molar mass of freshly prepared samples was also analysed. As shown in table

182   1, the M_w was similar for both the fresh sample and labelled samples indicating CCOA

183   labelling did not cause further degradation of the samples. The M_w of both BBG and OBG

184   decreased due to oxidation even though there were differences in the extent of degradation.

185   The M_w decrease of non-labelled BBG was 86% (from 500 000 g/mol to 69 000 g/mol) and

186   50% (from 500 000 g/mol to 250 000 g/mol) when oxidised with 70 mM H_2O_2 and 70 mM

187   Asc, respectively. The corresponding percentages for non-labelled OBG were 52% (from

188   420 000 g/mol to 200 000 g/mol) for both oxidants.

189   The CCOA method applied covers all aldehyde and keto groups present. If the

190   oxidation is accompanied by degradation also the number of reducing end groups increases.

191   The combination with SEC-MALLS allows to calculate those newly formed reducing ends

192   from the number average molar mass (M_n). The difference to the total amount of carbonyl

193   groups hence corresponds to the groups introduced by oxidative processes (*cf.* Table 1). The

194   carbonyl content was shown to increase due to oxidation and the increase was more

195   significant in samples oxidised with H_2O_2 than in those oxidised with Asc (Table 1). In both

196   BBG and OBG samples the carbonyl content increased only 2–4-fold when oxidised with

197   Asc but with different concentrations of H_2O_2 the increase was 17–28-fold and 9–14-fold in

198   BBG and OBG, respectively (Table 1, Figure 1 a-f). In BBG and OBG samples oxidised with

199   70 mM H_2O_2 (Figure 1 c, d) a shoulder was seen in LS signal in the area where larger

200   molecules elute indicating some aggregation in the samples.

201

202   **Table 1.** Molar masses (number average molar mass = M_n, weight average molar mass = M_w,

203   Z-average molar mass = M_z), total carbonyl content ($c(C=O)_{tot}$) and equivalent concentration

204   of reducing end groups ($c(C=O)_{reg}$) of non-oxidised and oxidised BBG and OBG analysed

205   after 4 days of oxidation at room temperature. The oxidation was initiated with different

206   concentrations (10/40/70 mM) of H_2O_2 or ascorbic acid (Asc) and 1 mM $FeSO_4 \cdot 7H_2O$ as a

207   catalyst.

| Sample	Treatment	Fresh samples[a]			Labelled samples[b]				
		M_n (x10³ g/mol)	M_w (x10³ g/mol)	M_z (x10³ g/mol)	M_n (x10³ g/mol)	M_w (x10³ g/mol)	M_z (x10³ g/mol)	$c(C=O)_{tot}$ (µmol/g)[c]	$c(C=O)_{reg}$ (µmol/g)[d]
BBG	Non-oxidised	440	500	560	460	560	660	2.4	2.2
	10 mM H_2O_2	100	150	310	97	160	270	40	10
	40 mM H_2O_2	65	98	190	67	120	320	57	15
	70 mM H_2O_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_2O_2	140	190	340	150	200	260	31	6.6
	40 mM H_2O_2	150	210	420	150	230	320	40	6.6
	70 mM H_2O_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.

208

209
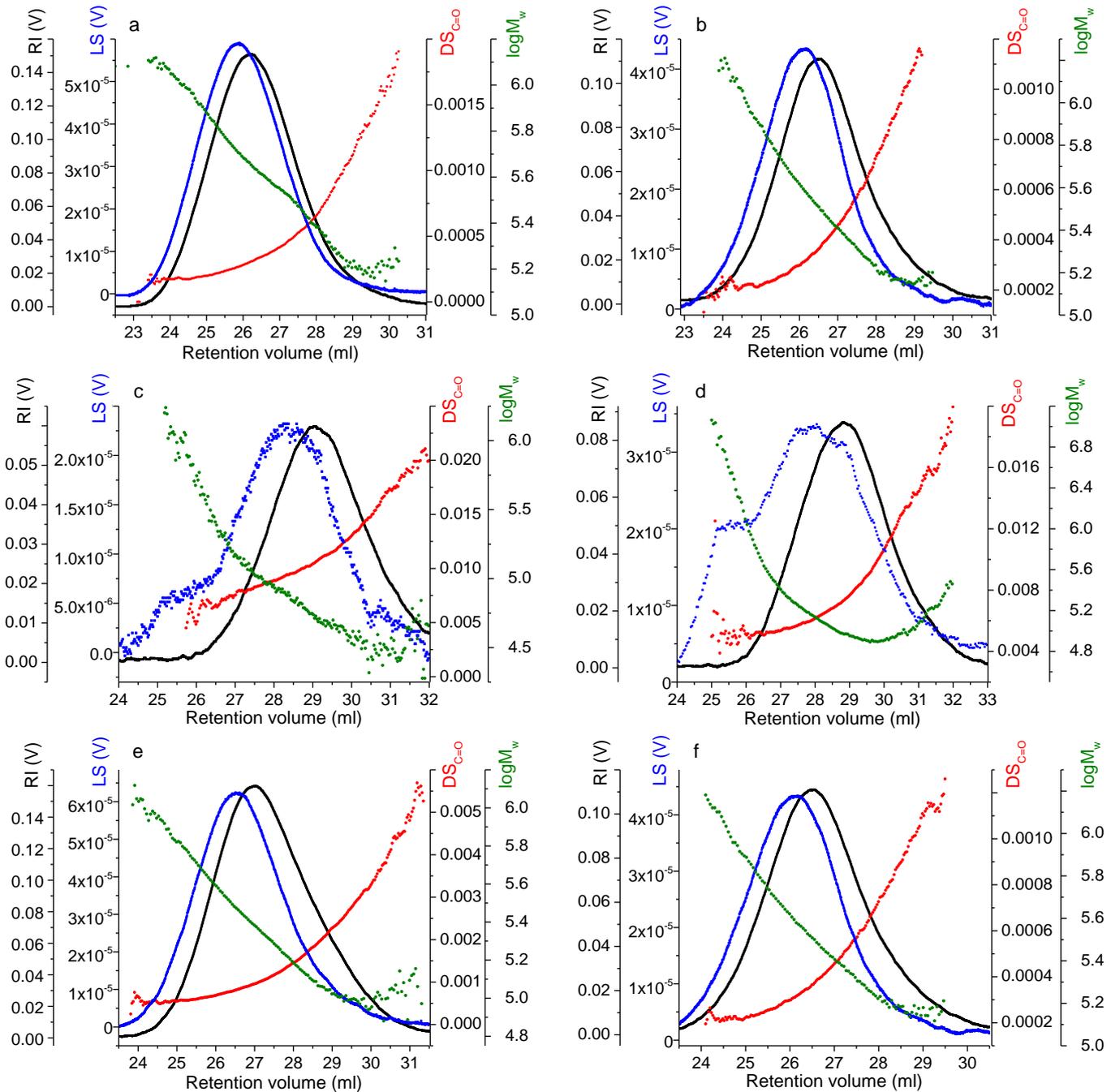
Figure 1. HPSEC chromatograms of barley (a, c, e) and oat (b, d, f) β-glucan before

oxidation (a, b) and after 4 days of oxidation with 70 mM H_2O_2 (c, d) and 70 mM ascorbic

acid (e, f). 1 mM $FeSO_4 \cdot 7H_2O$ was used as a catalyst in oxidised samples. Refractive index

(RI) peak is shown with black line, light scattering (LS) peak with blue line, logarithmic

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

215   with red line.

216

3.2 Formation of formic acid in oxidation of β-glucan

218   The 1D ^{1}H spectrum of non-oxidised BBG is shown in Figure 2a. Figure 2b and 2c show

219   BBG oxidised with 70 mM H_2O_2 and 1 mM $FeSO_4 \cdot 7H_2O$ as a catalyst for 2 and 3 hours,

220   respectively. In the spectra, the β-(1→4) and β-(1→3) anomeric signals occur at δ 4.53 ppm

221   and δ 4.78 ppm, respectively. The β-(1→3) signal is partially suppressed due to its close

222   proximity to the suppressed water signal. According to analysis, a peak at δ 8.23 ppm, which

223   was assigned to formic acid, was observed in the H_2O_2 oxidised sample (Figure 2). Neither

224   the control non-oxidised sample (Figure 2a) nor sample oxidised with Asc (data not shown)

225   showed the presence of formic acid. Formic acid accumulated with oxidation time as shown

226   in Figure 2b and 2c, which were evaluated after 2 and 3 hours of oxidation respectively.
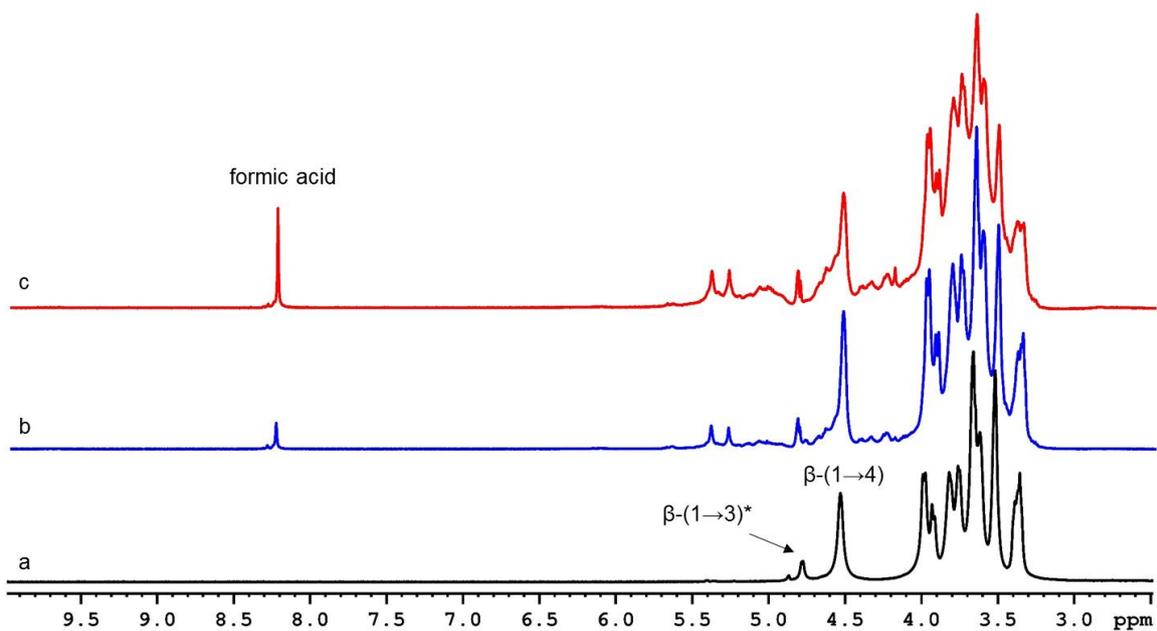

Figure 2. The 1D ^{1}H spectrum of barley β-glucan showing the formation of formic acid

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

231   H_2O_2 and 1 mM $FeSO_4\cdot 7H_2O$ as a catalyst after 3 hours. The spectra were recorded at 600

232   MHz in D_2O at 22°C. Peaks are referenced to internal acetone (^1H = 2.225 ppm). * In the

233   experiment, the anomeric proton of the β-(1→3)-linked glucosyl units is suppressed due to

234   close proximity to the suppressed residual water signal.

235

236   The formic acid content of the samples, measured at two time points (day 1 and day 4),

237   indicated that formic acid accumulated with oxidation time in samples oxidised with H_2O_2

238   (Figure 3). In non-oxidised samples and in the OBG samples oxidised with Asc, the formic

239   acid content was negligible at both time points. The formic acid concentration was

240   significantly higher in BBG samples than in OBG samples after 4 days of oxidation, the

241   difference being about 4-fold in samples oxidised with 70 mM H_2O_2 for 4 days (13.6 mg/g of

242   β-glucan in BBG compared to 3.3 mg/g of β-glucan in OBG). In OBG samples oxidised with

243   Asc there was no formic acid formed (concentrations <0.7 mg/g of β-glucan) and in BBG

244   samples the amount was minimal (varying from 0.87 to 1.50 mg/g of β-glucan).
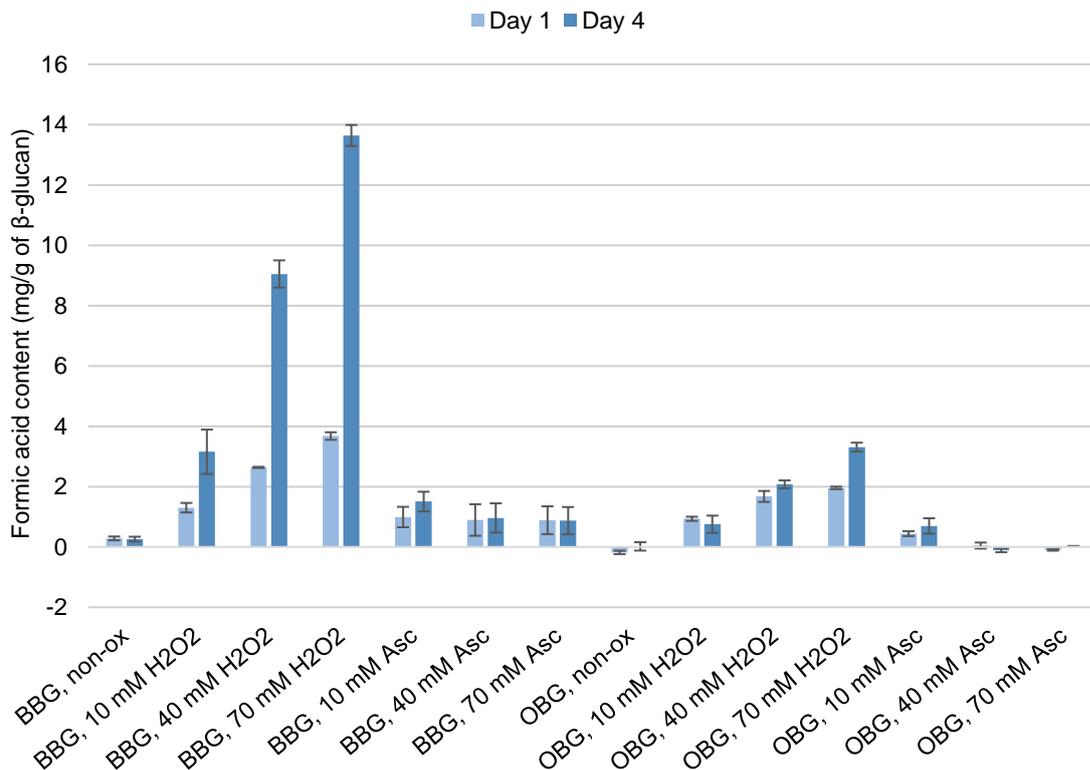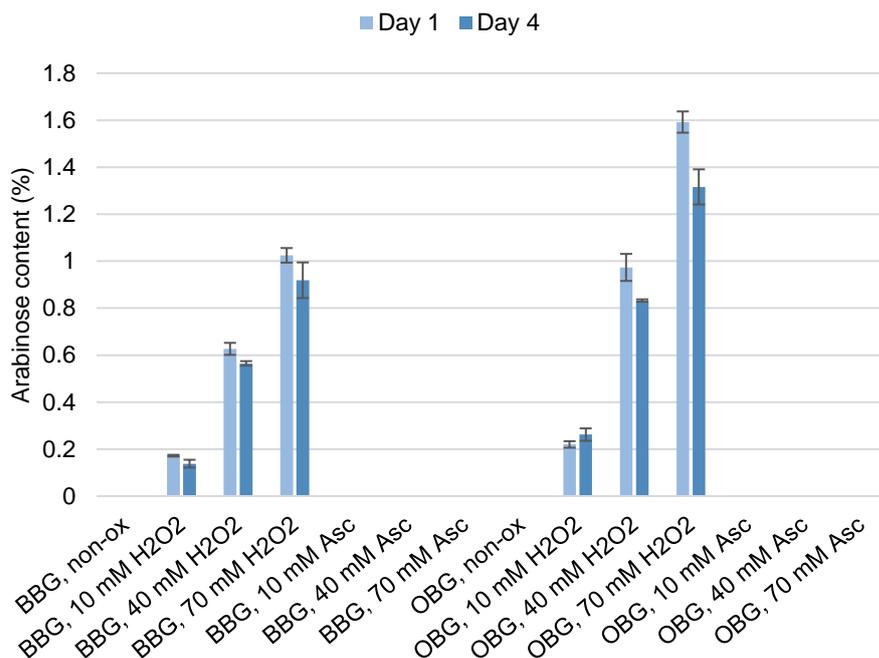

245

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 H_2O_2 or ascorbic acid

249 (Asc) and 1 mM $FeSO_4 \cdot 7H_2O$ 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 H_2O_2, 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 H_2O_2 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 H_2O_2, 40 mM H_2O_2 and 70 mM

261 H_2O_2 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 H_2O_2 (from 0.99% to 0.85% in BBG samples
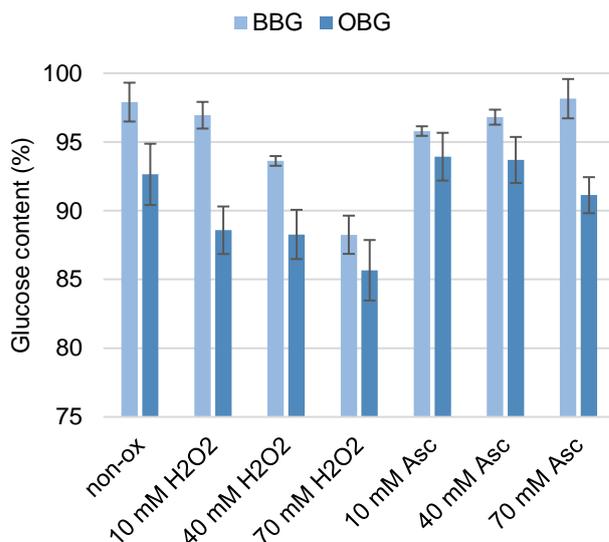
265 and from 1.54% to 1.30% in OBG samples).

266

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 H_2O_2 or ascorbic acid (Asc) and 1 mM $FeSO_4 \cdot 7H_2O$ as a catalyst.

Glucose contents of non-oxidised BBG and OBG were 98% and 93%, respectively

(Figure 5). When oxidised with 70 mM H_2O_2 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 H_2O_2 or ascorbic acid (Asc) and 1 mM $FeSO_4 \cdot 7H_2O$ 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 AsFlFFF analysis where single oxidised molecules and aggregates were separated (Mäkelä et al., 2015). A small portion of the samples oxidised with H_2O_2 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

295   process did not cause additional formation of reducing end carbonyl groups. The M_w results

296   of BBG were similar with the ones obtained in our previous study (Mäkelä et al., 2015) and

297   both non-oxidised BBG and OBG gave comparable M_w results to those reported by the

298   manufacturer (500 000 g/mol compared to 495 000 g/mol for BBG and 420 000 g/mol

299   compared to 361 000 g/mol for OBG). In OBG the oxidative degradation was shown to be

300   significant but still less extensive than in BBG.

301   The carbonyl content was higher in BBG oxidised with both H_2O_2 and ascorbic acid

302   compared to OBG. In OBG oxidised with Asc in the presence of Fe^{2+}, the carbonyl content

303   was at lower level than shown by Kivelä et al. (2012). Furthermore, Kivelä et al. (2012)

304   reported a 77% decrease in M_w when medium viscosity OBG was oxidised with 10 mM Asc

305   and 0.1 mM FeSO₄ for 4 days compared to the 52% decrease obtained in this study for high

306   viscosity OBG oxidised with 10 mM Asc and 1 mM FeSO₄·7H₂O. Faure, Werder and

307   Nyström (2013) have shown that increasing iron concentration will increase the formation of

308   hydroxyl radicals in oxidation reactions of β-glucan. In the study by Kivelä et al. (2012),

309   however, the M_w decrease was higher with lower iron concentration compared to our results.

310   The purity of the OBG used in these studies was different (99% in medium viscosity OBG

311   used by Kivelä et al. (2012) compared to >94% in high viscosity OBG used in this study),

312   and thus, the impurities may have affected the oxidation reactions.

313   A clear difference between the oxidants H_2O_2 and Asc was seen when the amount of

314   carbonyls in the oxidised samples were compared. Oxidation with Asc was not extensive and

315   did not result in a significant amount of internal carbonyl groups ($c(C=O)_{tot} - c(C=O)_{reg} < 4$

316   μmol/g). On the contrary, oxidation with H_2O_2 resulted in a substantial increase in the

317   number of reducing end and internal carbonyl groups. As shown in Table 1, with 70 mM

318   H_2O_2 the total amount of carbonyls was higher in BBG compared to OBG (66 μmol/g and 46

319   μmol/g in BBG and OBG, respectively) but the amount of internal carbonyl groups

320 $(c(C=O)_{tot} - c(C=O)_{reg})$ were relatively similar (42 μmol/g and 37 μmol/g in BBG and OBG,

321 respectively). This indicated that more glycosidic bond cleavage occurred in BBG and this

322 corresponds well with the M_w decrease. The difference between the two oxidants was in

323 accordance to our earlier results (Mäkelä et al., 2015), which showed the formation of large

324 aggregates in the samples oxidised with H_2O_2 but not in Asc treated samples. The aggregates

325 may form from intermolecular interaction of β-glucan chains due to the presence of internal

326 carbonyl groups. It has to be also noted that in our previous article (Mäkelä et al., 2015) we

327 showed that pH of the samples was not the reason for the differences that were seen between

328 the oxidants as the pH of the samples oxidised with H_2O_2 and Asc did not differ significantly

329 (pH was 3.0–3.2 in all oxidised samples).

330   The hydroxyl radicals attack the carbohydrate chains non-selectively, and for example

331 in the studies of oxidation of D-glucose altogether six different radical forms of glucose were

332 detected with electron spin resonance (ESR) spectroscopy (Schuchmann & von Sonntag,

333 1977; von Sonntag, 1980). With polysaccharides, the oxidation can lead to elimination

334 reactions and therefore formation of keto-groups on the anhydroglucose ring or the radical

335 can cause degradation of the chain, depending on the position of the peroxyl radical in the

336 glucosyl unit. Our results support previous studies, which suggest that hydroxyl radicals

337 attack different carbons on glucosyl units of the β-glucan resulting in both degradation and

338 formation of oxidised groups within the β-glucan chain. Iurlaro et al. (2014) used tritium

339 labelling to evaluate BBG oxidised with 1 μM $CuSO_4$/10 mM H_2O_2/10 mM Asc. Based on

340 their study, they suggested that more mid-chain carbonyl groups were formed compared to

341 new reducing end carbonyl groups (ratio of 3:1) during oxidation. With BBG the ratio of

342 internal carbonyls to reducing end carbonyls were at similar level. Interestingly, for OBG the

343 ratio was even higher, when oxidised with H_2O_2.

344

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

369   the oxidation time. The formation of formic acid was significantly lower in OBG samples

370   than in BBG, a trend that also correlated with the observed M_w decrease.

371   Jin et al. (2005) proposed an oxidation pathway where formic acid is cleaved from

372   reducing end glucosyl units leaving an aldonic acid with one less carbon than the original

373   aldose. The cleavage of formic acid from glucose was suggested to be sequential until six

374   molecules of formic acid are formed from one glucose unit. In our study, however, arabinose

375   rather than arabonic acid was observed. It has been shown that aldonic acids are

376   decarboxylated by the action of Fenton's reagent (Larsen & Smidsrød, 1967; Stapley &

377   BeMiller, 2007). The reaction (Ruff degradation) occurs when a complex between the aldonic

378   acid, H_2O_2 and ferric ions (Fe^{3+}) is formed depending on the reaction conditions. Our study

379   therefore suggests that oxidation of the β-glucan reducing end unit C1 results in formation of

380   gluconic acid which further undergoes Ruff degradation to form arabinose and formic acid.

381   Notably, in Asc induced oxidation, the Fe^{3+} formed during H_2O_2 decomposition is recycled

382   back to Fe^{2+} (Guo et al., 2002) and therefore not available for Ruff degradation. Hence, this

383   may explain why arabinose was not observed in samples oxidised with Asc.

384   According to the results obtained here, arabinose content increased in BBG and OBG,

385   and the increase was dependent on the H_2O_2 concentration. Interestingly, the arabinose

386   content was higher in OBG compared to BBG, although for the other markers of oxidation

387   (M_w decrease, glucose decrease and formic acid content) the behaviour was contrary since the

388   BBG seemed to oxidise more efficiently. This suggests that the formed arabinose undergoes

389   oxidation and therefore further degrades to form lower aldoses and formic acid. The formed

390   arabinose is most likely located in the reducing end of the oxidised β-glucan.

391   According to the results, OBG seemed to be less susceptible to oxidation than BBG.

392   Our current studies show that the mineral content is higher in OBG than in BBG, and

393   furthermore, OBG contains a 30-fold higher amount of phytic acid compared to BBG (data

394   not shown). This may explain the differences in the oxidation susceptibility of these β-

395   glucans, since the phytic acid can act as an antioxidant because of its ability to bind iron

396   (Graf & Eaton, 1990). Additionally, the results show that Asc oxidised β-glucan less

397   extensively than H_2O_2. In Asc initiated oxidation, the concentration of H_2O_2 (and thus also

398   radicals) is dependent on the amount of molecular oxygen that is used for production of

399   H_2O_2. Therefore, the availability and solubility of oxygen may act as a rate limiting factor in

400   Asc mediated oxidation. Additionally, this difference between Asc and H_2O_2 may be caused

401   by the formation of hemiacetal linkages between hydroxyl groups of Asc and carbonyl

402   groups of oxidised β-glucan. These carbonyl groups would still be measured in CCOA

403   labelling method as described by Potthast, Rosenau, Kosma, Saariaho and Vuorinen (2005)

404   but the interaction might decrease the amount of Asc that is free for oxidation reactions.

405   Furthermore, Asc has been shown to oxidise in the presence of H_2O_2 (Deutsch, 1998;

406   Grinstead, 1960), and thus, it is possible that part of the hydroxyl radicals formed in the

407   presence of Asc are actually consumed to oxidise Asc instead of β-glucan. More studies

408   should be done to clarify the differences in the oxidation pathways of these two oxidants and

409   the underlying factors.

410

411   **5 CONCLUSIONS**

412   The current study shows that depending on the oxidation conditions, several oxidation

413   pathways occur in oxidation of cereal β-glucan. When compared to H_2O_2, the oxidation with

414   ascorbic acid mainly resulted in glycosidic bond cleavage. However, with H_2O_2, the

415   formation of carbonyl groups within the chain was significantly more than the formation of

416   reducing end carbonyl groups. Nonetheless, with H_2O_2 this study for the first time evidences

417   the formation of formic acid during β-glucan oxidation with the consequential formation of

418   arabinose, most-likely at the reducing ends of the oxidised β-glucan chains. The more

419 significant oxidation of β-glucan with H_2O_2 compared to oxidation with ascorbic acid

420 possibly results from a higher initial amount of available hydroxyl radicals during H_2O_2

421 oxidation. The occurrence of these random non-specific oxidation reactions during food

422 processing and storage affect both the technological and physiological functionality of cereal

423 β-glucans.

424

429

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