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Modulating the structural properties of β-D-glucan degradation products by alternative reaction pathways

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ABSTRACT

The aim of the present study was to compare the degradation of β-D-glucan induced by hydroxyl radical to the degradation induced by heat treatment. β-D-Glucan was quickly and widely degraded by the action of hydroxyl radicals produced by a Fenton system at 85 °C, while thermal hydrolysis at 85 °C induced slow β-D-glucan depolymerization. The hydroxyl radical-induced degradation of β-D-glucan was accompanied by the formation of peroxyl radicals and new oxidized functional groups (*i.e.* lactones, carboxylic acids, ketones and aldehydes), as detected by ESR and NMR, respectively. In contrast, no changes in the monomer chemical structure of β-D-glucan were observed upon thermal hydrolysis. Therefore, different mechanisms are proposed for the oxidative cleavage of β-D-glucan, which are initiated by the presence of an unpaired electron on the anomeric carbon.

1. Introduction

Cereal (1→3),(1→4)-β-D-glucan, hereafter referred to as β-D-glucan, is a water soluble fiber occurring in the endospermic and aleuronic cell walls of barley and oat (Lazaridou & Biliaderis, 2007). It is a homopolysaccharide composed of blocks of consecutive β-(1→4) linked D-glucopyranosyl segments separated by single β-(1→3)-linkages (Lazaridou & Biliaderis, 2007). The β-D-glucan chain is mainly (90%) constituted of cellotriosyl and cellotetraosyl blocks linked by single (1→3)-linkages, however blocks of four to fifteen consecutive β-(1→4) linked glucose residues can also occur (Wood, 2010). In the last years, β-D-glucan has received significant interest from the scientific community due to its beneficial effects on human health, where it has been attributed the capacity to attenuate postprandial blood glucose, insulin level, as well as serum cholesterol (Braaten et al., 1994; Wood, 2004; Wood et al., 1994). These functionalities have been closely related to the ability of β-D-glucan of forming highly viscous solutions, a feature which is directly linked to its concentration in solution and molecular weight (Wood, 2007, 2010). However, β-D-glucan in solution can easily be subjected to molecular breakdown, due to different occurring processes, which in turn could modify the effectiveness of its viscosity-related health benefits.

Polysaccharides, including β-D-glucan can be oxidized by the hydroxyl radical (•OH) produced through the Fenton reaction (Eq. (1)).

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad (1)$$

Recently, it has been demonstrated that the exposure of β-D-glucan solutions to •OH generating systems such as H_2O_2 and Fe^{2+} or ascorbic acid (AH_2) and Fe^{2+} causes the viscosity loss of β-D-glucan solution and implicitly its degradation (de Moura et al., 2011; Faure, Andersen, & Nyström, 2012; Faure, Münger, & Nyström, 2012; Kivelä, Gates, & Sontag-Strohm, 2009; Kivelä, Nyström, Salovaara, & Sontag-Strohm, 2009; Kivelä, Sontag-Strohm, Loponen, Tuomainen, & Nyström, 2011). In our previous work, we have explored thoroughly the relation between AH_2/Fe^{2+} induced β-D-glucan degradation and •OH formation, and conclusively confirmed that the degradation of β-D-glucan was directly related to •OH formation in the system (Faure, Andersen, et al., 2012). The formation of hydroxyl radical is also promoted by the mere presence of iron by a series of reactions, which starts with the iron(II) autoxidation (Eq. (2)), followed by the self-dismutation of the superoxide radical ($O_2^{\bullet -}$) (Eq. (3)), and finally the formation of •OH *via* the Fenton reaction (Eq. (1)). The introduction of H_2O_2 in these systems amplifies the formation of •OH.

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet -} \quad (2)$$

Abbreviations: H_2O_2, hydrogen peroxide; •OH, hydroxyl radical.

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$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

The hydroxyl radical (•OH) attacks biomolecules such as carbohydrates in their direct vicinity at diffusion-controlled rates in a non-selective way. These radicals can rapidly oxidize polysaccharides by randomly abstracting carbon-bounded hydrogen atoms leading to the formation of carbon centered radicals (von Sonntag, 1980). Subsequently, the carbon centered radicals can react with O_2 to form peroxyl radicals ($R–O_2^{\bullet}$) which may further undergo a superoxide radical ($O_2^{\bullet -}$) elimination, resulting in the formation of carbonyl groups (von Sonntag, 1980) (C=O). Alternatively, peroxyl radicals formed in the proximity of glycosidic bonds may also induce cleavages that involves alkoxyl radical fragmentations (Schuchmann & von Sonntag, 1978).

Kivelä, Henniges, Sontag-Strohm, and Potthast (2012) have recently observed the formation of carbonyl groups in β-D-glucan solutions treated thermally in the presence of AH_2 and Fe^{2+}, and related these findings to an extensive degradation of the fiber (Kivelä et al., 2012). Furthermore, de Moura et al. (2011) have reported that applying a Fenton system (H_2O_2/Fe^{2+}) to a β-D-glucan solution promotes the generation of carbonyl and carboxyl functions (de Moura et al., 2011). Moreover, the content of carboxyl and carbonyl groups was shown to increase with increasing H_2O_2 concentration, and this was related with the degree of viscosity loss of the β-D-glucan solution which increased with H_2O_2 concentration (de Moura et al., 2011). Both studies demonstrated that oxidative treatments of β-D-glucan lead to structural changes of the fiber, such as the introduction of carbonyl groups, which in turn trigger its degradation. However, the mechanism of scission of the polysaccharide chain, which occurs subsequent to the formation of carbonyl groups, is still poorly understood. Hence, a physicochemical characterization of the oxidized fragments of β-D-glucan would provide valuable information, and an extensive understanding of the oxidative cleavage mechanism.

Alongside with oxidation, β-D-glucan in solution can be degraded by hydrolysis. Hydrolysis of polysaccharides is highly dependent on the pH media, since the process is catalyzed by H^+ and OH^-. Previous studies have reported that β-D-glucan is hydrolyzed in acidic media, and favored at elevated temperature (Johansson et al., 2006; Kivelä, Nyström, et al., 2009; Vaikousi & Biliaderis, 2005). Compared to the radical mediated degradation, β-D-glucan hydrolysis is considered specific because it leads to the cleavage of the glycosidic bonds, and thereby to the depolymerization of β-D-glucan. For instance, it has been shown that treatment with high acid concentrations at 120 °C leads to a total hydrolysis of β-D-glucan to glucose monomers (Johansson et al., 2006). However, moderate acidic treatments may simply lead to partial hydrolysis of the β-D-glucan which would result in the release of lower molecular weight β-D-glucan products with similar structural characteristics (Vaikousi, Biliaderis, & Izydorczyk, 2004).

As mentioned earlier, information about the mechanism of β-D-glucan oxidative cleavage induced by •OH are scarce. Therefore, in the present study the •OH-mediated degradation of β-D-glucan at elevated temperature in presence of the Fenton system (H_2O_2/Fe^{2+}) as •OH generator has been thoroughly characterized. For this purpose, we monitored the formation of •OH using ESR spin trapping in β-D-glucan solution, the viscosity loss of the solutions, as well as the molecular weight changes. Additionally, direct ESR detection was used to investigate the formation of radicals in the β-D-glucan chain. The structural changes occurring during the oxidative treatment were followed with FTIR and NMR. All results are compared to those obtained for mildly acidic hydrolysis at elevated temperature with the aim of clarifying the competition between oxidative cleavage of β-D-glucan chain and cleavage by hydrolysis.

2. Materials and methods

2.1. Material

High viscosity barley β-D-glucan (M_w = 495 kDa, purity >97%), medium viscosity barley β-D-glucan (M_w = 245 kDa, purity >97%) and low viscosity barley β-D-glucan (M_w = 179 kDa, purity >97%) were purchased from Megazyme (Ireland). The spin trap POBN (α-(4-pyridyl *N*-oxide)-*N*-*tert*-butylnitrone; 99%) and the reference TEMPO (free radical, sublimed, ≥99%) were bought from Sigma Aldrich (St. Louis, MO, USA) and were stored at -20 °C. Hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Merck (Germany).

2.2. Preparation of the β-D-glucan solution

The 1 wt.% solution of high viscosity barley β-D-glucan was prepared by dissolving 1 g of dry barley β-D-glucan in Milli-Q water in a 100 mL volumetric flask. The solution was heated for 3 h at 80 °C under continuous shaking at 100 rpm in a shaking water bath (Julabo SW 21, Allentown, USA). After complete dissolution of the β-D-glucan, the pH of the solution was adjusted to 4.5 with 1 mM HCl solution, and then the volume in volumetric flask was adjusted to 100 mL.

2.3. Solution treatment

The oxidative degradation of β-D-glucan was carried out with H_2O_2 treatment at 85 °C, collecting samples at 0, 2, 5and 24 h, and after 1 week. The reaction was initiated by the addition of H_2O_2 to the final concentration of 100 mM in a 1 wt.% barley β-D-glucan solution at pH = 4.5. Iron(II) was not added to the reaction mixture since the β-D-glucan powder contains intrinsic iron ($12.27 \pm 0.28\ \mu g\ g^{-1}$), which was sufficient to promote the formation of •OH and thereby β-D-glucan oxidation.

The hydrolysis of β-D-glucan was performed by storing the 1 wt.% barley β-D-glucan solution pH = 4.5 at 85 °C.

At different reaction time 2, 5, 24 h and 1 week, samples from each solution were collected, and frozen to −80 °C prior to freeze-drying them. Additionally, at reaction 0 min, 1 h, 24 h and 1 week 500 μL of the solutions were transferred to 1.5 mL Eppendorf for the ESR spin trapping procedure.

2.4. ESR spin trapping

The formation of free radicals in the different models was monitored using the spin trapping method together with ESR detection of spin adducts. The spin trapping method is based on an indirect detection of •OH using POBN in combination with EtOH (Faure, Andersen, et al., 2012).

The stock solution of POBN (4 M) was prepared by dissolving the spin trap in Milli-Q water. 500 μL of the solutions were transferred to 1.5 mL Eppendorf, and then 10 μL of POBN solution and 10 μL EtOH were added in order to reach the final concentration of 80 mM and 2% (v/v), respectively. The sample was mixed by vortexing (1 min), incubated for 1 h, and then the ESR spectrum was recorded. This procedure was used for each solution at 0 min, 1 h, 24 h and 1 week of incubation time. The ESR spectra were recorded at room temperature with a benchtop ESR Spectrometer MiniScope MS300 (Magnettech, Berlin, Germany), and using 50 μL micropipettes (Brand GMBH, Wertheim, Germany). The settings used were as follows: B0-field 3350 G; sweep width 100 G; sweep time 30 s; steps 4096; number of passes 4, modulation frequency 1000 mG; microwave attenuation 10 dB; receiver gain 900. The relative ESR signal or adduct concentration was obtained by calculating the ratio between the peak-to-peak-amplitude of the

first doublet in the ESR signal of POBN adduct and the peak-to-peak-amplitude of the first singlet in the ESR signal of a TEMPO solution (2 μM in H_2O), which was used as a standard and measured in triplicate on each day of measurements.

2.5. Direct ESR detection of radicals

The freeze-dried samples obtained were analyzed by direct ESR in order to study the formation of radicals during the degradation process. The ESR spectra were recorded at room temperature with a benchtop ESR Spectrometer MiniScope MS300 (Magnettech, Berlin, Germany). Approximately 50 mg of dried material was placed in quartz ESR tubes. The settings used were as follows: B0-field 3350 G; sweep width 100 G; sweep time 30 s; steps 4096; number of passes 4, modulation frequency 5000 mG; microwave attenuation 10 dB; receiver gain 900. In the case of the sample oxidized for 1 week, the ESR measurements were made with a wide magnetic field sweep (5000 G) due to the presence of Mn(II). The *g*-values of the ESR signal were calculated by using a calibrated Mn(II) standard ($g = 2.0024$).

2.6. Viscosity measurement

Viscosity measurements were performed using an AR-2000 rheometer (TA Instruments, New Castle, DE, USA) with computer control. A cone and plate geometry was used with a plate radius of 40 mm and a cone angle of 2°. The gap between the cone and plate geometry was set at 59 μm. The flow curves were obtained over a shear rate range of 20–2000 s^{-1}, and the temperature of measurements was 20 °C.

2.7. Light scattering

Light scattering (LS) experiments were performed in a 1 cm glass cuvette at 25 °C on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) dynamic light scattering device, and detecting the backscattered light ($\lambda_{He-Ne} = 633$ nm, 5 mW) at 173°. From the Debye plot, the mass-average molecular weight (M_w) and second virial coefficient (A_2) were determined.

2.8. NMR spectroscopy

^1H, ^{13}C and ^1H–^{13}C HSQC NMR experiments were carried out at 80 °C on a Bruker Avance Spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) operating at 400 MHz (^1H) and 100 MHz (^{13}C), and using DMSO-d_6 as solvent and as internal standard.

2.9. FTIR spectroscopy

Fourier-transform infrared (FTIR) spectra of solid samples (freeze-dried) were recorded at room temperature with a Varian 640 FTIR spectrometer (Agilent Technologies AG, Basel, Switzerland) and a MKII Golden Gate single Attenuated Total Reflection (ATR) system.

3. Results and discussion

3.1. Relation between the formation of hydroxyl radical, viscosity loss and molecular weight changes

The ESR-based spin trapping method, which consisted of addition of POBN in combination with EtOH to the systems, was used to monitor the formation of •OH in the β-D-glucan solutions. The formation of •OH in the systems leads to the formation of POBN–$CH(OH)CH_3$ adduct which can be detected by ESR, and therefore an increase of the intensity of the ESR signal revealed an increase of •OH formed (Faure, Andersen, et al., 2012). The ESR data shows that •OH radicals were formed in the sample without addition of H_2O_2 (Fig. 1).

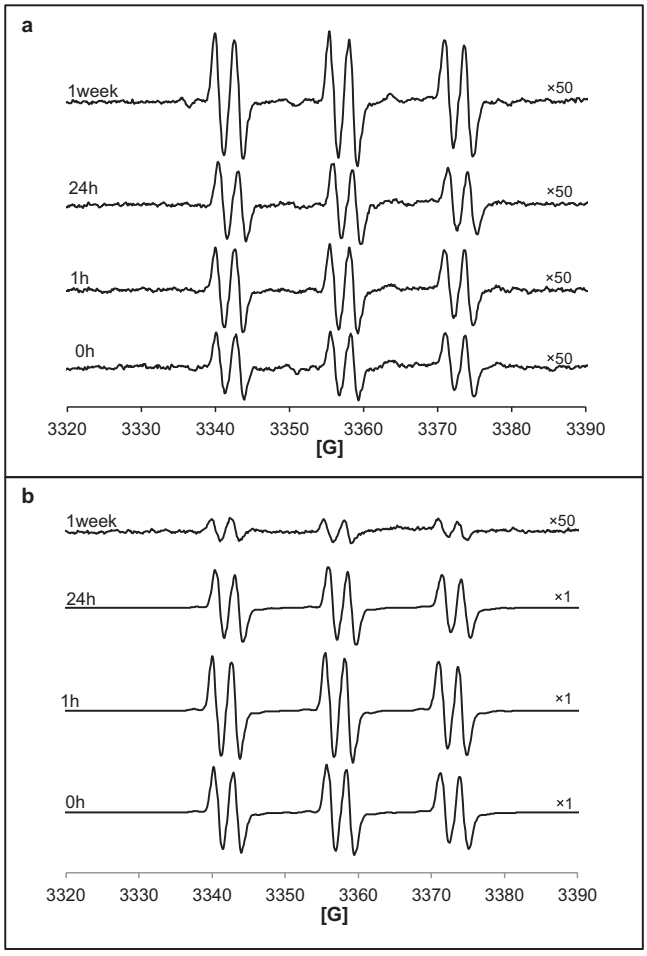

Fig. 1. ESR spectra of POBN–$CH(OH)CH_3$ adduct formed in 1 wt.% β-D-glucan solution containing (a) 80 mM POBN and 2% (v/v) EtOH and (b) 100 mM H_2O_2, 80 mM POBN and 2% (v/v) EtOH at 85 °C.

The radical formation is most likely due to the presence of iron contaminant in the β-D-glucan powder, which catalyzes the formation of •OH through a metal-catalyzed cycle. We have previously observed that the presence of iron(II) in β-D-glucan solution promoted the formation of •OH at elevated temperatures (unpublished data). Moreover, in the present study the formation of •OH was still observed after 1 week in a H_2O_2-untreated β-D-glucan solution (Fig. 1a). This is in agreement with a previous study where the generation of •OH was maintained during 24 h in a β-D-glucan solution containing iron(II). The sustainability of the •OH generation was readily explained by the reducing capacity of β-D-glucan, which allows regeneration of iron(III) to iron(II). The addition of H_2O_2 to the β-D-glucan solution led to a significant increase in the amount of •OH generated compared to the H_2O_2-untreated sample (Fig. 1b). For instance, after 0, 1 and 24 h the concentration of spin adducts in the sample containing H_2O_2 was 7–10 times higher than in the β-D-glucan solution without H_2O_2. This clearly shows the importance of H_2O_2 in the formation of •OH in the presence of iron(II). Furthermore, the ESR data showed that between 24 h and 1 week of storage time the amount of •OH formed in the H_2O_2-treated β-D-glucan solution decreased dramatically, with the ESR relative signal dropping from 60 to 0.4 (Fig. 1). The reason for this could be that after a certain time of storage (over 24 h) most of the added H_2O_2 had been consumed by the Fenton reaction.

Table 1
Apparent viscosity (η_{app}), average-weight molecular mass (M_w), and second virial coefficient (A_2) of 1 wt.% β-D-glucan solution treated with 100 or 0 mM H_2O_2 at 85 °C at different reaction times. The percentage of remaining viscosity is obtained by comparing the apparent viscosity at t_0 to the apparent viscosity at t_x of the β-D-glucan solution.

	100 mM H_2O_2			0 mM H_2O_2		
	2 h	24 h	1 week	2 h	24 h	1 week
η_{app} at 28 s^{-1} (mPa s)	3.7 ± 0.6 (1.5%)	1. 7 ± 0.5 (0.7%)	1.3 ± 0.1 (0.5%)	244 ± 17 (98.3%)	113 ± 11 (45.3%)	13.7 ± 1.9 (5.6%)
M_W (kDa)	ND	ND	ND	513 ± 6	469 ± 23	318 ± 7
A_2 (m^3 mol kg^{-2})	ND	ND	ND	$2.06 \pm 0.17 \times 10^{-4}$	$2.36 \pm 0.78 \times 10^{-4}$	$1.99 \pm 0.47 \times 10^{-4}$

Note: ND means not detected.

Furthermore, the relative ESR signal of the H_2O_2-untreated β-D-glucan solution after 1 week of storage time was higher than for the solution that originally contained added H_2O_2 (Fig. 1). Thus, iron(II) was less available in the sample treated with H_2O_2, possibly due to a depleted reducing capacity of the β-D-glucan oxidation products.

In order to check whether the formation of oxidizing species (•OH) was related to the viscosity loss in the β-D-glucan solution, the viscosity changes of two systems were investigated over a period of 1 week. The addition of H_2O_2 in the β-D-glucan solution caused a drastic viscosity loss of the solution (Table 1). After 2 h the β-D-glucan solution treated with H_2O_2 presented a viscosity of 3.7 mPa s (remaining viscosity of 1.5%) while the viscosity of the β-D-glucan solution without H_2O_2 did not significantly decrease and had a viscosity of 244 mPa s (98.3% remaining viscosity) (Table 1). This related well with the ESR data showing that a much higher amount of •OH were generated in the sample containing H_2O_2 compared to the one without H_2O_2 at 0 and 1 h. In the H_2O_2-untreated sample no significant viscosity loss was observed after 2 h, even though •OH were detected at 0 and 1 h (Fig. 1 and Table 1). Therefore, the amount of •OH formed during the first 2 h of incubation time in this solution was assumed to be too low to induce a significant viscosity loss. Furthermore, these results demonstrate that a 2 h of heat treatment at 85 °C did not significantly affect the viscosity of the β-D-glucan solution. Hence, the dramatic viscosity loss of the H_2O_2 containing β-D-glucan solution was mainly caused by a •OH-induced oxidative cleavage degradation. Additionally, our data are in agreement with the findings of de Moura et al. (2011) who showed that the addition of H_2O_2 in β-D-glucan solutions leads to a significant viscosity loss. Moreover, after 24 h the sample without H_2O_2 exhibited a viscosity of 113 mPa s, which was significantly lower than the original viscosity of 248 mPa s (Table 1). After 1 week, the same sample presented a viscosity of 13.7 mPa s (or remaining viscosity of 5.6%) approaching the viscosity value of the H_2O_2 treated sample at 2 h (3.7 mPa s) (Table 1). Although the ESR data showed that •OH radicals were continuously generated also in the H_2O_2-untreated sample, the viscosity decrease observed after 1 week in this sample cannot completely be attributed to an •OH-induced oxidative cleavage (Fig. 1).

In order to obtain a better understanding of the changes, the weight-average molar mass (M_w) changes of the β-D-glucan in the two systems at different incubation times were investigated by light scattering (LS) (Table 1). The LS experiments were not suitable to determine the molecular weight modifications of β-D-glucan during H_2O_2 treatment because the molecular weight of the degradation products was below the limit of detection at the chosen time of 2 h, 24 h and 1 week. This further indicates that the treatment of the β-D-glucan solution with H_2O_2 causes a fast (2 h) molecular breakdown of the polysaccharide into oligomers, in great agreement with the observed viscosity data, which show dramatic decrease in viscosity (249 mPa s down to 3.7 mPa s) after 2 h of incubation time.

In the case of the H_2O_2 untreated β-D-glucan solution, the average-weight molecular mass of β-D-glucan was determined after 2 h, 24 h and 1 week of incubation time. The second virial coefficient (A_2) obtained for the different analyzed sample showed a positive value in all cases, which indicated that water is a good solvent for this biopolymer at the measuring temperature. After 2 h of heat treatment at 85 °C, the M_w of β-D-glucan decreased slightly from 538 kDa (native β-D-glucan powder) to 513 kDa. This is consistent with the viscosity data, which did not show any significant decrease in viscosity of the β-D-glucan solution after 2 h of reaction. By increasing the time exposure to 24 h, the M_w of β-D-glucan decreased by 13% compared to the starting material, which is in accordance with the viscosity loss (54.7%) observed for the same sample after 24 h of incubation time. Finally, after 1 week of thermal treatment at 85 °C, the M_w of β-D-glucan (318 kDa) was 1.7 times lower that the starting material (538 kDa) explaining the low remaining viscosity (4.5%) observed for this sample at the same time point. These results show that with longer exposure to heat treatment the M_w of β-D-glucan decreased, and this change was linked to a viscosity loss of the β-D-glucan solution and degradation of the pristine polysaccharide backbone. Moreover, the viscosity data showed that heat treatment at 85 °C for 1 week – or a combined H_2O_2/heat treatment for 2 h on β-D-glucan solution – lead to similar low final viscosities (5.6% and 1.5%, respectively). However, the LS data demonstrated that the molecular mass of the degradation product obtained after these two treatments were very different. Indeed, after 1 week of heat treatment, the β-D-glucan was still in a relative long polymeric form presenting a M_w of 318 kDa, while β-D-glucan was denatured into oligomers after 2 h of H_2O_2 treatment. Thus, with a H_2O_2 treatment β-D-glucan is degraded to a greater extent than by heat treatment alone.

To summarize, the addition of H_2O_2 to a β-D-glucan solution with a natural level of iron induces a rapid formation of high level of oxidizing species (•OH) which attack rapidly the β-D-glucan monomeric units, and leads to the polymer backbone breakdown liberating oligomers after 2 h of incubation time. The exposure of β-D-glucan only to elevated temperatures leads to its slow degradation. Therefore, the degradation of β-D-glucan in the two different systems appears to be promoted by two different mechanisms.

3.2. Direct ESR detection of radicals

In order to monitor the formation of radicals (excluding •OH) in the β-D-glucan degradation products, direct ESR measurements (without addition of a spin trap) were performed at room temperature on the freeze-dried materials obtained from the two different systems after 2 h, 5 h, 24 h and 1 week of incubation. Radicals were not detected in freeze-dried samples obtained from H_2O_2-untreated β-D-glucan solutions. As explained earlier, •OH abstract H-atoms from the aliphatic moieties in the glucose units, which results in the formation of carbon-centered radicals. These carbon-centered radicals subsequently react with O_2 to form peroxyl radicals. Therefore, the absence of β-D-glucan radical formation in the H_2O_2-untreated solution indicates that •OH-induced oxidation did not occur. Thus, the β-D-glucan degradation taking place in the sample without H_2O_2 would not be caused by •OH-induced oxidative cleavage but rather by thermally induced hydrolysis.

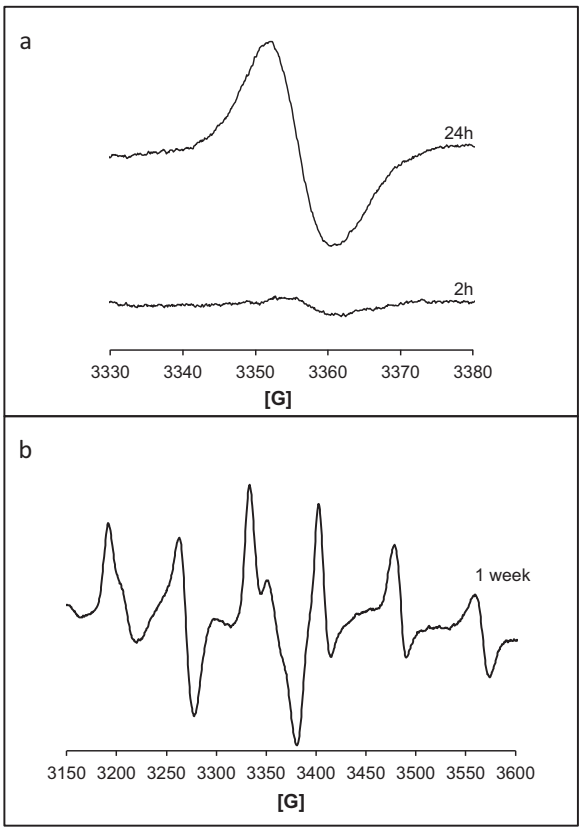

Fig. 2. ESR spectra of the freeze-dried samples obtained from a 1 wt.% β-D-glucan solution treated with 100 mM H_2O_2 at 85 °C for (a) 2 h and 24 h, and (b) 1week.

The ESR spectrum of the freeze-dried sample of β-D-glucan solution treated with H_2O_2 after 2 h, 5 h and 24 h gave a singlet with $g = 2.014$, and a peak-to-peak width of $\Delta H_{pp} = 9.1$ (Fig. 2a). The g-value of 2.014 obtained from the ESR signal is characteristic of peroxyl radical (Chamulitrat & Mason, 1989; Gunther, Hanna, Mason, & Cohen, 1995), which indicates that peroxyl radicals were formed in the H_2O_2-treated β-D-glucan solution. This is consistent with the mechanism of •OH-induced oxidation of carbohydrates, which includes the formation of peroxyl radical intermediates. The intensity of this ESR signal did not increase between 2 and 5 h of incubation (data not shown). However, the amplitude of the sample after 24 h storage was 17 times higher than after 2 h (Fig. 2a). Therefore, •OH-induced oxidation of β-D-glucan was amplified with increasing time of exposure to heat 85 °C.

The freeze-dried sample coming from the H_2O_2-treated β-D-glucan solution after 1 week of incubation exhibited a more complex ESR spectrum (Fig. 2b), where the singlet of the peroxyl radical ($g = 2.014$) and the characteristic sextet from Mn(II) ($g \approx 2$) could be easily identified (Bacić, Schara, & Ratković, 1993; Morsy & Khaled, 2002). Manganese is an essential element for plant growth, thus the presence and detection of this element in vegetal material such as β-D-glucan preparates should be expected. A well resolved sextet reveals the presence of unbound Mn(II) (Bacić et al., 1993), as observed in the ESR spectrum (Fig. 2b). The ESR detection of this element after 1 week of oxidation suggests that Mn(II) was initially complexed to β-D-glucan as an ESR-invisible complex, and that as the oxidation proceeded it was complexed in a different way that made it ESR-visible. However, the oxidation of β-D-glucan is assumed to cause the change on the Mn-complexation and not the mere hydrolytic process, which is confirmed by the absence of ESR Mn(II) signal.

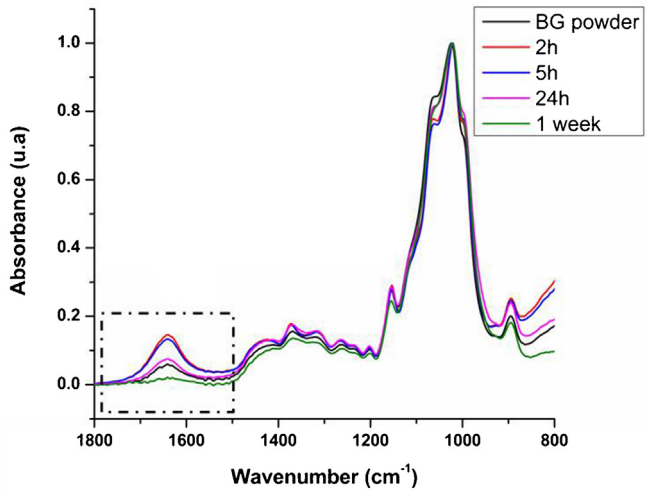

Fig. 3. FTIR spectra of β-D-glucan freeze-dried samples obtained from a 1 wt.% β-D-glucan solution heat treated at 85 °C for 2, 5, and 24 h, and 1 week, as well as for the pristine sample.

3.3. FTIR

In order to gain a deeper understanding of the molecular dissociation mechanism of β-D-glucan molecules during the two different degradation processes (*i.e.* hydrolysis and •OH mediated oxidation), FTIR spectroscopic analysis was performed on solid-state (freeze-dried) samples after different time of incubation (Figs. 3 and 4). Acid hydrolysis, a well-known and commonly used pathway toward the degradation of polysaccharides, *via* a thermally-induced breakage of the glycosidic linkages, is a process modulated by factors such as temperature or/and pH, which play however a very limited role on the molecular structure of the monomeric units.

Fig. 3 shows the in-time structural evolution of the high molecular weight β-D-glucan polymer during the process of acidic hydrolysis at a fixed temperature of 85 °C. The results clearly reveal that there are no major changes occurring within the chemical structure of the degraded polysaccharide, with the exception of the band at $\overline{\nu} = 1641\ cm^{-1}$ (Fig. 3), which absorption can be easily attributed to traces of H_2O remaining in the sample after the freeze-drying process (Lin, Xue, Ye, Zheng, & Xu, 2009). Furthermore, direct ESR data unambiguously shows the absence of either

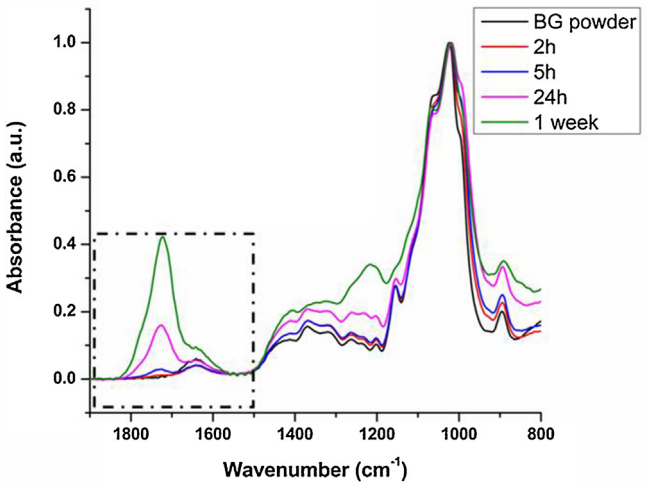

Fig. 4. FTIR spectra of the β-D-glucan freeze-dried samples obtained from a 1 wt.% β-D-glucan solution treated with 100 mM H_2O_2 at 85 °C for 2, 5, 24 h, and 1 week, as well as for the pristine sample.

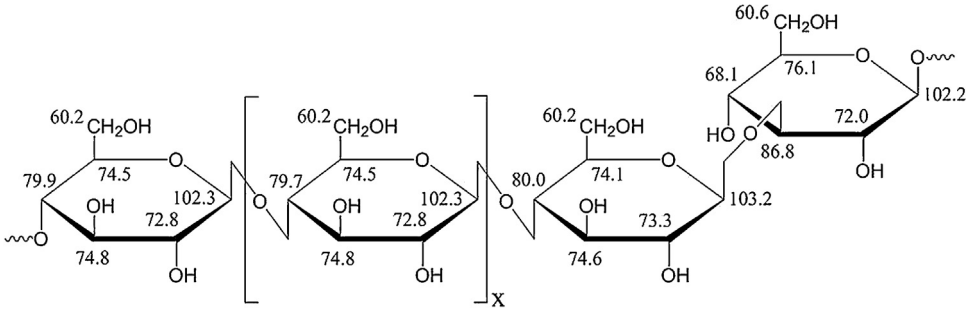

Fig. 5. ^{13}C NMR chemical shifts in DMSO-d_6 at 85 °C for the cellotetraosyl ($x = 1$) and the cellotriosyl ($x = 0$) units in the β-D-glucan polymer chain.

carbon-centered or peroxyl radicals – which would indicate •OH-induced oxidative process – during the thermal degradation of the polysaccharide. Therefore, thermally induced acidic hydrolysis of the β-D-glucan takes place without affecting the chemical structure of the monomers in the polymer backbone.

A qualitative analysis of the OH•-mediated degradation effects was performed by monitoring the time evolution of β-D-glucan samples treated with 100 mM H_2O_2 at pH = 4.5 and 85 °C (Fig. 4). Production of a large number of free •OH *via* the Fenton reaction that can randomly attack and oxidize the glucose-based backbone of the polysaccharide led to fast and clear changes in the FTIR spectra of the degraded β-D-glucan samples. These changes are most obvious in the region between $\overline{\nu} = 1900\,cm^{-1}$ and $1500\,cm^{-1}$ (Fig. 4), and correspond to variations in the intensity of the peak at $\overline{\nu} = 1641\,cm^{-1}$ – attributed to remaining H_2O – and the appearance of a new peak at $\nu = 1730\,cm^{-1}$. This new peak suggests a systematic altering in the chemical structure of the glucose backbone units, and is assigned to a C=O stretching, usually observed between $1740\,cm^{-1}$ and $1650\,cm^{-1}$ and therefore to the formation of carbonyl-based functional groups (*i.e.* carboxylic acids or lactones) (Pretsch, Buhlmann, & Affolter, 2000). Furthermore, a quantitative analysis based on the deconvolution of the peaks between $\overline{\nu} = 1550\,cm^{-1}$ and $1850\,cm^{-1}$ in a series of Lorentzian distributions revealed the systematic increase in time of the peak area corresponding to the C=O stretching and implicitly of the carbonyl-based functional groups – not observed during the hydrolysis process. Therefore, the formation of a large number of free •OH due to the presence of H_2O_2 leads to a different degradation pathway, as shown by the formation of C=O groups and characterized by the oxidative cleavage of the β-D-glucan chain.

3.4. NMR analysis

NMR experiments were performed to support and complement the results of the FITR analysis, furthermore to better understand the structural changes of the thermal and oxidative process at the molecular level. Solutions of β-D-glucan in DMSO-d_6 were analyzed by ^{1}H, ^{13}C and ^{1}H–^{13}C HSQC NMR, where ^{13}C NMR was the most sensitive technique for the analysis of such biopolymers (Colleoni-Sirghie, Fulton, & White, 2003; Dais & Perlin, 1982). Fig. 5 shows the ^{13}C NMR chemical shifts for the cellotetraosyl and the cellotriosyl units of the β-D-glucan starting materials. After thermal treatment (1 week at 85 °C), the β-D-glucan sample showed the same peaks in the ^{13}C NMR spectra as the reference starting materials. The anomeric carbons (C1) appear at $\delta = 103.2$ (β-(1→3)), 102.3 (β-(1→4)) and 102.2 (β-(1→4)) ppm. The peak related to the non-anomeric carbon participating in the β-(1→3) glycosidic bond (C3) is shifted to $\delta = 86.8$ ppm, and the corresponding non-anomeric carbons in the β-(1→4) glycosidic linkage (C4) are at $\delta = 80.0$, 79.9 and 79.7 ppm (Fig. 5). Thus, the thermal treatment of β-D-glucan leads to the partial hydrolysis of the biopolymer while keeping the chemical structure of the monomer units. These results agree to the decrease in the viscosity of the sample when thermal treatment is applied.

The oxidative treatment of β-D-glucan showed new peaks appearing, together with the vanishing of others from the starting material (Fig. 6). After 2 h at 85 °C, a tiny peak appeared at $\delta = 155.1$ ppm, which can be attributed to the oxidation of the secondary carbon C6 or the anomeric carbon C1 into the corresponding carboxylic acid or lactone, respectively. This results relates well with the FTIR data which show the formation of C=O. Moreover, already after 2 h of H_2O_2-treatment at 85 °C the β-D-glucan oxidized product were in oligomeric form and the viscosity measurements already showed a decrease in the viscosity of the sample, which might indicate that the preferred oxidation process happened in the anomeric carbon, and the corresponding fragmentation of the β-D-glucan polymer chain took place.

After 24 h of oxidative treatment, new peaks at $\delta = 172.4$, 162.1, 102.7, 76.5, 76.3, 76.1, 73.1, 72.9, 71.7, 71.5, 60.9, 60.0, 59.4 and 54.3 ppm appeared. These peaks are related to carbons with new chemical environments due the oxidation of aldehydes or alcohol groups to carboxylic functions as shown by the presence of the peaks at $\delta = 172.4$ and 162.1 ppm. Moreover, after 1 week at 85 °C, β-D-glucan showed numerous peaks in the low field region

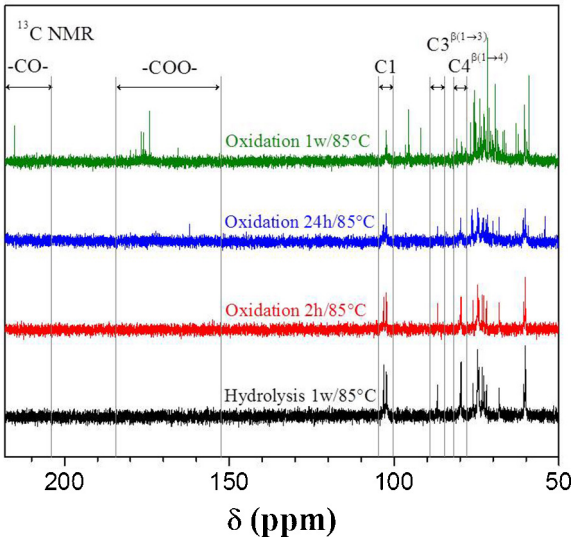

Fig. 6. ^{13}C NMR spectra at 80 °C in DMSO-d_6 for the β-D-glucan sample after 1 week hydrolysis at 85 °C (black), after 2 h oxidation at 85 °C (red), after 24 h oxidation at 85 °C (blue), and after 1 week oxidation at 85 °C (green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

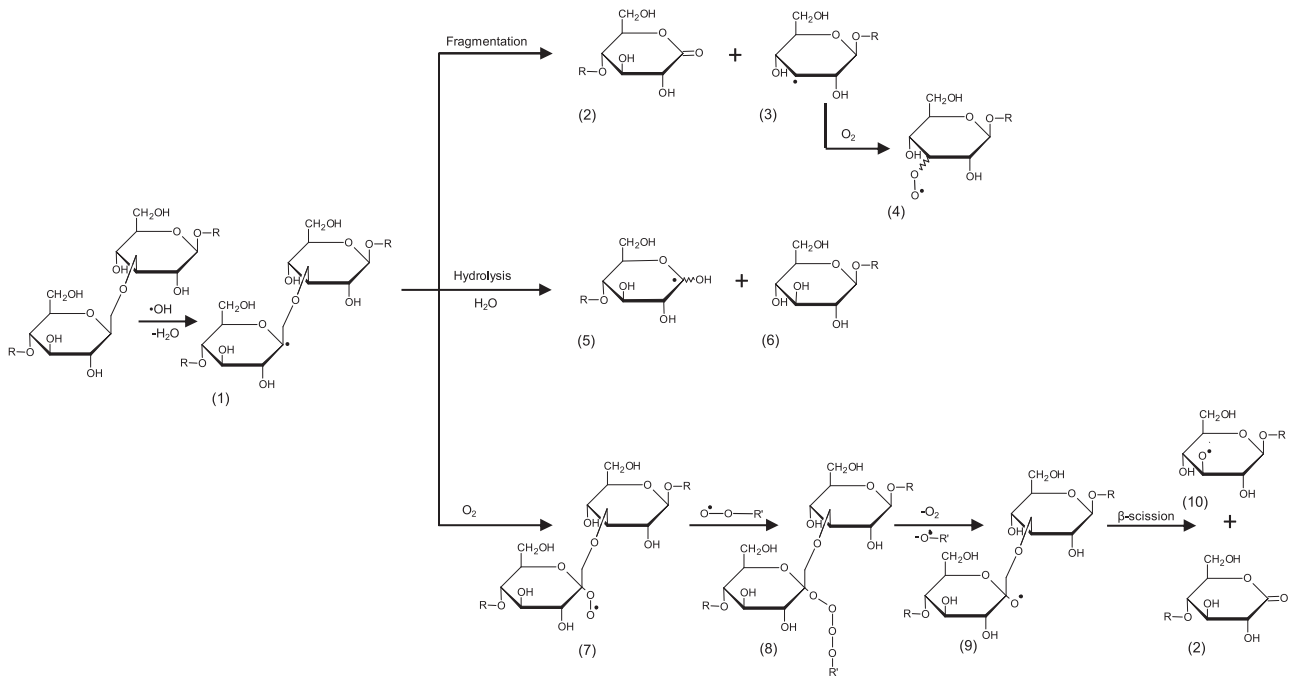

Fig. 7. Cleavage pathways suggested for the oxidative cleavage of the β-(1→3) linkage of β-D-glucan.

(δ = 215.2, 180.1, 179.1, 178.4, 176.2, 176.0, 174.3 and 165.5 ppm), which clearly indicated the presence of carbonyl (aldehydes and/or ketones) and carboxylic groups coming from the oxidation of the corresponding alcohols and aldehydes in the saccharide units. More importantly, the peak signals corresponding to the anomeric carbon in the β-(1→3) glycosidic bond (δ = 103.5 ppm) and the corresponding partner (δ = 86.8 ppm) disappeared. This result might support the idea that the β-(1→3) glycosidic linkage is more suitable to be attacked during the oxidative process than the corresponding β-(1→4) glycosidic bond. The presence of new peaks at δ = 95.7 and 91.9 ppm, together with a huge amount of extra peaks between δ = 80 and 50 ppm, is an indication that the oxidative process takes place at the different carbons in the repeating monomer unit. Thus, the anomeric carbon is oxidized and cleavage of the glycosidic bond happens – β-(1→3) preferred than β-(1→4) – while the secondary carbon and the rest of tertiary carbon atoms in the monomers are oxidized at lower rates. Furthermore, ^{1}H and ^{1}H–^{13}C HSQC NMR experiments showed and confirmed the shortening of the β-D-glucan chain after thermal treatment due to the narrowing of the peaks, and in the case of oxidative process, due to the presence of many narrow extra peaks (Supporting information).

3.5. β-D-Glucan degradation pathways

The thermal treatment (85 °C) of a mildly acidic (pH 4.5) β-D-glucan solution leads to a slow decrease of the molecular weight of the polysaccharide (41% decrease from the original M_w in 1 week at 85 °C), inducing the viscosity drop of the solution. Chemical analysis (FTIR and NMR analysis) of the resulting degradation materials at different reaction times did not reveal the introduction of new functional groups on the β-D-glucan fragments. It can therefore be concluded that thermal treatment induces β-D-glucan hydrolysis without affecting the structure of the glucose units. Additionally, the ESR spin trapping results show that •OH radicals were formed in the thermally-treated β-D-glucan solution are most likely due to the presence of residual iron. Therefore, it cannot be excluded that a small fraction of β-D-glucan was affected by oxidative cleavage; however, the absence of new functional groups indicates that the hydrolysis is the prevalent path to β-D-glucan degradation in absence of H_2O_2.

On the other hand, the H_2O_2-induced degradation of β-D-glucan leads to a fast breakdown of the polymer chain, accompanied by the introduction of new functional groups and peroxyl radicals on the β-D-glucan products. The chemical analysis performed on the β-D-glucan residues obtained at different oxidation times provides new insights on the possible pathways for the oxidative cleavage of β-D-glucan. For instance, the NMR data indicated that during the first 2 h of treatment, the oxidation of β-D-glucan was favored on C1 implicitly modulating the cleavage of the glycosidic bond and the release of a lactone in C1. Furthermore, the •OH-mediated oxidation of β-D-glucan induces the formation of peroxyl radical on the polysaccharide fragments already after 2 h. Based on these findings, two different scission pathways of the β-(1→3) glycosidic bond of the β-D-glucan are proposed, which lead to the release of a lactone in C1 (Fig. 7). These pathway are supposedly applicable for the cleavage of the β-(1→4) glycosidic bond.

The oxidative cleavage of β-D-glucan is initiated by the abstraction of a hydrogen atom from the anomeric carbon (C1) of the polysaccharide inducing the formation of an alkyl radical (1). To this point, there are two likely routes for the cleavage of the glycosidic bond with the generation of a lactone in C1 (2). The glycosidic bond may fragment, due to delocalization of the unpaired electron, which leads to the release of a β-D-glucan fragment with a lactone at C1 (2), and of a β-D-glucan fragment with an alkyl radical on C3 (3). Earlier studies have proposed this pathway for the radical induced scission of the glycosidic linkage of cellobiose after abstraction of an hydrogen atom on the anomeric carbon C1 (von Sonntag, 1980). Here, we suggest that the newly formed alkyl radical in C3 may react with O_2 to form the corresponding peroxyl radical (4), which can further undergo transformations.

Additionally, the glucan with an alkyl radical on the C1 (1) may also undergo hydrolysis. Indeed, a study on cellobiose radical-mediated cleavage suggested that the presence of an unpaired electron on the anomeric carbon (C1) could lead to a hydrolysis (von Sonntag, 1980). In the present case, this process would lead to the

release of a non-radical β-D-glucan fragment (6) and a β-D-glucan fragment containing a radical at C1 (5).

An alternative pathway for the cleavage of the β-(1→3) glycosidic bond of β-D-glucan involves the formation of peroxyl radical following the alkyl radical in C1 (1). The carbon centered radical in C1 would react rapidly with O_2 to give a peroxyl radical (7). It has been suggested that peroxyl radical can further combine with another peroxyl radical and fragment *via* an alkoxyl radical (R–O•) (Schuchmann & von Sonntag, 1978). The alkoxyl radical (9) formed would undergo a β-fragmentation, releasing a β-D-glucan fragment with a lactone in C1 (3) and a β-D-glucan fragment with an alkoxyl radical at C3 (10).

The described mechanisms based on initial hydrogen atom abstraction at C1 account well for our data. However it cannot be excluded that other pathways contribute to the scission of β-D-glucan, since the non-selective attack of •OH radicals most likely also generate radicals at other locations besides at C1. Moreover, the formation of carbonyl and aldehyde functional groups after 1 week of H_2O_2-treatment indicates that more complex degradation and oxidation pathways occur during later stages of oxidative degradation of β-D-glucan.

4. Conclusions

Our results demonstrated that the thermal treatment (85 °C) of an aqueous β-D-glucan solution induces the hydrolysis of the polymer, which in turn leads to a decrease of its average-weight molecular mass and viscosity. This degradation is a slow process, which does not affect the monomer structure of β-D-glucan. The presence of the Fenton reagents (H_2O_2 and Fe^{2+}) in β-D-glucan solution stored at 85 °C induces the generation of high amounts of •OH which promoted a fast and extensive degradation of β-D-glucan. Simultaneously to the degradation, new functional groups (*i.e.* lactone, carboxylic acid, aldehyde and ketone) were formed on the β-D-glucan residues. During the first 24 h of oxidation, lactones and carboxylic acid were formed, and as the time of exposure to the Fenton reagents increased (up to 1 week), new functional groups were generated such as ketones and aldehydes. Because of the detection of lactones at the beginning of the oxidation process of β-D-glucan, this functionality suggested to be the initial major product should be the result of the oxidative cleavage of the glycosidic bonds. Our results demonstrated the oxidative cleavage induces the formation of a lactone in C1 in the β-D-glucan fragment. This cleavage is initiated by the presence of a radical at the anomeric carbon (C1). Two pathways for the scission of the glycosidic bond leading to the liberation of a lactone at C1 were suggested: Fragmentation by delocalization of the unpaired electron at C1, or scission *via* formation of a peroxyl radical at C1 as observed by ESR. Furthermore, based on the NMR measurements we demonstrated the cleavage of the β-(1→3) glycosidic linkage was favored over the cleavage of the β-(1→4)-linkage.

Therefore, the treatment of β-D-glucan with •OH radicals generating system leads to its degradation, changing its structural properties with the introduction of new oxidized functional groups (*i.e.* lactones, carboxylic acids, ketones and aldehydes). A recent study showed that oxidized β-D-glucan has a better bile acid binding capacity than native β-D-glucan (de Moura et al., 2011) and thereby can potentially improve the cholesterol reducing capacity. The structural understanding of the chemical changes in β-D-glucan during oxidation processes is therefore also highly relevant for the study of its health related benefits.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.08.022.

References

Bacić, G., Schara, M., & Ratković, S. (1993). An ESR study of manganese binding in plant tissue. *General Physiology and Biophysics*, *12*, 49–54.

Braaten, J. T., Wood, P. J., Scott, F. W., Wolynetz, M. S., Lowe, M. K., Bradley-White, P., et al. (1994). Oat β-glucan reduces blood cholesterol concentration in hypercholesterolemic subjects. *European Journal of Clinical Nutrition*, *48*(7), 465–474.

Chamulitrat, W., & Mason, R. P. (1989). Lipid peroxyl radical intermediates in the peroxidation of polyunsaturated fatty acids by lipoxygenase. Direct electron spin resonance investigations. *Journal of Biological Chemistry*, *264*(35), 20968–20973.

Colleoni-Sirghie, M., Fulton, D. B., & White, P. J. (2003). Structural features of water soluble (1,3)(1,4)-β-d-D-glucans from high-β-D-glucan and traditional oat lines. *Carbohydrate Polymers*, *54*(2), 237–249.

Dais, P., & Perlin, A. S. (1982). High-field, ^{13}C-N.M.R. spectroscopy of β-d-D-glucans, amylopectin, and glycogen. *Carbohydrate Research*, *100*(1), 103–116.

de Moura, F. A., Pereira, J. M., da Silva, D. O., Zavareze, E. d. R., da Silveira Moreira, A., Helbig, E., et al. (2011). Effects of oxidative treatment on the physicochemical, rheological and functional properties of oat β-glucan. *Food Chemistry*, *128*(4), 982–987.

Faure, A. M., Andersen, M. L., & Nyström, L. (2012). Ascorbic acid induced degradation of beta-glucan: Hydroxyl radicals as intermediates studied by spin trapping and electron spin resonance spectroscopy. *Carbohydrate Polymers*, *87*(3), 2160–2168.

Faure, A. M., Münger, L. H., & Nyström, L. (2012). Potential inhibitors of the ascorbate-induced β-glucan degradation. *Food Chemistry*, *134*(1), 55–63.

Gunther, M. R., Hanna, P. M., Mason, R. P., & Cohen, M. S. (1995). Hydroxyl radical formation from cuprous ion and hydrogen peroxide: A spin-trapping study. *Archives of Biochemistry and Biophysics*, *316*(1), 515–522.

Johansson, L., Virkki, L., Anttila, H., Esselström, H., Tuomainen, P., & Sontag-Strohm, T. (2006). Hydrolysis of β-glucan. *Food Chemistry*, *97*(1), 71–79.

Kivelä, R., Gates, F., & Sontag-Strohm, T. (2009). Degradation of cereal beta-glucan by ascorbic acid induced oxygen radicals. *Journal of Cereal Science*, *49*(1), 1–3.

Kivelä, R., Henniges, U., Sontag-Strohm, T., & Potthast, A. (2012). Oxidation of oat β-glucan in aqueous solutions during processing. *Carbohydrate Polymers*, *87*(1), 589–597.

Kivelä, R., Nyström, L., Salovaara, H., & Sontag-Strohm, T. (2009). Role of oxidative cleavage and acid hydrolysis of oat beta-glucan in modelled beverage conditions. *Journal of Cereal Science*, *50*(2), 190–197.

Kivelä, R., Sontag-Strohm, T., Loponen, J., Tuomainen, P., & Nyström, L. (2011). Oxidative and radical mediated cleavage of β-glucan in thermal treatments. *Carbohydrate Polymers*, *85*(3), 645–652.

Lazaridou, A., & Biliaderis, C. G. (2007). Molecular aspects of cereal β-glucan functionality: Physical properties, technological applications and physiological effects. *Journal of Cereal Science*, *46*(2), 101–118.

Lin, Z., Xue, R., Ye, Y., Zheng, J., & Xu, Z. (2009). A further insight into the biosorption mechanism of Pt(IV) by infrared spectrometry. *BMC Biotechnology*, *9*(1), 62.

Morsy, M. A., & Khaled, M. M. (2002). Novel EPR characterization of the antioxidant activity of tea leaves. *Spectrochimica Acta. Part A, Molecular and Biomolecular Spectroscopy*, *58*(6), 1271–1277.

Pretsch, E., Buhlmann, P., & Affolter, C. (2000). *Structure determination of organic compounds. Tables of spectral data*. Berlin Heidelberg: Springer-Verlag.

Schuchmann, M. N., & von Sonntag, C. (1978). The effect of oxygen on the OH-radical-induced scission of the glycosidic linkage of cellobiose. *International Journal of Radiation Biology*, *34*(4), 397–400.

Vaikousi, H., & Biliaderis, C. G. (2005). Processing and formulation effects on rheological behavior of barley β-glucan aqueous dispersions. *Food Chemistry*, *91*(3), 505–516.

Vaikousi, H., Biliaderis, C. G., & Izydorczyk, M. S. (2004). Solution flow behavior and gelling properties of water-soluble barley (1→3,1→4)-β-glucans varying in molecular size. *Journal of Cereal Science*, *39*(1), 119–137.

von Sonntag, C. (1980). Free-radical reactions of carbohydrates as studied by radiation techniques. In R. S. Tipson, & H. Derek (Eds.), *Advances in carbohydrate chemistry and biochemistry* (pp. 7–77). Academic Press.

Wood, P. J. (2004). Relationships between solution properties of cereal beta-glucans and physiological effects – A review. *Trends in Food Science & Technology*, *15*(6), 313–320.

Wood, P. J. (2007). Cereal β-glucans in diet and health. *Journal of Cereal Science*, *46*(3), 230–238.

Wood, P. J. (2010). Oat and rye beta-glucan: Properties and function. *Cereal Chemistry*, *87*(4), 315–330.

Wood, P. J., Braaten, J. T., Scott, F. W., Riedel, K. D., Wolynetz, M. S., & Collins, M. W. (1994). Effect of dose and modification of viscous properties of oat gum on plasma glucose and insulin following an oral glucose load. *British Journal of Nutrition*, *72*(5), 731–743.