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Ascorbic acid induced degradation of beta-glucan: Hydroxyl radicals as intermediates studied by spin trapping and electron spin resonance spectroscopy

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ABSTRACT

The formation of hydroxyl radicals in beta-glucan solutions treated with ascorbic acid and iron(II) was demonstrated by ESR spin trapping based methods. Two different spin traps were tested, namely DMPO which is commonly used to detect hydroxyl radicals, and POBN often used to detect carbon centered radicals. The experiments performed showed that the presence of iron(II) with DMPO led to low DMPO–OH adduct stability and further to DMPO dimerization. The level of hydroxyl radicals formed during the beta-glucan radical mediated degradation was evaluated using two ESR spin trapping methods based on the use POBN together with either 2% (v/v) EtOH or DMSO. The addition of ascorbic acid together with iron(II) in beta-glucan solution led to an immediate maximal production of hydroxyl radicals while the presence of ascorbic acid alone led to a progressive production of radical. Further hydroxyl radicals were found to be formed when iron(II) was added alone in beta-glucan solutions. The viscosity loss observed in the three last mentioned beta-glucan solutions were found to relate with the formation of hydroxyl radicals. These data confirm the involvement of hydroxyl radical in the beta-glucan degradation.

1. Introduction

Beta-glucan has a great potential as food functional ingredient due to its two major health benefits, namely the improvement of glucose metabolism and the decrease in the level of cholesterol in the case of hypercholesterolemia (Wood, 2007, 2010) However, beta-glucan in solution may undergo non-enzymatic degradation when treated with ascorbic acid and iron(II), leading to decreases in molecular weight and viscosity and thereby modified functional properties (Kivelä, Gates, & Sontag-Strohm, 2009). This process is believed to be the result of an oxidative cleavage of the polysaccharide chain by hydroxyl radicals (•OH) (Kivelä, Gates, et al., 2009). Nonetheless there are only very few studies reporting analytical methods to detect and quantify the formation of hydroxyl radical in order to support their involvement in the degradation process.

Hydroxyl radicals are reactive oxygen species (ROS), and are highly reactive and may oxidize biomolecules such as proteins, DNA and other biopolymers. It has been demonstrated that cell wall polysaccharides are affected by degradation induced by •OH in the presence of ascorbate (Fry, 1998). More recent studies showed that the presence of ascorbic acid (AH_2) and Fe^{2+} in beta-glucan solutions lead to a loss of viscosity, resulting from a beta-glucan degradation (Kivelä, Gates, et al., 2009). The loss of viscosity was assumed to be the result of an oxidative cleavage of the beta-glucan chain by •OH. These radicals are formed by the Fenton reaction (Eq. (1)) which is promoted by AH_2 (Burkitt, 2003).

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

Although AH_2 is mostly considered an excellent antioxidant, it has a dual activity depending on its concentration, and at low amounts it may act as a pro-oxidant as a consequence of its reducing property (Buettner & Jurkiewicz, 1996; Kivelä, Nyström, Salovaara, & Sontag-Strohm, 2009). In biological systems AH_2 may replace superoxide ions as reducing agents in order to provide the necessary reduced Fe^{2+} for the Fenton reaction (process known as "ascorbate driven Fenton reaction") (Burkitt, 2003). During the process of radical formation, AH_2 reduces catalytic metals and dissolved O_2, thus providing the two substrates needed for the Fenton reaction (Eqs. (2) and (3)), which in turn induces the formation of •OH responsible for the polysaccharide degradation. The degradation has the consequence of breaking down its molecular integrity leading to a decrease of the molecular weight and therefore in its associated health benefits (Kivelä, Gates, et al., 2009).

$$AH_2 + Fe^{3+}/2Cu^{2+} \rightarrow A + 2H^{+} + Fe^{2+}/2Cu^{+} \quad (2)$$

$$AH_2 + O_2 \rightarrow A + H_2O_2 \quad (3)$$

Abbreviations: •OH, hydroxyl radical; AH_2, ascorbic acid; H_2O_2, hydrogen peroxide; ESR, electron spin resonance; BBG, barley beta-glucan; OBG, oat beta-glucan; EtOH, ethanol; DMSO, dimethylsulfoxide.

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The radical mediated degradation of beta-glucan has mostly been studied by means of rheology and size exclusion chromatography (SEC), which provides information about beta-glucan structural changes, with respect to changes in the viscosity of the solution or the size of the polysaccharide (Kivelä, Gates, et al., 2009; Kivelä, Nyström, et al., 2009; Paquet, Turgeon, & Lemieux, 2010). However, because there is a lack of data linking the formation of •OH to the beta-glucan degradation, studies are still required to reinforce the involvement of the •OH in the degradation in presence of ascorbic acid.

Electron spin resonance (ESR) is a highly sensitive tool that allows detection of molecules with unpaired electrons such as free radicals. ESR has a wide range of application in the fields of physics, chemistry, biology, earth sciences and other branches of science. ESR has been used to detect polysaccharide radical formation directly (caused by •OH oxidation) with substrates like cellulose, dextran, galacturonan or pectins in order to study the mechanism of hydroxyl radical attack (Arthur, Hinojosa, & Bains, 1968; Gilbert, King, & Thomas, 1984). However the direct detection of these radicals requires recording ESR spectra at very low temperature in order to stabilize the radicals, which are very reactive and therefore exist as short-lived intermediates.

Short-lived radicals such as •OH cannot be directly detected by ESR due to their low steady-state concentration. The spin trapping method allows overcoming this problem by using an indirect detection (Andersen & Skibsted, 2007). Nitrone and nitroso compounds are used to trap an unstable free radical, which in turn leads to the formation of a stable nitroxide radical (spin adducts) which may be detected by ESR (Alberti & Macciantelli, 2009). Two widely used water soluble spin traps are POBN and DMPO that are commonly used to detect carbon- and oxygen-centered radicals, respectively.

Spin trapping techniques have been extensively used in the past years to study lipid oxidation or for assessing the flavour stability of lager beer in the brewing industry (Andersen & Skibsted, 2006, 2007) At the same time ESR spin trapping techniques have also been applied to detect the formation of •OH involved in polysaccharides degradation. Previous studies based on ESR spin trapping showed the implication of •OH in the scission of xyloglucan in a ascorbate–H_2O_2–copper system (Tabbi, Fry, & Bonomoc, 2001) Moreover, this method has also allowed to link the formation of •OH to the degradation of polysaccharides from plant cell walls (Schweikert, Liszkay, & Schopfer, 2002). More recently ESR spin trapping has been used to show the formation of free radical (most likely •OH) in a thermally treated beta-glucan extract in the presence of AH_2 and Fe^{2+} (Kivelä, Sontag-Strohm, Loponen, Tuomainen, & Nyström, 2011).

No ESR studies are available which directly assess the involvement of •OH in the degradation of beta-glucan treated with AH_2 and Fe^{2+}. Therefore the goals of our work were to develop ESR spin trapping methods to investigate •OH formation, to use the methods to study the effect of the reagents on the •OH formation and the viscosity of beta-glucan solution, to relate the results obtained from ESR and rheology measurements, and finally to apply the method to compare the oxidative degradation in barley and oat beta-glucan solutions.

2. Materials and methods

2.1. Materials

High viscosity barley beta-glucan (BBG) (purity > 97%) and high viscosity oat beta-glucan (OBG) (purity > 97%) were purchased from Megazyme (Ireland). The spin traps POBN (α-(4-pyridyl N-oxide)-N-*tert*-butylnitrone; 99%), DMPO (5,5-dimethyl-1-pyrroline, ≥99%) and the reference TEMPO (free radical, sublimed, ≥99%) were from Sigma–Aldrich (St. Louis, MO, USA) and were stored at −20 °C, and in the case of DMPO under nitrogen gas. Iron(II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$) was bought from Sigma–Aldrich Chemie GmbH (Germany). Ascorbic acid (≥99.5%) and ethanol (puriss. p.a., ACS reagent, absolute alcohol, without additive, ≥99.8%) were purchased from Fluka (Germany). Hydrochloric acid (HCl) and Sodium hydroxide (NaOH) were obtained from Merck (Germany).

2.2. Preparation of beta-glucan solutions

The 0.6% beta-glucan solutions were prepared from high viscosity barley beta-glucan (BBG) or high viscosity oat beta-glucan (OBG) by adding 0.6 g of dry beta-glucan to Milli-Q water in a volumetric flask. The solution was heated in a water bath 3 h at 80 °C under continuous shaking. After complete dissolution of beta-glucan, the pH of the solution was adjusted to 4.5 with 1 mM HCl solution and the volume of the solution was adjusted to 100 ml. This pH condition was selected as previous studies have shown optimal degradation of polysaccharide in AH_2 system at this pH (Fry, 1998; Kivelä, Gates, et al., 2009)

2.3. Sample preparation

Samples were prepared with either 0.6% BBG or 0.6% OBG solution depending on the experiment. Stock solutions of the reagents AH_2 (10 mM), Fe^{2+} (1 mM) and H_2O_2 (0.1 M) were freshly prepared before sample preparation. The final concentrations of added AH_2, Fe^{2+} and H_2O_2 in samples analyzed in different combinations were 250 μM, 50 μM and 1 mM, respectively. Each sample was analyzed on three different days in order to obtain three independent results. All samples were incubated at room temperature.

2.4. ESR spin trapping

Solution stock of DMPO (5 M) and POBN (4 M) were prepared by dissolving the spin trap in Milli-Q water. Two different techniques (initial addition and periodic addition) of spin trap addition were used in the study.

Initial addition: DMPO stock solution was added to beta-glucan solution to reach a final concentration of 50 mM, and the solution was mixed by vortex 1 min before addition of AH_2 and/or Fe^{2+}. POBN was used at a final concentration of 80 mM together with either 2% (v/v) ethanol (EtOH) or 2% (v/v) dimethylsulfoxide (DMSO). POBN stock solution as well as EtOH or DMSO were added to beta-glucan solution, which was then mixed by vortex, followed by the addition of AH_2 and/or Fe^{2+}.

Periodic addition: 200 μL of a sample already containing the reactants was transferred in 0.5 mL eppendorf tube, and then 4 μL of POBN solution stock and 4 μL EtOH were added in order to reach respective final concentration of 80 mM and 2% (v/v), respectively. The sample was mixed by vortex for 1 min, incubated for 1 h, after which a spectrum was recorded.

Several spectra of the different samples were recorded at different time of incubation in order to check the evolution of the ESR signal.

2.5. ESR measurements

Samples (50 μL) were loaded in 50 μL micropipettes (Brand GMBH, Wertheim, Germany) and the ESR spectra were recorded with a Benchtop ESR Spectrometer MiniScope MS300 (Magnettech, Berlin, Germany) at room temperature. 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 or the peak-to-peak-amplitude of the second singlet in the ESR signal of DMPO and the peak-to-peak-amplitude of the first singlet in the ESR signal of a TEMPO solution (2 μM in H_2O). TEMPO was used as a standard and was measured in triplicate on each day of measurements.

2.6. Rheology measurements

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.

3. Results and discussion

3.1. Development of ESR spin trapping methods for the detection of hydroxyl radicals

3.1.1. DMPO as spin trap

DMPO has been widely used to trap •OH in biological systems as the DMPO–OH adduct has a half-life in the range of 12–156 min in neutral solutions (Rosen, Britigan, Helpern, & Pou, 1999). The detection of •OH by spin trapping with DMPO was tested in a BBG solution containing 50 mM DMPO, AH_2 and Fe^{2+}, however ESR signals were not observed up to 55 min after mixing. The same experiment was repeated several times, and consistently no ESR signals were observed. Previous works have suggested that the mixing of AH_2 and Fe^{2+} leads to a production of •OH and furthermore to the degradation of beta-glucan resulting in the viscosity loss of beta-glucan solution (Kivelä, Gates, et al., 2009; Paquet, Turgeon, & Lemieux, 2010). However, the absence of spin adduct ESR signals in our experiments indicate that the amount of DMPO–OH formed, was below the limit of ESR detection.

Experiments were carried out with a BBG solution with added H_2O_2 in addition to the AH_2 and Fe^{2+} in order to achieve a high level of •OH generated by the Fenton reaction (Eq. (1)). An intense ESR signal assigned to a DMPO–OH adduct (a_N = 14.9 G, a_H = 14.9 G) was immediately observed (Fig. 1a). However already after 10 min incubation the height of the signal was 50% smaller (Fig. 1b). This suggests a rather short half-life of DMPO–OH adducts in the BBG solution. Baga (1993) has reported that the stability of DMPO–OH adducts was decreased by the presence of copper. Furthermore Reinke, Moore, and McCay (1996) explained that the presence of Fe^{2+} would reduce the nitroxide functional group of the adducts into a hydroxylamine or ESR silent compounds explaining a fast decrease of the ESR signal. Thus the lack of stability of DMPO–OH adducts may also explain the absence of ESR signal in AH_2 containing sample, as the adducts did not accumulate up to the ESR detectable concentration.

It has also been showed that iron or copper can interact directly with DMPO (Finkelstein, Rosen, & Rauckman, 1980). Thus in order to check if Fe^{2+} reacts with DMPO, experiments were carried out without addition of AH_2 and H_2O_2. A mixture of DMPO, BBG solution and Fe^{2+} gave a small ESR signal after 30 min incubation, and the signal increased during further incubation (Fig. 2a). A similar ESR signal was observed in an analogous experiment where only DMPO and Fe^{2+} without BBG were mixed (data not shown). Therefore it was assumed that beta-glucan was not involved in the appearance of this signal. By removing one compound at a time, it was deduced that the recorded spectra appeared due to an interaction between the spin trap and Fe.

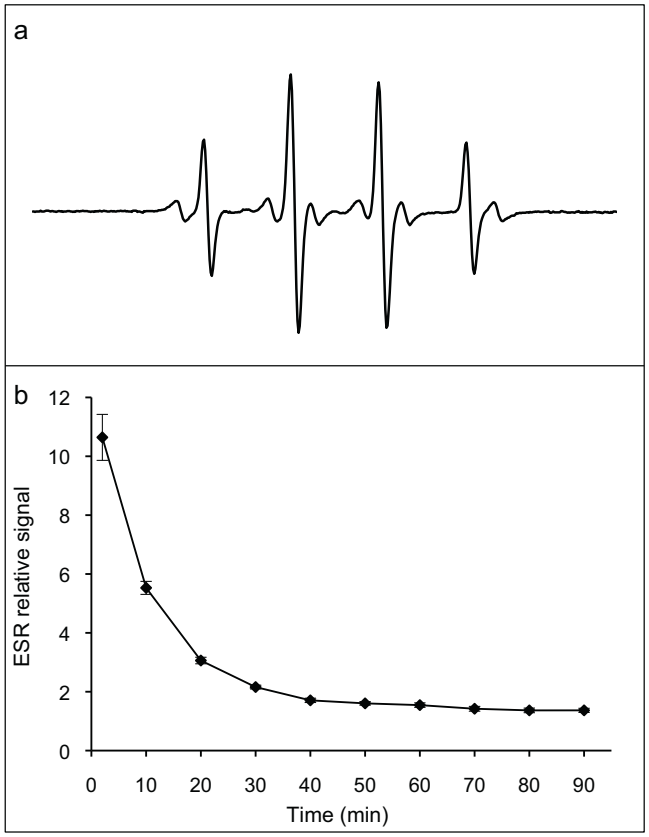

Fig. 1. (a) ESR spectrum of DMPO–OH adduct formed in 0.6% BBG solution containing 250 μM AH_2, 1 mM H_2O_2 and 50 μM Fe^{2+} with 50 mM DMPO at 2 min incubation. (b) Intensity of ESR signals in function of the time of the same sample as (a).

The effect of the DMPO concentration was tested by using three different concentrations, 100 mM, 200 mM and 300 mM, in the BBG solutions with added Fe^{2+}. Immediate intense ESR signals were observed, and the intensities increased as the concentration of DMPO was increased (Fig. 2b). By performing an ESR simulation, the hyperfine coupling constants for the six-line signal were found to be a_N = 15.4 G and a_H = 22.5 G, which are similar to the values expected for carbon-centered radical adducts of DMPO. Fe^{2+} may oxidize DMPO to DMPOX (a_N = 7.1 G and a_H = 4.0 G) (Rosen, Britigan, Helpern, & Pou, 1999), however the measured coupling constants for the detected signal did not match with the ones of DMPOX. Pou, Hassett, Britigan, Cohen, and Rosen (1989). have reported that DMPO could also be oxidized into a nitroxide by EDTA-iron giving rise to a sextet signal with the coupling constants a_N = 15.3 G and a_H = 22.0 G. Finkelstein, Rosen, and Rauckman (1980), proposed that the signal is caused by a dimer of DMPO. It is therefore likely that the observed DMPO adduct in the BBG-solutions could be due to formation of DMPO-dimers.

Experiments with BBG solution with added AH_2 and Fe^{2+} were repeated with a higher concentration of DMPO (300 mM) and ESR spectra were recorded every hour until 6 h incubation to see if the signal of DMPO dimer could be detected. A weak ESR signal corresponding to a carbon-centered radical adduct (a_N = 15.4 G and a_H = 22.7 G) was observed after 4 h incubation, increasing significantly after 5 h incubation, and reached a plateau region (at 6 h incubation). At this point the sample showed the same intensity as the signal of 300 mM DMPO, BBG solution and Fe^{2+} without AH_2 (Fig. 2c). However, ESR-signals that could be assigned to the

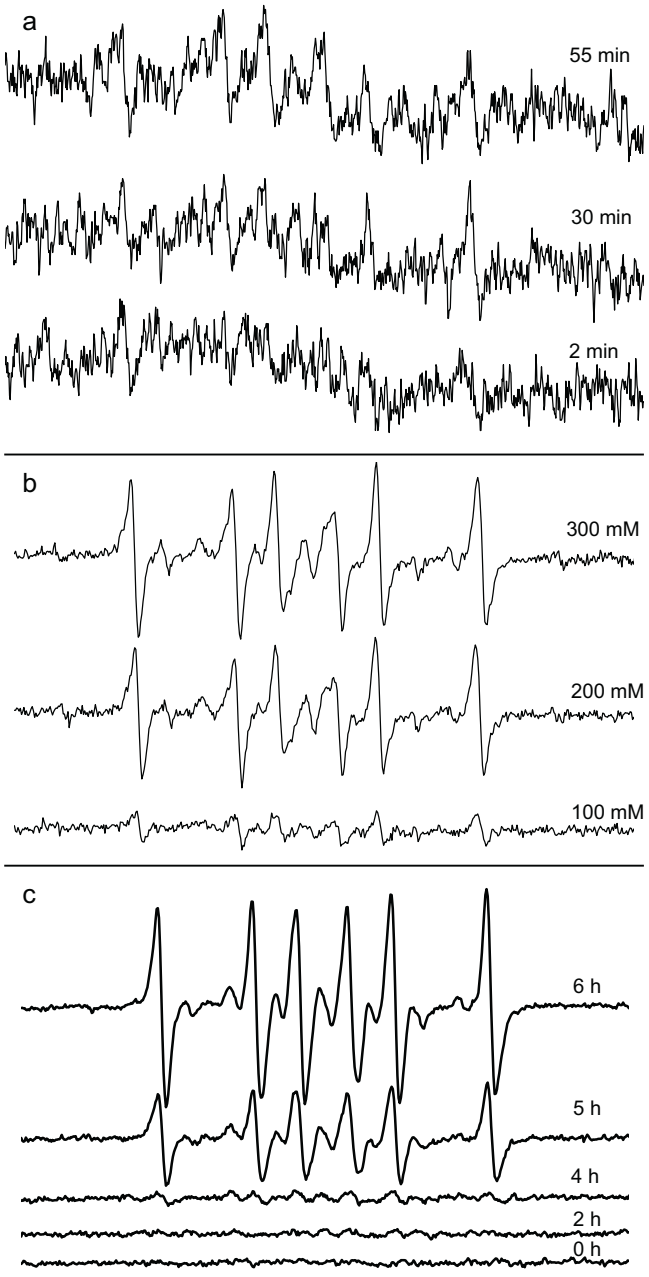

Fig. 2. (a) ESR spectra of 0.6% BBG solution containing 50 μM Fe^{2+} with 50 mM DMPO at different time of incubation. (b) Same sample as (a) but with 3 different concentration of DMPO at 2 min of incubation. (c) ESR spectra of 0.6% BBG solution treated with 250 μM AH_2, 50 μM Fe^{2+} with 300 mM DMPO at different times of incubation.

DMPO–OH adduct did not appear at any time during this experiment, thereby not proving the existence of hydroxyl radicals, despite the high concentration of DMPO and the final high level of accumulated spin adducts.

The results demonstrated that DMPO is not suited for detection of radicals in the glucan solutions. Spin adducts derived from carbon centered radicals were observed, however, it could not be excluded that they were DMPO degradation products generated by direct interaction between DMPO and the added iron species. Moreover, the short half-life of the DMPO–OH adducts and the low rate of formation of •OH in systems without added hydrogen peroxide most likely result in too low steady-state concentrations of the adducts that allowed the detection by ESR.

3.1.2. POBN used as spin trap

3.1.2.1. POBN in conjunction with EtOH. The spin trap, POBN is often used to detect carbon centered radicals due to the good stability of the adducts, however the half-life of the POBN–OH adduct is less than 1 min (Andersen & Skibsted, 2007; Pou et al., 1994). Thus it would appear that POBN is not suitable for the direct detection of •OH. It has been previously reported that POBN in conjunction with ethanol (EtOH) is a sensitive system for detecting •OH due to the great reactivity of $•CH(CH_3)OH$ with POBN and the stability of the adduct POBN-$CH(CH_3)OH$ (Pou et al., 1994). The oxidation of EtOH by •OH (Eq. (4)) produces $•CH(CH_3)OH$ radicals, which is further trapped by POBN (Eq. (5)) to give a stable adduct (Pou et al., 1994).

$$CH_3–CH_2–OH + •OH \rightarrow •CH(CH_3)OH + H_2O \quad (4)$$

$$•CH(CH_3)OH + POBN \rightarrow POBN–CH(CH_3)OH \quad (5)$$

The detection of •OH in a solution containing a viscous polymer by this method was carried out by adding POBN together with EtOH to BBG solution before AH_2 and Fe^{2+} (referred to as *initial addition*, since the spin trap is present during the incubation). The ESR spectra of sample aliquots at different times of incubation (from 2 min up to 60 min) were recorded. The recorded spectra exhibited a signal with hyperfine coupling constants (a_N = 15.5 G and a_H = 2.6 G) characteristic to the spin adduct POBN–$CH(CH_3)OH$, and the intensity of the signal increased as the reaction proceeded (data not shown). These results suggested that using POBN together with EtOH was a suitable system due to the fact that it generated stable spin adducts able to accumulate in sufficient amount to be detectable by ESR.

Therefore ESR measurements were carried out on the same sample composition on a longer time scale (from 0 min up to 7 days) to monitor the height of the ESR signal as a function of time. It was observed that the adduct concentration progressively increased up to 24 h without presence of a lag phase, and after that decreased very slowly until 72 h and then remained relatively stable (Fig. 3a). The fact that the intensity of the ESR signals only increased during the first 24 h implied that the spin adducts either stopped accumulating or began to degrade. Therefore having POBN/EtOH present in the samples for extended periods of time cannot be used to evaluate the level of •OH formed after 24 h of storage. In order to overcome this problem the method of *periodic addition* of POBN/EtOH was introduced. In this method an aliquot of sample of BBG solution during incubation was drawn, and POBN was added together with EtOH to the sample and the mixture was further incubated for a certain time.

Accordingly to the data reported by Kivelä, Gates, et al. (2009), it is during the first 6 h of incubation that a BBG solution treated with AH_2 and Fe^{2+} loses most of its viscosity. Therefore in order to follow the formation of •OH precisely during the first 6 h of reaction, the time of incubation of POBN/EtOH should not be too long. At the same time the POBN/EtOH incubation should allow the spin adducts to be formed in a concentration sufficient to be detectable by ESR. It was considered that a time of incubation between 1 h and 2 h would be suitable, however the difference of adduct concentration between 1 h and 2 h was found not to be significant. Therefore 1 h of POBN/EtOH incubation was preferred (Fig. 3b).

When using the POBN/EtOH peridodic addition, the rate of formation of the adducts was found to be stable from 0 min up to 8 h. Between 8 h and 24 h the formation of spin adducts decreased with 41% and then remained relatively stable over the following days (Fig. 3b). Unlike the results obtained with the initial addition of POBN/EtOH showing a decrease of the ESR signal at 24 h, these results showed that the production of •OH may continue even after 24 h.

The method developed (POBN/EtOH with *periodic addition*) quantifies the tendency of •OH formation at a specific time point

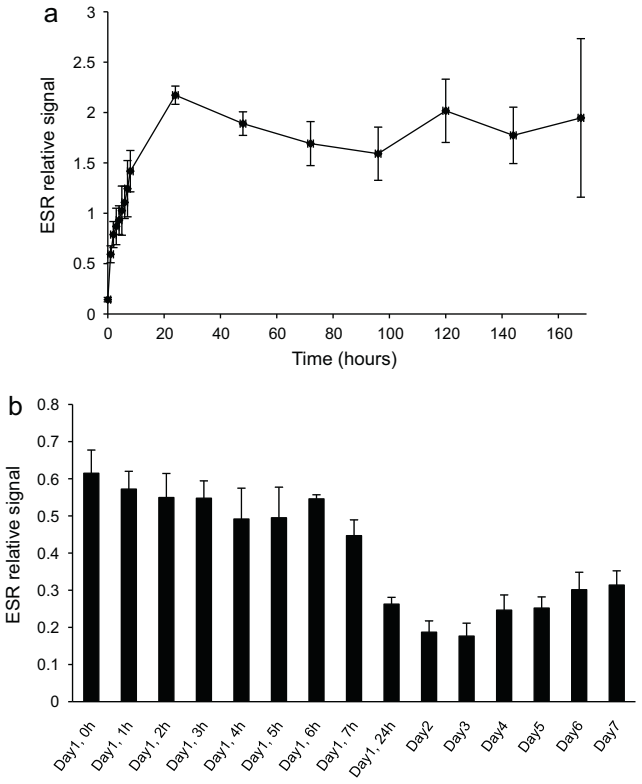

Fig. 3. Intensity of the ESR signal overtime of 0.6% BBG solution containing 250 μM AH_2, 50 μM Fe^{2+} with 2% EtOH and 80 mM POBN with (a) initial addition and (b) periodic addition at different times of incubation. In periodic addition the time indicated is the time of sampling. After the POBN addition, the sample was incubated for 1 h to allow the formation of the spin adduct.

(Frederiksen, Festersen, & Andersen, 2008). Our results showed that the rate of •OH formed decreased significantly between 8 and 24 h, which would suggest that AH_2 lost part of its proxidative potential. The oxidation of AH_2 lead to formation dehydroascorbate (A) (Eqs. (2) and (3)). It has been demonstrated that A is much less effective than AH_2 in the xyloglucan degradation (Fry, 1998). Therefore it appears that the maximal production of •OH involved in the degradation of beta-glucan would occur during the first hours of the reaction until AH_2 becomes less abundant in the system, and the radical formation decreases.

3.1.2.2. POBN in conjunction with DMSO. In addition to EtOH, also dimethylsulfoxide (DMSO) can be used as an initial •OH target molecule for spin trapping (Grace & Logan, 2000; Gunther, Hanna, Mason, & Cohen, 1995) Hydroxyl radicals oxidize DMSO (Eq. (6)) generating methyl radicals ($^{\bullet}CH_3$), which then react with POBN (Eq. (7)) to give stable adducts. The spectrum corresponding to the spin adduct POBN–CH_3 consists of a 6 lines spectrum with hyperfine constants (a_N = 15.9 G and a_H = 2.8 G) (Gunther, Hanna, Mason, & Cohen, 1995).

$$CH_3\text{–}SO\text{–}CH_3 + {}^{\bullet}OH \rightarrow {}^{\bullet}CH_3 + CH_3SOOH \quad (6)$$

$$^{\bullet}CH_3 + POBN \rightarrow POBN\text{–}CH_3 \quad (7)$$

POBN was added together with DMSO in the BBG solution, before the addition of AH_2 and Fe^{2+}, and ESR spectra were recorded from 0 min to 7 days. The ESR spectra of the samples exhibited a signal characteristic of the POBN–CH_3 spin adduct. The results obtained with the POBN/DMSO addition were compared with the results obtained with the POBN/EtOH addition (Fig. 4). The relative intensities of the ESR signals obtained with POBN/DMSO increased with time but had very low amplitudes

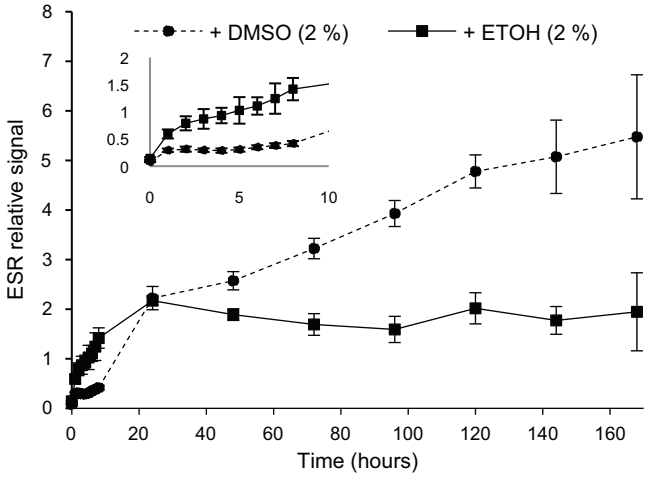

Fig. 4. Comparison of the POBN adduct formation with 2% DMSO and 2% EtOH in a 0.6% BBG solution containing 250 μM AH_2, 50 μM Fe^{2+} with 80 mM POBN.

between 0 and 8 h compared to the similar BBG solution containing POBN/EtOH (Fig. 4). The •OH reacts 2.8 times faster with DMSO than with EtOH (k'' = 5.4 × 10^9 M^{-1} s^{-1} and k' = 1.9 × 10^9 M^{-1} s^{-1}, respectively) (Buxton, Greenstock, Helman, & Ross, 1988; Milne, Zika, & Saltzman, 1989). However our results showed that the combination of POBN/EtOH produced adducts earlier in the experiment than the system POBN/DMSO, which suggested that the $^{\bullet}CH(CH_3)OH$ radicals were either more stable than $^{\bullet}CH_3$ or reacted faster with POBN than $^{\bullet}CH_3$. Bartels and Lawler (1985) reported that the lifetime of $^{\bullet}CH_3$ and $^{\bullet}CH(CH_3)OH$ were 0.2 μs and 1.3 μs respectively, thus $^{\bullet}CH(CH_3)OH$ are more stable than $^{\bullet}CH_3$. Unfortunately no study was available concerning the reaction rate between $^{\bullet}CH_3$ and POBN.

For a longer storage time (from 0 min up to 1 week), it was observed that the ESR signal of the BBG solution with POBN/EtOH decreased after 24 h incubation while the ESR signal of the BBG solution with POBN/DMSO kept increasing up to 7 days (Fig. 4). Based on these results it was assumed that the POBN–CH_3 adducts were more stable at longer time scales than the POBN–$CH(CH_3)OH$ spin adducts.

The POBN/DMSO system gave weak ESR signals during the first hours of the BBG reaction system (Fig. 4, zoomed detail), and during this initial phase it is preferred to use the method with POBN/EtOH and *periodic addition* if the production of •OH is expected to be high. On the other hand, POBN in conjunction with DMSO with *an initial addition* lead to the formation of spin adducts of good stability, which may be useful in cases where the production of •OH is expected to be low and a 1 h POBN/EtOH incubation is not enough to accumulate an ESR detectable amount of spin adduct.

3.1.2.3. Effect of sodium azide on the ESR signal. Sodium azide (NaN_3) is often added to water solution to prevent bacterial growth relevant for longer storage times. Bacteria may degrade beta-glucan through the action of enzymes, which in turn would lead to a decrease in viscosity. Thus we wanted to check whether the addition of sodium azide in beta-glucan solution might influence the oxidation processes and thereby also the intensities of the ESR signals. Three solutions of BBG with 0.01%, 0.02% and 0.06% NaN_3 were prepared and POBN together with EtOH, and further AH_2 and Fe^{2+} were added. ESR spectra were recorded after 60 min of incubation.

The ESR relative signal decreased as the NaN_3 concentration was increased (Fig. 5) meaning that NaN_3 has a significant lowering effect on the height of the ESR signal. It has been demonstrated earlier that in a •OH generating system, the presence of NaN_3 gives rise to formation of azide radicals, which could be then trapped

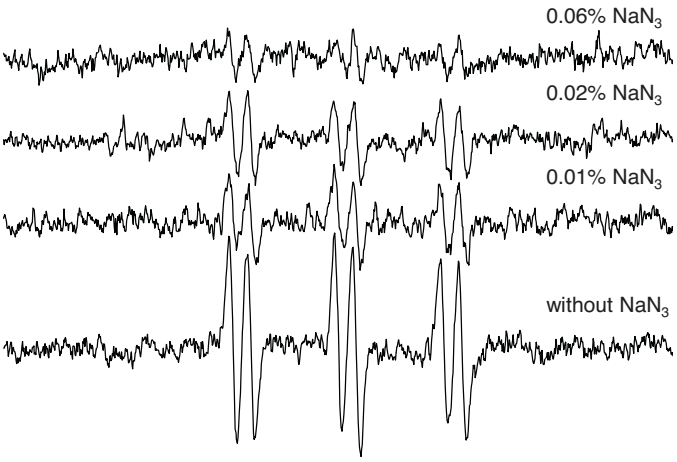

Fig. 5. ESR spectrum of 0.6% BBG solution containing 250 μM AH_2, 50 μM Fe^{2+}, 80 mM POBN and 2% ETOH with different concentration of NaN_3 at 60 min of incubation.

by DMPO, PBN and PyBN leading to the appearance of characteristic ESR signals (Calle, Fernández-Arizpe, & Sieiro, 1996; Kremer & Singh, 1980; Yamamoto & Kawanishi, 1989). The formation of the azide radical was attributed to an •OH scavenging activity of the azide ion by an electron transfer mechanism (Eq. (8)) (Calle, Fernández-Arizpe, & Sieiro, 1996).

$$N_3^- + {}^\bullet OH \rightarrow {}^\bullet N_3 + OH^- \quad (8)$$

Although the obtained spectra did not show the presence of POBN–N_3 adduct, the partial inhibition of POBH–CH(CH_3)OH formation suggested an •OH scavenging activity of NaN_3. In the simultaneous presence of the two •OH scavengers (EtOH and NaN_3), the POBN–CH(CH_3)OH adduct formation results of a competition between these two radical scavengers in quenching •OH. Therefore, a decrease of the ESR signal demonstrates the efficiency of NaN_3 in scavenging •OH. Moreover the decrease of the POBN–CH(CH_3)OH formation with an increase of NaN_3 concentration, shows that higher is the level of NaN_3 applied to the system higher is the proportion •OH quenched (Fig. 5).

A previous study has reported the great potential of NaN_3 as oxidation inhibitor due to its •OH scavenger activity (Yoon, Lee, & Kang, 2010). It was described that NaN_3 could prevent prevent lipid peroxidation in a ferritin/H_2O_2 system (Yoon, Lee, & Kang, 2010). Even though our system (water based) is different from this study, both results corroborate to show that azide is able to efficiently scavenge •OH and in turn prevent oxidation of molecules. Therefore researchers dealing with biomolecule oxidation should be cautious when using azide as an antibacterial agent.

Our results showed that an attempt to follow •OH formation over time during radical mediated degradation of beta-glucan using ESR in the presence of azide in beta-glucan solution may lead to wrong conclusions. Moreover the •OH scavenging capacity of azide may also lead to erroneous results when studying the viscosity loss of beta-glucan solution during radical mediated degradation.

3.2. Relationship between hydroxyl radical formation and viscosity loss of beta-glucan solution

3.2.1. Effects of the reagents on the radical mediated degradation of beta-glucan

The involvement of the reagents (AH_2 and Fe^{2+}) in the formation of •OH and the consequent viscosity loss were studied using BBG solution of different compositions and analyzing the changes in viscosities and radical contents. Since the amount of •OH formed was expected to be high in solutions containing AH_2, they were analyzed by ESR using POBN/EtOH with *periodic addition*. The samples without AH_2 were first analyzed using the system POBN/EtOH with *periodic addition*, but no signals were obtained even after several repetitions. Thus in these samples DMSO/POBN with *initial addition* were employed in order to detect the formation of •OH in BBG solution and in BBG solution with Fe^{2+}.

Adding only AH_2 to BBG solutions led to production of •OH, which was most probably due to the presence of iron contaminants in the BBG solution (Fig. 6a). The measurement of Fe content by atomic absorption spectroscopy revealed the presence 12.27 ± 0.28 μg/g Fe in the BBG powder. Moreover the formation of POBN adducts during the first hour was found to be 23% lower with only AH_2 added as compared to having both AH_2 and Fe^{2+} present (Fig. 6a). However the formation of adducts in the sample treated with AH_2 alone increased progressively until 8 h exceeding the adduct concentration of the sample with AH_2 and Fe^{2+} at 3 h. From 24 h storage and over the next days the same trend of adduct formation was observed for both solutions (Fig. 6a).

The addition of AH_2 together with Fe^{2+} led to a decrease of viscosity of 51.4% and 81.5% of the BBG solution, after 2 h and 8 h, respectively, which coincide with the phase of stable and high formation of •OH observed between 0 and 8 h by ESR (Fig. 6a and c). From day 1 and over the following days of storage the viscosity decreased from 88% to 94% relating to the phase of stable and low •OH formation occurring from the first day of storage (Fig. 6a and c). The BBG solution where only AH_2 was added showed a viscosity loss of only of 8.6% in 2 h while the BBG solution with AH_2 and Fe^{2+} gave a decrease of 54.1% (Fig. 6a and c). These results match with the ESR data where a lower •OH formation during the first hour was observed in the BBG solution with only AH_2 added than in the solutions with both AH_2 and Fe^{2+} (Fig. 6a and c). However after 8 h the viscosity loss of the BBG solution containing AH_2 and Fe^{2+}, and the BBG solution containing AH_2 were 81% and 74% respectively, therefore most of the viscosity loss of the BBG solution with only AH_2 occur after 2 h of storage (Fig. 6a and c). Moreover the ESR data demonstrated that the •OH formation in the BBG solution with only AH_2 increased 55% between 0 and 8 h, exceeding the •OH level of the BBG solution with AH_2 and Fe^{2+} at 3 h (Fig. 6a). Therefore the increase of •OH formation in the BBG solution with AH_2, lead to a faster decrease of the viscosity. The two solutions had the same apparent viscosity (6.5 mPa s) at 24 h and in the next days the viscosity decrease only of few mPa s, which coincide with the decrease of •OH formation between 8 and 24 h, followed by a stable and low adduct formation over the next days (Fig. 6a and c).

These results demonstrate that AH_2 together with Fe^{2+} promoted the production of •OH which caused viscosity loss of the BBG solution resulting of the beta-glucan degradation. In addition the ESR data show that adding Fe^{2+} with AH_2 in beta-glucan solution lead to an initial •OH formation higher than without addition Fe^{2+}, which was related to a faster decrease of the viscosity between 0 and 2 h. Thus the addition of Fe^{2+} with AH_2 accelerates the process of beta-glucan degradation. It has been reported that beta-glucan is able to complex with Fe^{2+}, and we also hypothesize that the Fe^{2+} contaminant in BBG solution could be complexed to beta-glucan. To this point the complexation would make the intrinsic Fe^{2+} more difficult to access than the Fe^{2+} added free in the BBG solution (Platt & Clydesdale, 1984).

Fe^{2+} alone in BBG induced the formation of POBN–CH_3 spin adducts and the formation of adducts seemed to be linear in time (Fig. 6b). The spin adduct POBN–CH_3 were generated in BBG solution in the absence of the reagents (AH_2 and Fe^{2+}), however in smaller amounts than with addition of Fe^{2+} (Fig. 6b). This was most likely due to the presence of Fe^{2+} contaminant in the BBG solution. The viscosity of BBG solution alone and the BBG solution with Fe^{2+}

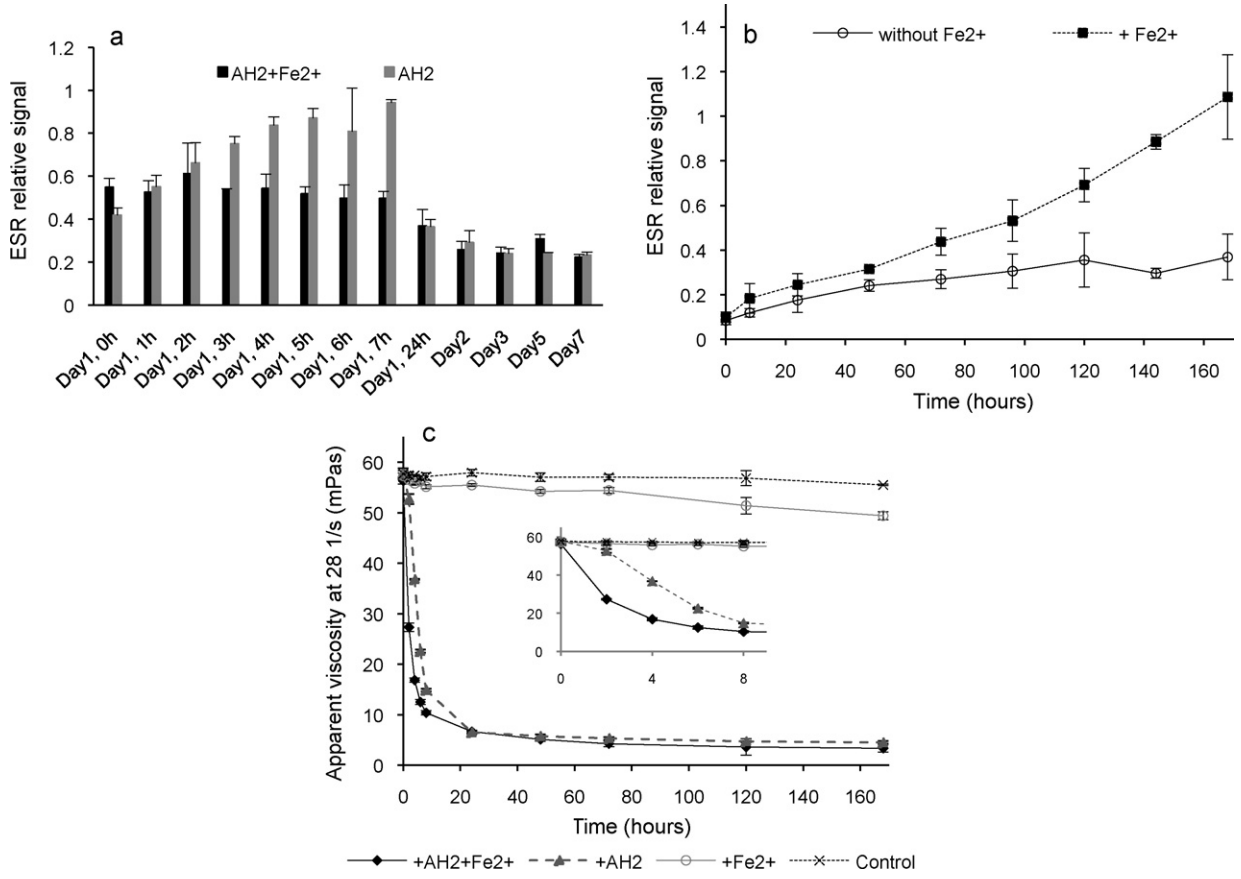

Fig. 6. (a) Intensity of the ESR signal of POBN–CH(CH_3)OH adduct (periodic addition) in BBG solutions containing 2% EtOH, 80 mM POBN, 250 μM AH_2 with and without 50 μM Fe^{2+} at different time of storage. (b) Formation of POBN–CH_3 adduct (initial addition) over time in 0.6% BBG solutions containing 2% DMSO, 80 mM POBN with and without 50 μM Fe^{2+}. (c) The effect of Fe^{2+} (50 μM) and AH_2 (250 μM) alone and together on the viscosity of 0.6% BBG.

decreased by 3% and 14% in 7 days, respectively, which agreed with the ESR data (Fig. 6b and c).

Based on these data the presence of mere Fe^{2+} induced •OH formation, although in much lower degree than in presence of AH_2 in the BBG solution. It has been reported that Fe^{2+} and O_2 could lead to •OH formation due to their ability in producing superoxide and H_2O_2 (Qian & Buettner, 1999). Previous studies have demonstrated that deoxyribose is degraded in presence of Fe^{2+}, and this was attributed to an oxidation of Fe^{2+} by O_2 which in turn would produce superoxide radical, and further on this radical would generates H_2O_2. Therefore all the substrates required for Fenton reaction become available in the solution (Gutteridge, 1984). We saw that the higher •OH formation was correlated with a higher viscosity loss, which clearly shows that there is a relationship between •OH formation and viscosity loss.

3.2.2. Comparison between oat beta-glucan and barley beta-glucan

One of the aims of this work was to compare the formation of •OH radical in OBG and BBG solutions and to link radical formation with the loss of viscosity in the two types of beta-glucan solutions. Therefore the production of •OH, as well as the viscosity loss of OBG and BBG solutions containing Fe^{2+} and AH_2 were monitored over time. When using POBN/EtOH with a periodic addition, the ESR data showed that the production of •OH in the OBG solution was two times lower than in the solution of BBG during the first 6 h (Fig. 7b). However after 24 h of incubation the •OH formation was in the same level in both solutions. Radical formation decreased significantly in the BBG solution, whereas in the case of the OBG a slight but significant decrease in •OH could only be noticed after 3 days (Fig. 7b).

It was initially assumed that the difference in •OH formation rate in the two beta-glucan solutions could be due to a difference of Fe contaminant in the dry beta-glucan powders. Therefore, the Fe content of both beta-glucan dry powders was measured. Surprisingly these results showed a higher Fe contamination in OBG (121.5 ± 3.2 μg/g) than in BBG (12.3 ± 0.3 μg/g). The final added concentration of Fe^{2+} was 50 μM in both solutions, and so the total Fe content of the solution of OBG was much higher than in the BBG solution. Therefore the difference in the intrinsic Fe was obviously not responsible for the difference in •OH production. Since it has previously been shown that beta-glucan can form a complex with Fe, it seemed reasonable to assume that the degree of Fe complexation with the beta-glucan may differ between OBG and BBG, which could lead to the difference in complexed/free Fe (Platt & Clydesdale, 1984).

The flow curves showed approximately 4 times lower viscosities for OBG solution compared to the BBG at 0 min (Table 1; Fig. 7a). Previous studies have reported that OBG solutions are generally more viscous than BBG solution due to a structural difference tri/tetrasaccharide ratio (Mikkelsen et al., 2010). Furthermore, Li, Cui, Wang, and Yada, 2011 explained than beta-glucan in aqueous solution can (within 30 min after sample preparation) form relatively fast aggregates, and also that the degree of aggregation of OBG is higher than the one of BBG. Therefore the higher aggregation of OBG in aqueous solution may contribute to the observed lower viscosity of OBG solutions. In terms of viscosity loss, OBG solution lost 56% of its viscosity at 8 h incubation while the BBG solution already lost 51% of its viscosity after 2 h incubation (Table 1). The viscosity

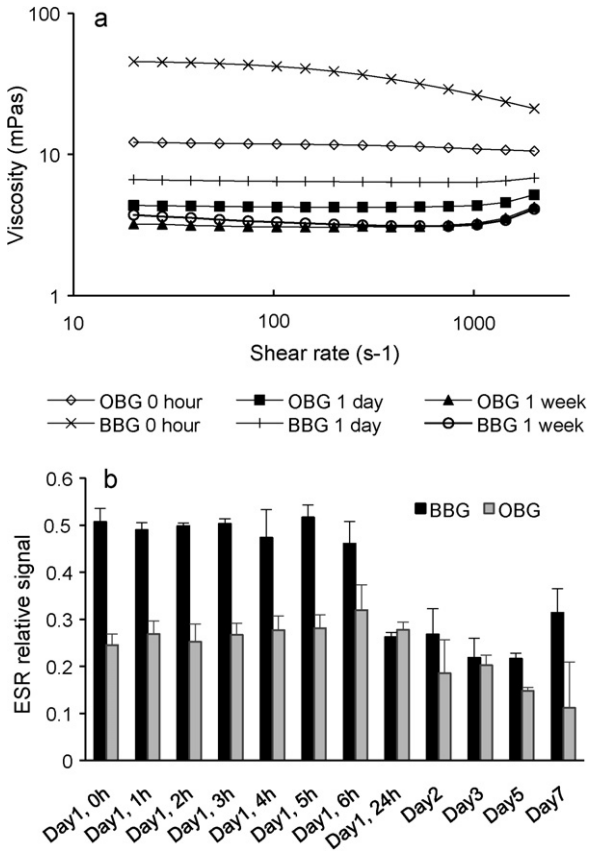

Fig. 7. (a) Flow curves of 0.6% OBG solution and 0.6% BBG solution treated with 250 μM AH_2 and 50 μM Fe^{2+} at 0 h, 1 day and 1 week. (b) Comparison between the intensities of the ESR signal of POBN–CH(CH_3)OH adduct formed (periodic addition) in 0.6% OBG and 0.6% BBG solutions containing 2% EtOH, 80 mM POBN, 250 μM AH_2 and 50 μM Fe^{2+} at different time of storage.

Table 1
Apparent viscosity (at 28 s^{-1}) at different times of incubation of 0.6% OBG solution and 0.6% BBG solution treated with 250 μM AH_2 and 50 μM Fe^{2+}. The percentage of remaining viscosity is obtained by comparing the apparent viscosity at t_0 to apparent viscosity at t_x of the beta-glucan solution.

Apparent viscosity at 28 s^{-1} (mPa s) in function of t		
Time	BBG + AH_2 + Fe^{2+}	OBG + AH_2 + Fe^{2+}
0 h	49.2 ± 3.6 (100%)	12 ± 0.2 (100%)
2 h	24.1 ± 0.6 (49%)	8 ± 0.3 (66%)
8 h	10.6 ± 1.5 (22%)	5.3 ± 0.3 (44%)
1 day	6.9 ± 0.9 (14%)	4.3 ± 0.09 (36%)
3 days	4.4 ± 0.2 (9%)	3.8 ± 0.06 (32%)
5 days	3.9 ± 0.2 (8%)	3.5 ± 0.06 (29%)
1 week	3.9 ± 0.3 (8%)	3.3 ± 0.1 (28%)

of OBG and BBG solution reached similar values after 1 week incubation: 3.3 mPa s and 3.9 mPa s, respectively. These results indicate that the OBG solution loses its viscosity slower than the BBG solution, which is in good relation with the found lower formation of •OH observed in the same solution. The viscosity loss of the OBG solution supported greatly the ESR data.

In order to explain the difference of •OH formation in BBG and OBG solution further work would be needed. However, we showed that for both BBG and OBG solutions, the formation of •OH radical is well related with the viscosity loss of the two solutions.

4. Conclusion

DMPO in the presence of Fe^{2+} could not be reliably used for the spin trapping in BBG solutions, due to DMPO dimerization and poor stability of DMPO–OH spin adducts. ESR spin trapping methods were therefore developed using POBN together with either EtOH or DMSO in order to investigate the AH_2-induced formation of •OH in beta-glucan solutions over time. The results obtained with these spin trapping methods confirmed the formation of •OH in the beta-glucan solutions in presence of AH_2 together with Fe^{2+}, but also with Fe^{2+} alone. It was observed that the formation of •OH was smaller in OBG solution treated with AH_2 and Fe^{2+} than BBG solution treated with the same reagents. However, more work is needed in order to understand these differences. Nevertheless, it was possible to link the viscosity loss of the OBG solution to the level of •OH formation.

The formation of •OH in beta-glucan solution treated with AH_2 lead to a viscosity loss of the solution, demonstrating that beta-glucans are degraded in the presence of •OH radicals.

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