Carbohydrate Polymers 115 (2015) 739–743

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Iron(II) binding by cereal beta-glucan

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ARTICLE INFO

Article history:
Received 12 June 2014
Received in revised form 14 July 2014
Accepted 18 July 2014
Available online 4 August 2014

Keywords:
Beta-glucan
Iron(II) binding
Fenton reaction
Hydroxyl radicals
Kinetics

ABSTRACT

Beta-glucan is a dietary fiber, which possesses several health benefits, such as cholesterol lowering, however this fiber is easily degraded in the presence of Fenton reagents. In the present study, the iron binding capacity of oat beta-glucan and barley beta-glucan was evaluated by investigating the kinetics of the Fenton reaction at pH 2.7 and 4.7 using stopped flow spectroscopy. The rate constant of the Fenton reaction is not affected by the presence of the beta-glucans in a solution pH 2.7, hence none of the beta-glucans bind iron(II) at this pH. However, at pH 4.7, the kinetics of the Fenton reaction vary between acetate buffer ($k = 2.8 \times 10^2\ M^{-1}\ s^{-1}$), barley beta-glucan ($k = 2.2 \times 10^2\ M^{-1}\ s^{-1}$) and oat beta-glucan ($k = 1.2 \times 10^2\ M^{-1}\ s^{-1}$), which demonstrates that barley beta-glucan and oat beta-glucan form complexes with iron(II). Moreover, oat beta-glucan shows a stronger affinity for iron(II) than barley beta-glucan, and may thereby reduce the formation of hydroxyl radicals and diminish the rate of viscosity loss of the oat beta-glucan solution, as shown by the ESR and rheological data. The results presented in this study suggest that cereal beta-glucans can potentially reduce the bioavailability of iron.

1. Introduction

Cereal beta-glucan is a dietary fiber, which occurs naturally in the cell walls of the endosperm and aleurone layer of cereals grains such as barley, oat, rye or wheat (Lazaridou & Biliaderis, 2007). In the last years, beta-glucan has increasingly attracted the interest from the food industry and the scientific community due to its beneficial effect on the human health, most importantly the improvement of glucose metabolism and lowering of cholesterol (Wood, 2007, 2010). Both the European Food Safety Authority (EFSA, 2010) and the U.S. Food and Drug Administration (FDA, 2002) allow the use of a health claim for the role of beta-glucan in reducing the risk of coronary heart disease (EFSA, 2010; FDA, 2002). Although beta-glucan is thus a promising functional food ingredient, its integration into liquid food products remains a challenge due to its instability in solution in the presence of metals. Indeed, beta-glucan in aqueous system is oxidized by hydroxyl radicals produced through the Fenton reaction in the presence of metals such as iron (Faure, Andersen, & Nyström, 2012a; Faure, Sánchez-Ferrer, Zabara, Andersen, & Nyström, 2014). However, no studies on the kinetics of the Fenton reaction in beta-glucan solutions have been reported.

In our last study, we showed that the addition of the Fenton reagents (H_2O_2 and iron(II)) in beta-glucan solutions stored at 85 °C induces the generation of appreciable amounts of hydroxyl radical that causes a quick viscosity loss of the solutions and an extensive degradation of the polysaccharide (Faure et al., 2014). Moreover, the hydroxyl radical-mediated degradation of beta-glucan was accompanied by the formation of peroxyl radical and new, oxidized, functional groups (*i.e.* lactones, carboxylic acids, ketones and aldehydes) on the beta-glucan residues. Several studies have also reported that the treatment of beta-glucan solutions with ascorbic acid and iron(II) leads to a drastic loss of viscosity of the solutions, which demonstrates a degradation of the polysaccharide (Faure et al., 2012a; Faure, Münger, & Nyström, 2012b; Faure, Werder, & Nyström, 2013; Kivela, Gates, & Sontagstrohm, 2009; Kivelä, Nyström, Salovaara, & Sontag-Strohm, 2009). This degradation was directly related to the generation of hydroxyl radicals promoted by the presence of the system ascorbic acid/iron(II) (Faure et al., 2012a). In addition, it was shown that the formation of hydroxyl radicals in beta-glucan solutions treated with ascorbic acid/iron(II) was higher in barley beta-glucan that in oat beta-glucan, therefore it was suggested that oat beta-glucan and barley beta-glucan might have different iron(II) binding capacities (Faure et al., 2012a).

Until now, only one study has demonstrated that beta-glucan binds iron (Platt & Clydesdale, 1984), therefore, a study assessing the capacity of beta-glucan to bind iron(II) is still needed. In the present paper, we studied by stopped flow spectrophotometry the

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http://dx.doi.org/10.1016/j.carbpol.2014.07.038
0144-8617/

kinetics of the Fenton reaction in oat and barley solutions at two different pH values, 2.7 and 4.7. We used ESR spin trapping methods to monitor the formation of hydroxyl radicals in oat and barley beta-glucan solutions, and rheology measurements to monitor the degradation of beta-glucan. In the following, we refer to the reactive product of the Fenton reaction as the hydroxyl radical, although, instead, a higher oxidation state of iron, oxidoiron(IV), may be formed (Koppenol & Bounds, 2011). Indeed, it may well be that near neutral pH the oxidant is oxidoiron(IV) (Bataineh, Pestovsky, & Bakac, 2012).

2. Material and methods

2.1. Material

High viscosity barley beta-glucan (purity >97%) and high viscosity oat beta-glucan (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. Iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from Sigma-Aldrich Chemie GmbH (Germany). Hydrogen peroxide (30%), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were obtained from Merck (Germany).

2.2. Stopped flow spectroscopy

The kinetic experiments were carried out at ambient pressure and 25 °C with a SX17MV stopped flow spectrophotometer made by Applied Photophysics Ltd (Leatherhead, UK). The mixing time was less than 1.2 ms, the cell volume is 80 μl, and the optical path length 10 mm.

2.3. Preparation of beta-glucan solution

The 0.6% solutions of high viscosity barley and oat beta-glucan were prepared by adding 0.6 g of dry beta-glucan to either acetate buffer (40 mM) pH 4.7 or Milli-*Q* water. The solutions were heated in a water bath for 3 h at 80 °C under continuous shaking. After complete dissolution of beta-glucan the volume in volumetric flask was adjusted. In solutions with Milli-*Q* water the pH was adjusted to 2.7 with sulfuric acid.

2.4. ESR spin trapping

The formation of radicals was monitored using a ESR spin trapping method. The spin trapping method used consisted in an indirect detection of the hydroxyl radicals using POBN in combination with EtOH. We have previously shown that this method is suitable for the detection of hydroxyl radicals in beta-glucan solution (Faure et al., 2012a). The mechanism involved is an oxidation of EtOH by hydroxyl radicals that leads to the formation of hydroxyethyl radicals, which are trapped by POBN, resulting in the formation of stable POBN-CH(CH_3)OH spin adducts detectable by ESR. A stock solution of POBN (4 M) was prepared in Milli-*Q* water, and POBN together with EtOH were used at final concentrations of 80 mM and 2% (v/v), respectively. 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. 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 a week of storage time.

2.5. ESR instrument

The samples were loaded in 50 μl micropipettes (Brand GmbH, Wertheim, Germany) and the ESR spectra were recorded with a High Sensitive Benchtop EPR 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 corresponding to the content of spin adducts 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). TEMPO was used as a standard and was measured in triplicate on each day of measurements.

2.6. Viscosity

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

3.1. Effect of barley and oat beta-glucan on the rates of reaction between iron(II) and hydrogen peroxide

The reaction between iron(II) and hydrogen peroxide in oat and barley beta-glucan solutions was monitored at two different pH values, 2.7 and 4.7 by stoppedflow spectrometry. The experiments were performed with hydrogen peroxide concentrations at least 5 times higher than the concentration of iron(II) (500 μM). The time *vs* absorbance traces deviated from a first-order rate law (example shown Fig. 1), hence, a double exponential function was necessary to fit the experimental curve. Under all the conditions tested the pseudo–first-order formation rates of iron(III) (fast phase) increased linearly with increasing hydrogen peroxide concentration (Figs. 2 and 3).

At pH 2.7, no significant differences between the k_{obs} values measured in solutions of oat beta-glucan (1.1×10^2 M^{-1} s^{-1}), barley beta-glucan (1.0×10^2 M^{-1} s^{-1}) and sulfuric acid solution

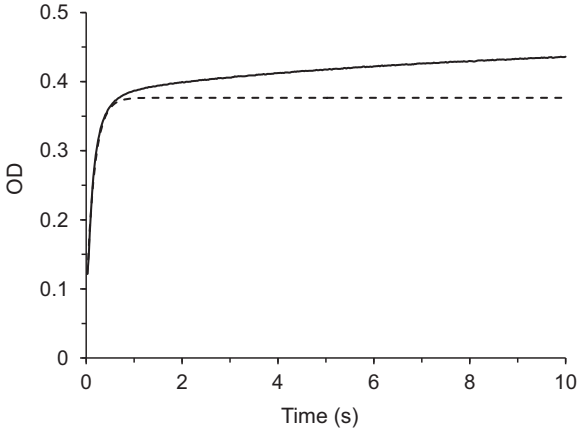

Fig. 1. Kinetic trace observed at 300 nm from the reaction of 0.5 mM iron(II) with 25 mM H_2O_2 in 0.6% barley beta-glucan and 40 mM acetate buffer (4.7). The dashed line indicates the first order kinetic plot closest to the theoretical data.

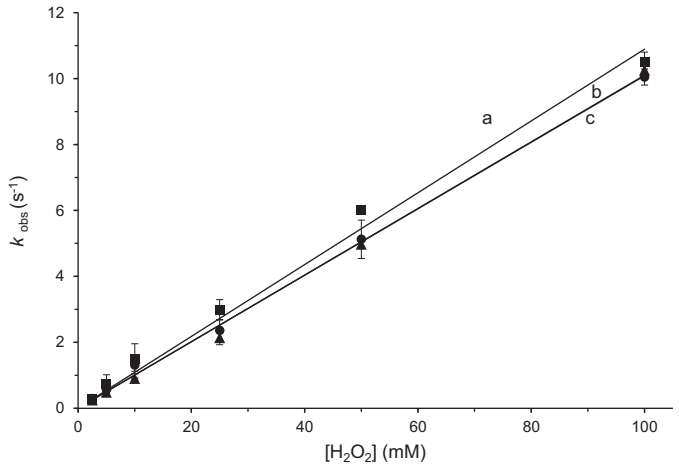

Fig. 2. Plot of the pseudo-first-order formation rates of iron(III) observed at 300 nm as a function of H_2O_2 concentration in (a) sulfuric acid solution (pH 2.7) (b) 0.6% barley beta-glucan, (pH 2.7) and (c) 0.6% oat beta-glucan (pH 2.7) and 0.5 mM iron(II).

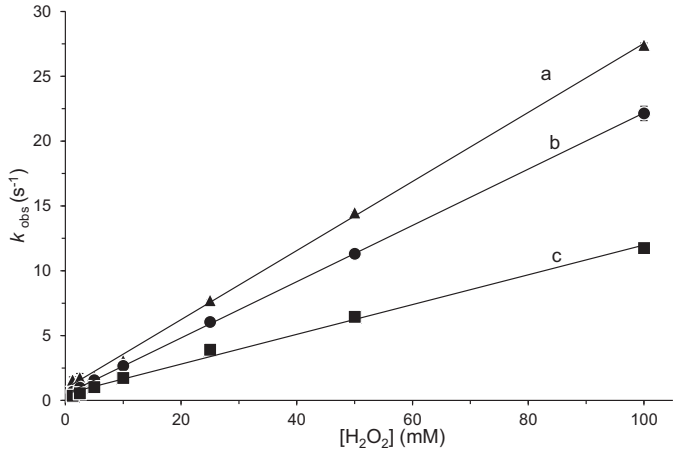

Fig. 3. Plot of the pseudo-first-order formation rates of iron(III) observed at 300 nm as a function of H_2O_2 concentration in (a) 40 mM acetate buffer (pH 4.7) (b) 0.6% barley beta-glucan, 40 mM acetate buffer (pH 4.7) and (c) 0.6% oat beta-glucan, 40 mM acetate buffer (pH 4.7) and 0.5 mM iron(II).

(1.0×10^2 M^{-1} s^{-1}) are observed. This demonstrates that, at pH 2.7, oat and barley beta-glucan do not affect the rate of the Fenton reaction and thus do not bind iron ions. At pH 4.7 the slopes of the regression lines obtained from the plot of the pseudo-first-order formation rates of iron(III) as a function of hydrogen peroxide differ significantly between the different solutions (Fig. 3). Indeed, the rate of the Fenton reaction increases in the following order: oat beta-glucan solution < barley beta-glucan solution < acetate buffer, which indicates that oat beta-glucan binds iron(II) more tightly than barley beta-glucan. As no difference between the solutions was observed at pH 2.7, it appears that the binding of iron(II) to beta-glucans is pH dependent. The highest rate constant for the Fenton reaction was found in acetate buffer, 2.8×10^2 M^{-1} s^{-1}, which is about 4.6 times higher than the one reported in the literature (63 M^{-1} s^{-1}) (De Laat & Gallard, 1999). This result demonstrates that the binding of iron ions to acetate enhances the kinetics of the Fenton reaction. The introduction of beta-glucan (0.6%) in acetate buffer pH 4.7 resulted in a significant decrease of the rate of the Fenton reaction. Indeed, the measured rate constant obtained in the barley beta-glucan solution and in the oat beta-glucan solutions were 2.2×10^2 M^{-1} s^{-1} and 1.2×10^2 M^{-1} s^{-1}, respectively. This indicates that barley and oat beta-glucan form complexes with iron(II) that react slower with H_2O_2 than iron(II)–acetate complexes.

Table 1
Apparent viscosity (at 28 s^{-1}) at different time of incubation of 0.6% oat and barley beta-glucan solutions treated with H_2O_2 (100 mM) and iron(II) (0.5 mM). The percentage of remaining viscosity is obtained by comparing the apparent viscosity at t_0 to the apparent viscosity at t_x of the beta-glucan solutions. Different letters within one time point denote a statistically significant difference ($p < 0.05$).

Apparent viscosity at 28 1/s (mPa s) in function of *t*		
Time (h)	Barley + H_2O_2 + Fe^{2+}	Oat + H_2O_2 + Fe^{2+}
0	61.6 ± 1.3^a (100%)	13.9 ± 0.4^b (100%)
2	3.9 ± 0.3^c (7%)	4.0 ± 0.2^c (30%)
6	2.5 ± 0.2^d (4%)	2.8 ± 0.3^d (21%)
24	1.3 ± 0.5^e (3%)	1.8 ± 0.1^e (14%)

3.2. Hydroxyl radical mediated degradation of oat and barley beta-glucan at pH 4.7

The aim of the present study was to evaluate whether the difference observed between the rate constant of the Fenton reaction in oat and barley beta-glucan at pH 4.7 was related to the rate of beta-glucan degradation. Therefore, the formation of hydroxyl radicals as well as viscosity changes in oat and barley beta-glucan solutions at pH 4.7 treated with H_2O_2 (100mM) and iron(II) (0.5 mM) were monitored in parallel. The ESR data show that the spin adduct concentration was constant up to 24 h in both oat and barley beta-glucan solutions, which indicates that during that time formation of hydroxyl radical took place (Fig. 4). Furthermore, at all the time points the spin adduct concentration was higher in the barley beta-glucan solution than in the oat beta-glucan solution (Fig. 4). For instance at 1 h, the ESR relative signal obtained in the barley beta-glucan solution was 1.6 times higher than the one obtained in oat beta-glucan solution. Barley and oat beta-glucan solutions at similar concentration (0.6 wt%) exhibit different initial apparent viscosities, 61.6 and 13.9 mPas, respectively. Therefore the percentage of remaining viscosity was used to compare the viscosity changes between the two solutions (Table 1). After 2 h, the viscosity of an oat beta-glucan solution treated with H_2O_2 (100mM) and iron(II) (0.5 mM) is 30% of the original solution, while the barley beta-glucan solution treated with the same reagents had a remaining viscosity of only 7% (Table 1). After 24 h, the apparent viscosities of oat and barley beta-glucan were almost equal, 1.3 and 1.8 mPas, respectively (Table 1). These results demonstrate that the viscosity of the barley beta-glucan solution decreases significantly faster than that of the oat beta-glucan solution, which fits with the observation of a faster generation of hydroxyl radicals observed in barley beta-glucan.

4. Discussion

In the present study, we determined the kinetics of the oxidation of iron(II) by H_2O_2 in oat and barley beta-glucan solutions, and investigated whether these cereal beta-glucans bind iron(II). We carried out stopped flow spectroscopy experiments to analyze the kinetics of the iron(III) formation observed at 300 nm. At all the conditions tested, the formation of iron(III) did not follow a first-order rate law and a double-exponential function was required to fit the kinetics trace. The two phases could be clearly distinguished: a fast phase, which corresponds to the Fenton reaction, and a slow phase that indicates the presence of a secondary process after the Fenton reaction. The k_{obs} of the secondary process were always lower than the k_{obs} of the Fenton reaction. Because all the solutions were saturated with argon, it seems unlikely that the secondary reaction corresponds to the auto-oxidation of iron(II), also given that H_2O_2 was present in excess. However, iron(III) at pH > 2

forms iron hydroxide complexes, which undergo further condensation; these mixed iron(III)-oxido and iron(III)-hydroxido complexes have higher extinction coefficients than iron(III)$_{aq}$ between 290 and 400 nm (Faust & Hoigné, 1990). Therefore, we assume that the slow process corresponds to the condensation of iron(III)-hydroxido complexes. In the case of the beta-glucan containing solutions, it is also possible that, in addition to the condensation process, iron(III) forms complexes with the fiber. Because these processes occur on a slower time scale, we are still able to determine the rate constants of the Fenton reactions.

The iron binding capacity of cereal beta-glucans is dependent on the pH of the beta-glucan solutions. Indeed, at pH 2.7, none of the beta-glucans bound iron since the kinetics of the Fenton reaction in the various solutions (control, barley and oat beta-glucan solutions) was identical, with k values around $1.0 \times 10^2\ M^{-1}\ s^{-1}$. However, at pH 4.7, the kinetics of the Fenton reaction were slower in both oat beta-glucan ($k = 1.2 \times 10^2\ M^{-1}\ s^{-1}$) and barley beta-glucan ($k = 2.2 \times 10^2\ M^{-1}\ s^{-1}$) than in acetate buffer ($2.8 \times 10^2\ M^{-1}\ s^{-1}$), hence at this pH both beta-glucans formed complexes with iron(II). These results are in good agreement with the findings of Platt and Clydesdale (1984), who showed that in acidic barley beta-glucan solution (pH 2.0) the portion of iron bound to the fiber was about 60% while it was about 95% at pH 5.7. There is no further data available in the literature concerning the metal chelating properties of beta-glucan, however, a considerable amount of literature has been published on the metal binding capacity of other dietary fibers showing that, in general, dietary fibers release iron at lower pH and bind it again at higher pH. For instance, Thompson and Weber (1979) showed that part of the iron bound to brans, hulls and cellulose become soluble after acidic treatment, but when the pH was increased up to pH 6.8, the metal was bound again to the polysaccharides. Similarly, Torre, Rodriguez, and Saura-Calixto (1995) found that at pH > 3 the iron binding capacity of dietary fiber material increases. However, it is difficult to directly compare our results with those obtained with other types of dietary fibers since they all differ in structure and charge. Our results are highly relevant to the physiological effect of beta-glucan, because they give a first indication of the effect of beta-glucan on the bioavailability of iron. One may assume that beta-glucan releases iron(II) in the stomach since the pH is about 2, but as soon as the pH is increased in the duodenum (pH 6 to 6.5), beta-glucan would bind iron again, which leads to a reduction in iron absorption. Therefore, although beta-glucan possesses health benefits such as decreasing the level of cholesterol, it may potentially decrease the bioavailability of nutritionally important minerals such as iron. However, this is only a hypothesis that needs further investigation.

The stopped flow data did not only reveal that beta-glucan binds iron(II) in a pH-dependent manner, but also showed a difference in iron binding between oat beta-glucan and barley beta-glucan. The kinetics of the Fenton reaction were faster in the barley beta-glucan solution than in oat beta-glucan solution, from which we concluded that oat beta-glucan has a higher affinity for iron than barley beta-glucan. The major difference between the structures of oat beta-glucan and barley beta-glucan is that they present different molar ratio of cellotriose (DP3) and cellotetraose (DP4) units (DP3/DP4). For instance, Wood, Weisz, and Blackwell (1994) have reported that oat beta-glucan exhibits a molar DP3/DP4 value (2.1–2.4) that is lower than barley beta-glucan (2.8–3.4) . This implies that oat beta-glucan exhibits higher amount of beta-(1 → 4)-linkages compare to barley beta-glucan and is, therefore, closer to the structure of cellulose than barley beta-glucan. Platt and Clydesdale (1984) showed that, at pH 5.7, cellulose possesses a higher capacity to bind iron; however, most of the complexes formed were insoluble. During our investigation, the presence of iron(II) with oat beta-glucan led to the formation of beta-glucan precipitates (data not shown), hence insoluble material, which

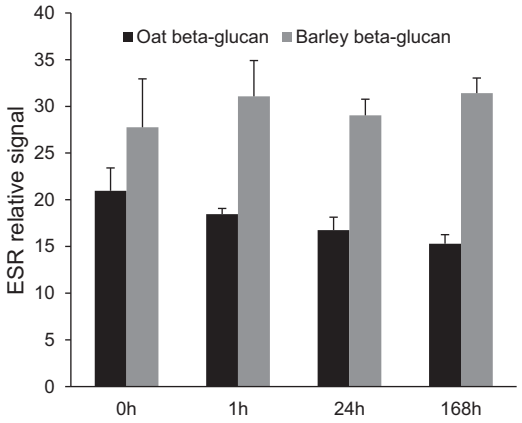

Fig. 4. Radical formation in beta-glucan solutions treated with H_2O_2 (100 mM) and Fe^{2+} (0.5 mM).

was not observed in barley beta-glucan solution that contained iron(II). This relates well to the work of Platt and Clydesdale (1984), who showed that barley beta-glucan forms soluble complexes with iron at pH 5.8. The present results also suggest that the beta-(1 → 4)-linkages are responsible for the difference in iron affinity between oat beta-glucan and barley beta-glucan. Moreover, the higher affinity of oat beta-glucan for iron should reduce the amount of hydroxyl radicals generated *via* the Fenton reaction and thereby slow down beta-glucan degradation, as was demonstrated in this study. In general, the rate constant of the Fenton reaction increases with the number of coordinated water molecules (Graf, Mahoney, Bryant, & Eaton, 1984). In order to check this hypothesis, the formation of hydroxyl radicals and the degradation of beta-glucan as demonstrated by loss of viscosity was monitored in oat and barley beta-glucan solutions pH 4.7.

The amount of hydroxyl radicals generated by the Fenton reaction (H_2O_2, 100 mM, and iron(II), 0.5 mM) was always higher in the barley beta-glucan solution than the oat beta-glucan solution (Fig. 4). These ESR data are in qualitative agreement with the stopped flow spectroscopy results, which show that the rate constant of Fenton reaction was greater in barley beta-glucan ($k = 2.2 \times 10^2\ M^{-1}\ s^{-1}$) than in oat beta-glucan ($k = 1.2 \times 10^2\ M^{-1}\ s^{-1}$) solutions. Furthermore, the barley beta-glucan solutions loose their viscosity faster than the oat beta-glucan solutions, which further demonstrates that barley beta-glucan is degraded more rapidly than oat beta-glucan. The present results are also in agreement with our previous work, where we showed that the amount of hydroxyl radical generated by the ascorbic acid (250 μM) and iron(II) (50 μM) system was higher in barley beta-glucan than in oat beta-glucan solutions (during the first 8 h of incubation), and this was related to a more rapid decrease in viscosity of the barley beta-glucan solution (Faure et al., 2012a). Therefore, because oat beta-glucan binds iron(II) better than barley beta-glucan, it produces hydroxyl radical more slowly and thereby protects itself from degradation. The implication of these results is highly relevant to the use of beta-glucans as food-functional ingredients, as it suggests that oat beta-glucan enriched aqueous foods may be more stable than barley beta-glucan ones.

Another interesting aspect of this study is that the formation of hydroxyl radical was sustained for 24 h. Because initially H_2O_2 was present in excess, all iron(II) was oxidized rapidly. In previous studies, we observed that the formation of hydroxyl radical could be sustained for up to a week in beta-glucan treated with solely iron(II). We assumed that beta-glucan has the ability to reduce iron(III) (Faure et al., 2013). Here, too, we may speculate that beta-glucan acts as a reducing agent. Moreover, as iron acts catalytically

in the Fenton reaction (Fenton, 1894), it is possible that beta-glucan radicals reduce iron(III).

In summary, oat beta-glucan and barley beta-glucan do not affect the kinetics of the Fenton reaction at pH 2.7, however at pH 4.7 both beta-glucans reduce the rate of the Fenton reaction. We concluded that both beta-glucans bind iron(II), although oat beta-glucan does so more strongly than barley beta-glucan, most likely due to an higher proportion of beta-(1 → 4)-linkages. The iron binding differences between oat and barley were supported by the ESR data and viscosity data, which demonstrated that the rate of formation of hydroxyl radicals was lower in oat beta-glucan solutions compared to that in barley, and this related with a slower loss of viscosity of the oat beta-glucan solution. In conclusion, the present study demonstrates that cereal beta-glucan complexes iron(II), and this could potentially have an effect on the bioavailability of iron(II) in case of high dietary fiber intake diets.

Acknowledgement

This study was funded by ETH Zurich.

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