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1 **Co-migration of phytate with cereal β -glucan and its role in starch hydrolysis *in-vitro***

2

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Highlights

- Intrinsic phytic acid in β -glucan sample reduced starch hydrolysis *in vitro*.
- Viscosity of β -glucan also contributed to the reduction of starch hydrolysis.
- Phytic acid co-precipitated with β -glucan when using ethanol in isolation.
- Phytic acid did not co-precipitate with β -glucan when using 20% $(\text{NH}_4)_2\text{SO}_4$.

Co-migration of phytate with β-glucan during extraction (precipitation) & Contribution of phytate to reduction of starch hydrolysis *in vitro*

1 **Co-migration of phytate with cereal β -glucan and its role in starch hydrolysis *in-vitro***

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10 **Abstract**

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12 This study investigated the mechanisms of the co-migration of phytic acid during β -glucan isolation
13 and its contribution to the retardation of starch hydrolysis *in vitro*. During the isolation, phytic acid
14 precipitated together with β -glucan when ethanol was added as the precipitation solvent. The
15 precipitation of phytic acid was reduced by lowering the pH or the ethanol concentration. When 20%
16 $(\text{NH}_4)_2\text{SO}_4$ was used as the precipitation solvent, only minor phytic acid was found in isolated β -
17 glucan, because phytic acid did not precipitate by this solvent. In the *in vitro* starch hydrolysis test,
18 the isolated oat β -glucan (OBG) containing 3.9% co-migrated phytic acid showed better retardation
19 effect than OBG containing 0.6% phytic acid. Therefore, we concluded that the co-migration of phytic
20 acid was dependent on the chosen isolation procedure and conditions, and both intrinsic phytic acid
21 and viscosity contributed to the retardation of starch hydrolysis.

22

23 **Keywords**

24 Cereal β -glucan, phytic acid, phytate, ethanol precipitation, starch hydrolysis

1 Introduction

Cereal β -(1 $\rightarrow$ 3),(1 $\rightarrow$ 4)-D-glucan is a water soluble polysaccharide that has been shown to reduce starch digestion and post-prandial glucose and insulin responses (Kim et al., 2013; Makelainen et al., 2007; Regand et al., 2011). This effect was suggested to be attributed to the ability of β -glucan to form viscous solution at low concentrations. In the digestive tract, the viscous solution of β -glucan acts as a barrier, reducing absorption and diffusion of the nutrients such as glucose, lipid and bile acid directly or via limiting the contact between the digestive enzymes and the food substrates.

When considering health functionality of fiber-rich fractions, quite often the role of compounds that co-migrate with β -glucan are neglected. In cereals, phenolic compounds are mainly associated with water-unextractable non-starch polysaccharides (Rao and Muralikrishna, 2004). On the contrary, more than 90% phytic acid has been found in water-soluble fiber fraction of oats, which is mainly β -glucan (Frolich and Nyman, 1988). Significant amount of phytate in oat β -glucan extract (6% in dw) and in purified oat β -glucan (1.2% in dw) has been reported (Kivela, 2011; Wang et al., 2017a). Wang et al. (2017a) have shown that the phytic acid content in purified barley β -glucan was considerably lower (<0.3%). Nonetheless, how phytic acid associates with β -glucan, and the different phytic acid content in oat and barley β -glucan have not been understood.

Phytic acid is regarded as an anti-nutrient which deteriorates mineral absorption. It is also a natural antioxidant which chelates transition metals and/or shifts the redox of oxidation. Recently, Wang et al. (2017a,b) have found that the residual phytate protected the oat β -glucan from oxidative degradation and retarded the lipid oxidation in emulsions containing β -glucan. Phytic acid has been shown to inhibit enzymes (e.g. trypsin and α -amylase) within the digestive tract therefore leading to inhibition of digestion and absorption of dietary components. Phytic acid may bind to starch by hydrogen bond, bind to enzymes like α -amylase and starch granule proteins by electrostatic interaction, or bind to enzyme cofactors such as Ca^{2+} , therefore reducing the rate of digestion and absorption of starches (Schlemmer et al., 2009; Thompson et al., 1987). The role of phytic acid and viscosity of oat β -glucan on reduction of starch digestion has been shown separately. Nonetheless, the contribution of residual phytate in oat β -glucan to the reduction of starch digestion has not been considered and evaluated. This study aimed to investigate 1) the role of residual phytate in oat β -glucan, in addition to the viscosity by β -glucan, in the starch hydrolysis and 2) the factors influencing the phytic acid content in isolated β -glucans.

2 Materials and methods

2.1 Isolation of β -glucan

Oat β -glucan (OBG) and barley β -glucan (BBG) were extracted from oat bran concentrate (14% β -glucan “as is” basis, Oatwell, Swedish Oat Fibre, Sweden) and barley bran concentrate (16% β -glucan “as is” basis, Bonafiber Oy Lahti, Finland) using MilliQ water (7g bran/100 ml water) at 40°C in a shaking incubator for 30 min. After centrifuging the dispersions (10 000 rpm, 10 min), the supernatant was collected and placed in a boiling water bath for 10 minutes to precipitate proteins. After boiling, the dispersions were centrifuged again, and the supernatant was collected. Porcine pancreatin (18.75 mg/ml in 150 mM NaHCO₃) (Sigma, USA) was then added to the supernatant and incubated at 37°C for 10 min. The enzymes were inactivated by placing the dispersions in a boiling water bath for 10 minutes before undergoing another centrifugation. The β -glucan in the collected supernatant was precipitated by gradually adding two volumes of 96% ethanol. The collected precipitate was washed by ethanol at least 3 times before oven dried at 60°C overnight to obtain the isolated β -glucan. The moisture content of the isolated β -glucan was around 5-7%.

2.2 Phytate removal

Phytate in OBG samples was removed by ion exchange resin before ethanol precipitation according to Wang et al. (2017a). The β -glucan solution was acidified (pH 3) and stirred vigorously with activated resin (Amberlite IRA-410 Chloride form 20-25 mesh, Sigma-Aldrich, Saint Louis, USA) at 4°C for 2 hours before filtrated and transferred to a dialysis bag for dialysis against water (48h). Dialyzed β -glucan solution was then used for ethanol precipitation and drying.

2.3 Characterization of isolated oat β -glucan

The content of β -glucan, starch and phytate in the oat β -glucan extract were measured using mixed-linkage β -glucan assay, total starch assay, phytic acid (total phosphorus) assay, respectively, obtained from Megazyme, Ireland. The protein content was analyzed using Dumas combustion method (N $\times$ 6.25, Vario, Germany). Quantification of minerals including calcium and phosphorus were performed with inductively coupled plasma mass spectrometer (ICP-MS, Perkin-Elmer Elan 6000, USA) with an external standard.

2.4 Starch hydrolysis by α -amylase

Wheat starch (C-gel, Cargill) was dispersed in 0.05 M sodium phosphate buffer (pH 6.9) containing 0.05 M NaCl and gelatinized by boiling for 15 min. Isolated oat β -glucans with and without phytate (OBG and OBGPR) were used to study the role of residual phytate in starch hydrolysis. OBGPR contained a higher amount of β -glucan due to the phytate removal, which was considered when preparing β -glucan solution to give the same concentration. Phytic acid sodium salt (0.04%, w/v,

88 Sigma, USA) and calcium chloride (80 μ M, VWR International Oy, Finland) in similar amount as in
89 isolated β -glucan were used as controls. In order to study the effect of viscosity and intrinsic phytic
90 acid on the starch hydrolysis, OBG and OBGPR were treated with lichenase and phytase to degrade
91 β -glucan and phytic acid, respectively. Beta-glucan stock solutions (1%) were incubated with
92 lichenase (2 U/ml β -glucan, mixed-linkage β -glucan assay, Megazyme) or phytase (0.01 ml/ml β -
93 glucan, Phytic acid Total Phosphorus kit, Megazyme) at 50°C for 1 hour. Enzyme treated solutions
94 were heated at 100 °C for 10min to inactivate the enzymes. Viscosity value of 0.43% (w/v) β -glucan
95 and hydrolyzed β -glucan solution were also measured using a rheometer (Haake Rheostress 600,
96 Thermo Electron GmbH, Germany) with a cone and plate geometry (35 mm, 2°). The measurement
97 was carried out with shear rate ranges from 10 s⁻¹ to 100 s⁻¹ at 20°C. The viscosity value at a shear
98 rate of 10 s⁻¹ was used for comparison of samples. Triplicates were prepared for this measurement.

99 Three ml of oat β -glucan solution (1%, w/v) or phytic acid sodium salt solution (0.04%) were pre-
100 incubated with 0.1 ml of α -amylase (24U/ml, Sigma, USA) with addition of 1.9 ml 0.05 M sodium
101 phosphate buffer (pH 6.9) at 37°C for 30 min before adding to 2 ml gelatinized starch (7% w/v, 37°C).
102 The mixtures were incubated at 37°C for 20 min with continuous shaking. The amount of reducing
103 end was then analyzed with DNS (3,5-dinitrosalicylic acid) assay. 0.5 ml of hydrolyzed starch was
104 taken and added with 0.5 ml sodium phosphate buffer (0.05 M, pH 6.9) and 1.5 ml DNS reagent, after
105 which the mixture was heated at 100°C for 5 min. The absorbance was measured at 540 nm using
106 spectrophotometer (UV-1800, Shimadzu, Japan). The DNS reagent was prepared by gradually adding
107 30 g potassium tartrate tetrahydrate in pre-dissolved 3,5-dinitrosalicylic acid (1g/50ml H₂O, Sigma,
108 USA). Then 20 ml 2M NaOH (Sigma, USA) was added and the volume was adjusted to 100 ml with
109 water before stored at dark in room temperature. Standard curve was made by using 0.1-1.2 g/L
110 maltose solution to react with DNS reagent. The amount of reducing sugar in original OBG, degraded
111 OBG and starch without hydrolysis were measured to differentiate the amount of reducing sugar
112 released by the starch hydrolysis. The content (%) of digested starch in β -glucan samples were
113 calculated by comparing the amount of reducing sugar content in the tested samples with the control
114 sample which was the sample containing only starch and α -amylase. Thus the digested starch in this
115 control sample is regarded as 100%.

116 2.5 Manipulation of β -glucan isolation steps

117 To investigate the critical factors that influences the content of phytic acid in β -glucan, treatments
118 including heating (Factor **I**), changes of pH (Factor **II**) and precipitation solvent (Factor **IV**), ion
119 exchange (Factor **III**) and dialysis (Factor **V**) were separately done during β -glucan isolation (Figure
120 1). In Factor **I**, barley bran concentrate was refluxed with hot ethanol at 70°C for 2 hours to inactivate

121 endogenous enzymes, dried in the oven before water extraction and compared with samples without
 122 refluxing (Figure 1). Commercial oat bran concentrate was enzyme inactivated, therefore additional
 123 refluxing with ethanol was not done. Phytate is insoluble at neutral pH and tends to be more soluble
 124 at lower pH (Persson et al., 1998), therefore in Factor **II**, pH was adjusted to 3 before ethanol
 125 precipitation to compare with the samples without pH adjustment (pH 7). In Factor **III**, phytate was
 126 removed by ion exchange resin before ethanol precipitation, which was described in 2.2. It has been
 127 shown that β -glucan also precipitates with 20% $(\text{NH}_4)_2\text{SO}_4$ (Westerlund et al., 1993), therefore, the
 128 isolated β -glucan containing intrinsic phytate was dissolved in water (0.4%, w/v) at 80°C for 2 hours
 129 and re-precipitated with 20% $(\text{NH}_4)_2\text{SO}_4$ after which the re-precipitated β -glucan was dissolved and
 130 dialyzed to remove $(\text{NH}_4)_2\text{SO}_4$ (Factor **IV**). After dialysis, β -glucan solution was ethanol precipitated
 131 and oven dried. To study the effect of dialysis alone on the phytic acid content, the isolated β -glucan
 132 was dissolved (0.4% w/v) and dialyzed at pH 3 (HCl) and pH 7 (water) (Factor **V**).

133 **2.6 Precipitation of pure phytic acid with different solvents**

134 To understand the behaviour of phytic acid in different solvents, phytic acid sodium salt (1%, w/v)
 135 was dissolved in water and the pH of the solution was adjusted to pH 3.0, 4.6 (as such), and 7.0 by
 136 HCl and NaOH solutions. Phytic acid solution (10ml) was mixed with 1 volume (10ml), 1.5 volumes
 137 (15ml) or 2 volumes (20ml) of 96% ethanol. The phytic acid solution (pH not adjusted) was also
 138 mixed with 2 volumes of 30% $(\text{NH}_4)_2\text{SO}_4$ to reach 20% in the final mixture. Additionally, *myo*-
 139 inositol solution (1%, Sigma, USA) was mixed with 2 volumes 96% ethanol. All the mixtures were
 140 centrifuged at 7000 rpm for 10 min. The supernatant in each tube was carefully descanted and the
 141 precipitate in the bottom of the tube was oven dried at 60°C overnight before weighing. The
 142 percentage of precipitated phytic acid in each condition was calculated as:

$$143 \text{ Phytic acid precipitate (\%)} = (W_{\text{total}} - W_{\text{tube}}) / W_{\text{phytic acid}} * 100\%$$

144 where W_{total} is the weight of tube and precipitate, W_{tube} is the weight of empty tube, and $W_{\text{phytic acid}}$ is
 145 the total weight of added phytic acid in the solution which is 0.1g (10ml * 1%).

146 **2.7 Statistical analysis**

147 Statistical analyses were performed on Statistical Package for the Social Science (SPSS Statistics
 148 version 23, IBM), using one-way analysis of variance (ANOVA) followed by post hoc Turkey test.
 149 Differences were considered as significant at $p < 0.05$. All the results were presented as means $\pm$
 150 standard error.

151 **3. Results and Discussion**

152 3.1 Starch hydrolysis *in vitro*

153 3.1.1 Role of phytic acid and calcium from β -glucan in starch hydrolysis

154 OBG contained 3.9% phytic acid and OBGPR contained 0.6% phytic acid. The starch and protein
155 content in OBG was 2.0% and 8.2%, respectively. The intrinsic phytate in OBG played a role in the
156 retardation of starch hydrolysis. OBG (0.43%) significantly reduced the starch hydrolysis (79%) after
157 20 min comparing to the control samples without OBG addition (100%) (Figure 2). Pre-incubation
158 of OBG samples or phytic acid with α -amylase was needed for this retardation effect, which was also
159 shown by earlier studies (Deshpande and Cheryan, 1984). When phytate was removed from OBG,
160 the starch hydrolysis was 94%. Addition of pure phytic acid (in similar amount as in OBG) in OBGPR
161 contributed to the reduction of starch hydrolysis (84%), whereas the reduction extent was less than
162 OBG which contained intrinsic phytate. Pure phytic acid alone reduced the starch hydrolysis to 91%.
163 This agrees with the results shown by Knuckles and Betschart (1987) that 5mM phytate reduced
164 starch digestion (salivary α -amylase induced) to 86% at pH 6.9. Similarly, Thompson et al. (1987)
165 have shown that endogenous and added phytic acid in navy bean flour reduced the *in vitro* rate of
166 starch digestion and blood glucose response in humans. They suggested that phytate reduced starch
167 digestion by directly binding with starch or interaction with amylase enzymes.

168 Björck and Nyman (1987) showed that the addition of phytic acid inhibited the early stage of starch
169 hydrolysis (<30min) but had negligible influence on α -amylase activity and starch hydrolysis after 1h
170 even though preincubation of phytic acid and enzyme was performed. Later on in 1989, the same
171 authors showed that phytic acid did not inhibit the *in-vivo* starch digestion with a rat model (Nyman
172 and Björck, 1989). The diet contained raw wheat starch, sugar-beet fiber and casein as protein source,
173 which are all possible substrates to interact with phytic acid. When the interactive sites of phytic acid
174 are fully occupied by the substrates, the chelation or binding ability of phytic acid to enzymes and
175 metals are reduced.

176 Calcium is known to enhance α -amylase activity, and pure calcium at 0.8 μ M (same amount in OBG)
177 increased the starch hydrolysis to 250% (Figure 3) comparing to control samples without calcium
178 addition. According to Thompson et al. (1987), molar ratio of Ca: PA as 0.57 reduced the starch
179 digestion, the increase of this ratio to 2.2 reduced starch digestion but had less reduction. Yoon et al.
180 (1983) have shown that high Ca concentration (18.35 Ca/ PA molar ratio) enhanced the *in vitro*
181 starch digestibility. This agrees with our observation that when 0.5 mM CaCl_2 was added, starch
182 hydrolysis was not reduced by OBG containing intrinsic phytate and OBGPR with phytic acid
183 addition.

184 Ion exchanging and dialysis removed phytic acid as well as minerals including Ca. When the same
185 amount of phytic acid and Ca were added back to OBGPR, the starch hydrolysis was not inhibited
186 but enhanced. The added pure phytic acid and Ca could not form the same complex as intrinsic phytate
187 in OBG. The enhancing effect of Ca overwhelmed the inhibitory effect of phytic acid. Wang et al.
188 (2017b) also reported that the added phytic acid was not as effective as intrinsic phytic acid retarding
189 β -glucan oxidative degradation.

190 **3.1.2 Role of viscosity in starch hydrolysis**

191 OBG and OBGPR at 0.43% concentration had similar viscosity (300 mPa·s at shear rate 10 s^{-1}) and
192 phytate removal did not affect the viscosity of β -glucan. OBGPR reduced the starch hydrolysis
193 compared to the control samples although the phytate was removed, which indicates that other factors
194 such as viscosity contributed to the reduction. Addition of lichenase in OBG and OBGPR
195 significantly degraded the β -glucan molecules and decreased the viscosity to 10 mPa·s. After β -glucan
196 degradation, the starch hydrolysis was enhanced (Figure 3). This was expected as the previous studies
197 have shown that viscosity of β -glucan inhibits enzyme accessibility to starch and reduces starch
198 hydrolysis (Symons and Brennan, 2004; Zhang et al., 2017). After hydrolysis, OBG increased starch
199 hydrolysis more than OBGPR likely because the higher content of minerals in OBG which were
200 released and promoted the starch hydrolysis.

201 Phytase was used to degrade the endogenous phytate and release minerals. Additionally, phytase also
202 degraded β -glucan and lowered its viscosity to 16 mPa·s. Jaskari et al. (1995) also observed that
203 phytase preparation used for hydrolysis of phytic acid caused a reduction in viscosity of oat bran
204 slurries due to the side effect of phytase. Phytase addition in OBG considerably increased the starch
205 hydrolysis (2 times) because of the viscosity loss, phytate degradation and mineral (Ca) release.

206 **3.2 Changes of phytic acid content during isolation of β -glucan**

207 Oat and barley bran concentrates contained 1.3% and 0.6% phytic acid (w/w), respectively. Previous
208 studies have reported that the phytic acid content in oat and barley grain is 0.4-1.2% dry matter basis,
209 and higher amount can be found in bran fractions (Garci'a-Esteva et al., 1999). The isolated oat β -
210 glucan (OBG) produced with regular water extraction protocol contained 66% β -glucan and 3.9%
211 phytic acid ("as is" basis) (Table 1). With the same protocol, the isolated barley β -glucan (BBG) had
212 similar level of β -glucan (62%) but significantly lower content of phytic acid (0.5%). We investigated
213 the influencing factors on the phytate content of β -glucan samples, and found that the procedure for
214 β -glucan isolation was of importance for the co-migration of phytate.

215 **Refluxing** the barley bran concentrate with hot ethanol before water extraction (Factor **I**, Table 1)
216 effectively increased the phytic acid content to 2.2%, and increased the viscosity of the extract from
217 9 to 50 mPa·s. Studies have used heat treatment to inactivate the endogenous β -glucanases and obtain
218 β -glucan with high molecular weight (Zheng et al., 2011). According to the current results, the ethanol
219 refluxing also inactivated the endogenous phytase, therefore increased the amount of phytate in the
220 isolated barley β -glucan. This was also evidenced by a considerable reduction of the free phosphorus
221 content from 634 to 106 mg/100g in BBG after refluxing (Table 1). We could infer that the heat
222 treatment of the commercial oat bran concentrate contributed to the high amount of phytate in OBG.
223 The phytic acid content in isolated β -glucan was more than 2 times higher than in the starting raw
224 material after heat treatment, which indicated that phytic acid accumulated during β -glucan isolation.
225 Phytic acid also carried the cations (most abundantly Ca and Mg), which resulted in an increase of
226 mineral content in isolated β -glucan compared to the starting bran concentrates (Table 1). Wang et
227 al. (2017a) also reported that the mineral content in commercial β -glucans was higher in the samples
228 containing higher amount of residual phytate.⁷

229 **Precipitation solvents** chosen for β -glucan collection after extraction made a difference on phytate
230 content in the final extract. Ethanol (50-70%) is commonly used as a solvent to precipitate soluble
231 fibers in lab scale as well as in industrial scale. In our study, we used 2 volumes of ethanol (67%) to
232 precipitate β -glucan after extraction. Surprisingly, we found that phytic acid (sodium salt) itself also
233 precipitated (100%) under the same condition (Table 2). This largely explained the high phytic acid
234 content in isolated β -glucan. The extracted phytate from raw material co-precipitated with β -glucan
235 by ethanol. When 1.5 volumes of ethanol was added, the content of precipitated phytic acid reduced
236 to 24%, and no precipitation of phytic acid was observed when adding 1 volume ethanol (Table 2).
237 In addition, ammonium sulphate (20%, w/v) has been known to precipitate β -glucans, and used in the
238 purification and separation of β -glucan and other cereal gums (Izydorczyk et al., 1998; Westerlund et
239 al., 1993). The phytic acid content in OBG precipitated by 20% $(\text{NH}_4)_2\text{SO}_4$ was only 0.2% (Factor
240 **IV**, Table 1) because we found that pure phytic acid did not precipitate with 20% $(\text{NH}_4)_2\text{SO}_4$ (Table
241 2). This indicates that phytic acid and β -glucan are not covalently linked and the co-migration can be
242 altered by changing precipitation solvents. The co-migration of phytic acid with water soluble fiber
243 from oat was first reported by Frolich and Nyman (1988) and later Kivelä et al. (2011) observed 6%
244 of phytic acid in the isolated β -glucan⁶. In fact, both studies have used ethanol in the collection of β -
245 glucan, which caused the co-precipitation of phytic acid and resulted in the high phytic acid content
246 in the final β -glucan extracts. This also explained the presence of high amount of minerals in the
247 soluble fraction of oats noticed by Frolich and Nyman (1988). Myo-inositol without phosphorylation

248 did not precipitate with ethanol (Table 2) which indicates that the co-precipitated phytates in β -glucan
249 extract are mostly highly phosphorylated inositol phosphate esters. It has been shown that higher
250 phosphorylation degree correlated to better metal binding capacity and antioxidant activity
251 (Miyamoto et al., 2000; Sandberg et al., 1999). Therefore, co-precipitated phytate in β -glucan can
252 play a big role in metal binding and antioxidant activities as shown by Wang et al. (2017b). Other
253 precipitation solvents such as 50% isopropyl alcohol and 50% acetone have been used (Lee et al.,
254 2017) and the behaviour of phytic acid in these solvents should be investigated before using.

255 On the other hand, the precipitation behaviour under ethanol is not exclusive to phytic acid and
256 polysaccharides. Previous studies have shown that calcium and magnesium phosphate also
257 precipitated with ethanol (Babaie et al., 2015). Moreover, ethanol (>64%) has been used to precipitate
258 DNA which contains high amount of phosphate groups in the backbone (Green and Sambrook, 2016).
259 The highly charged phosphate makes DNA polar and water soluble. It is suggested that adding ethanol
260 to solution enhanced the electrical attraction between phosphate groups and any positive ions present
261 in solution and the formation of DNA precipitation. The precipitation of phytic acid by ethanol may
262 have the same mechanisms as for DNA. We showed that the myo-inositol which does not contain
263 phosphorus group did not precipitate with ethanol.

264 **Acidifying** the β -glucan solution before ethanol precipitation reduced the phytate content from 3.9%
265 to 2.6% for oat β -glucan and from 0.5% to 0.2% for barley β -glucan (Factor II, Table 1). It is well-
266 known that lowering the pH largely enhances the solubility of phytate in water (Persson et al., 1998),
267 however, the solubility of phytic acid in ethanol and the role of pH were not reported in literature.
268 We found that pure phytic acid (sodium salt) precipitated with the highest extent (about 100%) at
269 neutral pH when 2 volumes of ethanol were added (Table 2), which was also the conditions used for
270 regular β -glucan extraction. The precipitation of phytic acid reduced to 64% at pH 4.6 and 40% at pH
271 3.0. This explained the reduction of phytate content in OBG and BBG when the β -glucan solutions
272 were acidified before ethanol precipitation.

273 **Dialysis** at neutral pH before ethanol precipitation reduced the phytic acid content to 3.2% in OBG,
274 whereas dialysis at pH 3 reduced it to 0.6% (Factor V, Table 1). Phytate at neutral pH is mostly
275 insoluble and may complex with proteins, minerals and β -glucan forming aggregates that could not
276 effectively pass through dialysis bag (cut-off 14 kDa). At low pH, phytate is more soluble and phytate
277 complex disassociates and could pass through the dialysis bag. Zielke et al. (2018) have reported that
278 protein and β -glucan formed aggregates via electrostatic interactions depending on pH and the minor
279 phosphorus in β -glucan was suggested to contribute to the interaction. In their study, only the free
280 phosphorus content was measured without considering phytate which is the major storage of

phosphorus and contributor to electric charges. Therefore, the role of phytate in aggregation behaviour of β -glucan and its interactions with protein at different pH should be considered.

Ion exchange followed by dialysis (pH 3) removed 85% of the phytic acid in OBG (Factor **III**, Table 1). With the same anion exchange resin, Kumagai et al. (2002) removed 90% of the phosphorus in defatted soybeans. In our study, only dialysis without ion exchanging at low pH removed similar amount of phytic acid. Ion exchanging may reduce the time used for the phytate removal but unnecessarily the amount of removed phytate. Phytate removal also reduced considerably the amount of intrinsic minerals. The content of Ca and Mg in OBG reduced from 330 to 90 mg/100g and from 610 to 160 mg/100g, respectively, after phytate removal (OBGPR) (Table 1). The mechanical reduction of phytate e.g. by milling also causes substantial loss of minerals.

We showed that the phytate content in β -glucan varied largely due to the isolation procedure. The differences of phytate content in β -glucan material may influence the results and conclusions. The co-migrated phytate can offer β -glucan sample such properties as metal binding ability and antioxidant activity. Wang et al. (2017a,b) have shown that the differences of oat and barley β -glucans in iron binding capacity and oxidative stability were due to their phytate content instead of structural differences.

4 Conclusion

Our study demonstrated that not only the viscosity but also the co-migrated phytic acid and minerals in β -glucan play a role in the retardation of starch hydrolysis *in vitro*. The phytic acid content in β -glucan samples varied substantially according to the isolation procedure. The precipitation behavior of phytic acid in ethanol was the main reason for the high content of phytic acid in isolated β -glucan produced by ethanol precipitation. Ammonium sulphate, another precipitation solvent used in β -glucan isolation, could not precipitate phytic acid leading to a low content of phytic acid in the isolated β -glucan. Inactivation of the endogenous phytase contributed to the high content of phytic acid. pH lowering before ethanol precipitation, dialysis of the extract at low pH, or anion exchanging reduced the phytic acid content. The contribution of the intrinsic phytate should be taken into consideration when evaluating the physico-chemical and physiological properties of β -glucans.

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Captions of figures:

Figure 1. The procedure of β -glucan isolation and the treatments (factors) in various steps during isolation. To investigate the effect of each factor on phytic acid content, the corresponding treatment was independently done.

Figure 2. Starch hydrolysis in the presence of isolated β -glucan or phytic acid after 20 min at 37°C. Starch hydrolysis (starch+ α -amylase) without addition of any other compounds are control samples regarded as base line (100%) for comparison. OBG is isolated oat β -glucan containing phytate, OBGPR is phytate removed OBG, PA is phytic acid sodium salt (0.04 w/v).

Figure 3. Digested starch in the presence of hydrolysed β -glucan or calcium after 20 min at 37°C. Starch hydrolysis (starch+ α -amylase) without addition of any other compounds are control samples regarded as base line (100%) for comparison. OBG is isolated oat β -glucan, OBGPR is phytate removed OBG, PA is phytic acid sodium salt.

Table 1. Phytate and mineral content of oat and barley bran concentrates and isolated β -glucan.

β -Glucan samples		Factor	β -Glucan %	Phytic acid %	Free P mg/100g	Ca mg/100g	Mg mg/100g
Oat	Bran concentrate		14	1.27 $\pm$ 0.02 ^e	88.4 $\pm$ 0.7 ^f	120 ^b	290
	Isolated β -glucan (OBG)		66 $\pm$ 2 ^{bc}	3.86 $\pm$ 0.09 ^a	109.6 $\pm$ 0.8 ^c	330	610
	OBG						
	+ acidified before ethanol precipitation (pH 3)	II	69 $\pm$ 2 ^b	2.59 $\pm$ 0.02 ^c	77.5 $\pm$ 0.1 ^g		
	OBGPR (phytate removal)	III	72.7 $\pm$ 0.3 ^a	0.60 $\pm$ 0.05 ^f	88 $\pm$ 2 ^f	86	160
	OBG						
	+ (NH ₄) ₂ SO ₄ re-precipitation	IV	NM	0.23 $\pm$ 0.01 ^g	73.1 $\pm$ 1.0 ^h		
Barley	OBG + dialysis (pH as such)	V	NM	3.20 $\pm$ 0.05 ^b	120 $\pm$ 2 ^b		
	OBG + dialysis at pH 3	V	NM	0.57 $\pm$ 0.01 ^f	97 $\pm$ 2 ^e		
	Bran concentrate		16	0.61 $\pm$ 0.06 ^f	26.4 $\pm$ 0.9 ^j		
	Isolated β -glucan (BBG)		62 $\pm$ 2 ^d	0.51 $\pm$ 0.04 ^f	634 $\pm$ 11 ^a		
	BBG						
	+ hot ethanol refluxing before isolation	I	69.2 $\pm$ 0.9 ^b	2.24 $\pm$ 0.07 ^d	106.4 $\pm$ 0.8 ^d		
	BBG						
	+ acidified before ethanol precipitation (pH 3)	II	75 $\pm$ 2 ^a	0.21 $\pm$ 0.01 ^g	60.5 $\pm$ 1.2 ⁱ		

^{a-j} means within a column with different superscripts differ ($p < 0.05$)

Table 2. Precipitation of phytic acid sodium salt (1%, w/v) with ethanol or (NH₄)₂SO₄ at different pH.

Solvent	pH	Precipitated phytic acid
2 volumes EtOH	3.0	39.9 $\pm$ 0.2 ^c
	4.6	64.1 $\pm$ 0.6 ^b
	7.0	101 $\pm$ 0.4 ^a
1.5 volumes EtOH	4.6	24.0 $\pm$ 1.0 ^d
1 volume EtOH	4.6	ND
20% (NH ₄) ₂ SO ₄	4.6	ND
2 volumes EtOH	4.6	ND (Myo-inositol)

Note: ND means not detected. One volume means the same volume as phytic acid solution. Three replicates were prepared for each sample. ^{a-d} means within a column with different superscripts differ ($p < 0.05$).

Figure 1. The procedure of β-glucan isolation and the treatments (factors) in various steps during isolation. To investigate the effect of each factor on phytic acid content, the corresponding treatment was independently done.

Figure 2. Starch hydrolysis in the presence of isolated β -glucan or phytic acid after 20 min at 37°C. Starch hydrolysis (starch+ α -amylase) without addition of any other compounds are control samples regarded as base line (100%) for comparison. OBG is isolated oat β -glucan containing phytate, OBGPR is phytate removed OBG, PA is phytic acid sodium salt (0.04 w/v). ^{a-c} means bars with different superscripts differ ($p<0.05$).

Figure 3. Digested starch in the presence of hydrolysed β -glucan or calcium after 20 min at 37°C. Starch hydrolysis (starch+ α -amylase) without addition of any other compounds are control samples regarded as base line (100%) for comparison. OBG is isolated oat β -glucan, OBGPR is phytate removed OBG, PA is phytic acid sodium salt. ^{a-f} means bars with different superscripts differ ($p<0.05$).

Statement

This is to state that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder.

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