NATURAL FLOCCULANTS AFFECT PURITY AND PROPERTIES OF β-GLUCAN EXTRACTED FROM BARLEY AND OAT

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Abstract

In this study, β-glucan was extracted from wholegrain oat and barley flours by a novel extraction and purification method employing natural flocculants (chitosan, guar gum and gelatin). The use of flocculants decreased the total amount of extracted gum, which was highest in control samples (9.07 and 7.9% for oat and barley, respectively). The β-glucan specific yield, however, increased with the use of chitosan and guar gum, which were able to remove protein and ash impurities resulting in gums with a higher purity. The highest concentration of chitosan (0.6%) resulted in gums with the highest β-glucan content (39.6 and 69.0 % for oat and barley, respectively) and highest β-glucan specific yield (96.9 and 93.3 % for oat and barley, respectively). The use of gelatin was not successful. All gum samples had a high content of total dietary fiber (>74%) and a high water holding capacity (4.6 – 7.4 g/g), but differed in apparent viscosity, which was highest for the oat sample extracted with 0.6% chitosan. This sample also showed the highest β-glucan molecular weight among the oat samples, which were in general 10-fold higher than for the barley samples. Among the barley samples, β-glucan molecular weight was highest for the control.

Keywords: beta-glucan, extraction, flocculant, chitosan, guar gum

1. Introduction

Cereal β-glucan is a constituent of dietary fiber present mainly in barley and oat. It is a polymer, which consists of linear β-(1,3) and β-(1,4) linked glucose monomers. β-glucan is a valuable food constituent that reduces serum LDL-cholesterol levels (AbuMweis, Jew, & Ames, 2010; Wolever et al., 2010) and plays a significant role in lowering post-prandial glucose levels (Behall, Scholfield, & Hallfrisch, 2017; Brummer, Duss, Wolever, & Tosh, 2012). The ability of β-glucan to lower plasma cholesterol concentrations and post-prandial glucose levels is mainly influenced by its molecular weight and viscosity (Wang and Ellis, 2014, Wolever et al., 2010, Brummer et al, 2012). High molecular weight β-glucans, which are able to form viscous solutions, are most effective. These are mainly found in native forms of oat or barley (≥1000 kDa) (Wilson et al., 2004), while food processing or extraction may reduce β-glucan molecular weight (Rieder, Ballance, Løvaas, & Knutsen, 2015; Rimsten, Stenberg, Andersson, Andersson, & Åman, 2003; Tosh et al., 2010). Consumption of β-glucan with its specific physiological benefits may be beneficial for people with diabetes or cardiovascular diseases (Chen & Raymond, 2008), which makes β-glucan an interesting food ingredient. β-glucan has also been shown to have antioxidant and pre-biotic properties (Harasym, Suchecka, & Gromadzka-Ostrowska, 2015; Shah et al. 2017). The FDA and EFSA are two main authority bodies that have authorized the use of health claims for β-glucan from barley and oat. For example, the claim " lowering LDL-cholesterol levels" can be used for food products providing one gram per serving and over three grams per person per day (Izydorczyk, Chornick, Paulley, Edwards, & Dexter, 2008; EFSA, 2010). Apart from being beneficial for human health, β-glucan could also be a valuable ingredient in food applications. Due to its viscosity, it is perceived as a non-caloric thickener for various foods (Limberger-Bayer et al., 2014). Moreover, it is used as a stabilizing agent in foams and emulsions (Lazaridou & Biliaderis, 2007) a texturizing agent (Kodama et al., 2015) and a fat substitute (Pintado et al. 2016).

In oat and barley, which are the two main sources of cereal β-glucan, β-glucan is mainly located in the cell walls of endosperm and aleurone. While oat is usually subjected to a kilning process to inactivate lipases and thereby ensure lipid stability, barley does not normally undergo a heat treatment before further processing. However, kilning as a heat process may inactivate other enzymes besides lipases (Londono et al., 2015). The presence of active β-glucanases in barley flour has been shown to result in β-glucan degradation during processing (Rieder, Ballance, & Knutsen, 2015; Rieder, Ballance, Løvaas, & Knutsen, 2015). However, β-glucans are only a minor constituent in grains, which also consist of non-starch polysaccharides, starch, proteins, and lipids that need to be washed out during β-glucan extraction. Therefore, extraction processes of β -glucan have to be multistage taking into consideration the other substances present in grains (Hematian Sourki, Koocheki, & Elahi, 2017).

The extraction and purification of β-glucan from cereals are mainly divided into two main groups: wet and dry processes (Benito-Román, Alonso, & Lucas, 2011). Extraction performed in dry conditions is carried out by milling and sieving and wet conditions usually involve an enzymatic treatment, alkaline solvents, or ultrasounds (Ahmad, Anjum, Zahoor, Nawaz, & Ahmed, 2010; Benito-Román, Alonso, & Cocero, 2013). Generally, β-glucan extraction with wet-methods results in an efficient purification because it achieves up to $> 80\%$ pure β-glucan and various molecular weight distributions could be obtained depending on the pH and solvents used (from 60 000 to 2 200 000 g/mol) (Harasym, Suchecka, & Gromadzka-Ostrowska, 2015; Limberger-Bayer et al., 2014). Due to the health advantages and benefits for food technology, there is still a constant demand to extract and purify β-glucan taking into consideration its yield, purity and molecular weight.

During β-glucan extraction water or other solvents are used. However, impurities (protein-based or non-starch polysaccharides) are present in solutions during enzymatic treatment, though these fine particles could be removed by adding external flocculants, which aggregate solids to form flakes that can easily be removed from solutions (Meraz et al., 2016). Natural flocculants are very

promising agents in improving food processing operations due to their safety and ecological benefits. Several flocculants could be used in foods: guar gum, alginates, chitosan or gelatin, which are used in extractions or the isolation of different food constituents (Jain et al., 2017; de Oliveira, Mahl, Simões, & Silva, 2012; Wang, Wang, Li, Cao, & Sun, 2013).

There is a need to examine the possible applications and differences in the properties of extracted β-glucan with the aid of natural flocculants. Therefore, the objective of this study was to evaluate the characteristics of extracted β-glucan with the application of chitosan, guar gum, and gelatin as examples of natural flocculants.

2. Materials and methods

2.1. Materials

For the purpose of this study, we used barley flour (Rastik variety) and oat flour (Poseidon variety) milled using KitchenAid with 5KGM of grain mill (KitchenAid, MI, USA). The flours were produced using whole grains and the oat flour was milled after previous dehulling. Then, flours were sieved through 0.5mm screen with rotor mill. β -glucan content for barley flour was 4.58 $\pm$ 0.15 g/100g dry basis and for oat flour was 3.56 $\pm$ 0.21 g/100g dry basis. Molecular weight for β -glucan in oat flour was 1 127 x 10^3 (g/mol) and in barley flour 845 x 10^3 (g/mol). Deacetylated chitosan was purchased from Oxford Vitality as a dietary supplement (UK). Guar gum and gelatin from porcine source (Bloom 180) were supplied as food grade material from Agnex (Poland) Flocculants – guar gum and gelatin – were prepared in 3% solutions in distilled water with heating in 45°C and stirring for 60 min with 300 rpm using magnetic stirrer. Chitosan was dissolved in the same concentration as guar gum and gelatin but instead of water 1% of acetic acid solution was employed.(w/w).

2.2. Extraction of β-glucan

We based β-glucan extraction on an enzymatic treatment. 10g flour were placed in a 50 ml falcon tube and 30ml of water at pH 9.5 was added. The pH level was achieved with 10% sodium carbonate to prevent extraction of non-glucan polysaccharides, arabinoxylan and proteins.

Samples were mixed on a rotator for one hour with shaking (70 rpm) (IntelliMixer RM-2, Elmi Ltd., Latvia). Then, the samples were centrifuged at 8000g (Hettich Universal 320R, Germany) for 10 minutes and the supernatant was collected in beakers. Flocculant solutions were added to the supernatant in amounts appropriate to obtain a final concentration of 0.2 or 0.6% of chitosan (CT 0.2 and CT 0.6), guar gum (GG 0.2 and GG 0.6) and gelatin (GT 0.2 and 0.6) in the supernatant-flocculant solution mixture. Incubation in 45°C was conducted for 30 min and all impurities were discarded after centrifuging as above mentioned and the supernatant was once more collected. The temperature of flocculation in our study was chosen based on results by Limberger-Bayer et al. (2014), who reported that β-glucan could be solubilized at 45°C without the risk of starch gelatinization (Limberger-Bayer et al. 2014). Control samples had no added flocculant and were treated directly by α-amylase (BCN and OCN). All samples were treated with thermostable α-amylase at 80°C (Termamyl SC, Novozymes, Denmark) after adjusting the pH to the optimal value (pH 7.0). After a negative iodine test, solutions were cooled down and the pH was decreased to 3.5 by addition of 2M acetic acid to reduce protein solubility and placed in a 95°C water bath for protein denaturation and precipitation. Proteins were collected as pellets during centrifuging and the supernatant was added to ethanol in a ratio of 1:1,5. After storage for 24h at 4°C, the β-glucan precipitate was collected by centrifugation and washed on the filter with 96% ethanol to obtain a clean gum. Where it was needed, gum was dried in vacuum using vacuum oven (V500, Mammert Co., Germany).

2.3. Production yield and β-glucan extraction yield

The yield of production was assessed following a study by Ahmad, Anjum, Zahoor, Nawaz, & Ahmed (2010), where the yield was perceived as the mass of gum extracted from 100g of flour and calculated to a percentage. The β-glucan extraction yield was calculated as (β -glucan content in extracted dried matter/ β -glucan content in dried matter of oat or barley flour)· 100%.

2.4. Chemical analysis

The β-glucan content in flours and dried precipitate after extraction was determined using a specific enzymatic method from a Megazyme β-glucan Assay Kit (Mixed Linkage) (Megazyme Inc., Ireland). Total dietary fiber (TDF) was determined as the sum of insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) using the method developed for the Total Dietary Fiber Assay Kit (Megazyme Inc., Ireland). The nitrogen content was assessed using the Kjeldahl method and converted to protein content using a 6.25 conversion factor. Total ash content was determined with the AACC 08.01.01 method (AACC, 1999). All the results were converted to content in 100g of dry weight.

2.5. Physical properties

2.5.1. Color analysis

The color of β-glucan gums was measured using a Minolta CR-400 colorimeter (Konica Minolta Inc., Japan) (illuminant D65, measurement area ø=8mm, standard observers 2°), and the results were expressed in accordance to the CIELab color space. Determined parameters were L* (L = 0 (black) and L = 100 (white)), a* (- a = greenness and +a = redness), b* (-b = blueness and +b = yellowness). The differences between the control samples and extracts obtained with the aid of flocculants were assessed using ΔE parameter:

$$\Delta E = \sqrt{(\Delta L^2 + \Delta a^2 + \Delta b^2)}$$

2.5.2. Water holding capacity (WHC)

The WHC was determined using the method described by Liu et al. (2015). The 2.5% dispersions of β-glucan were prepared with distilled water in centrifuge tubes. Aliquots of 0.1 g dried sample were mixed with 25 mL distilled water in a 50 mL pre-weighted centrifuged tube and agitated thoroughly by a vortex mixer. Samples were placed at 4 °C for 24 h, The tubes were centrifuged at 3000g for 30min. The supernatant was discarded and the wet pellet was weighed. WHC was calculated using the equation below:

$$WHC\ \left(\frac{g}{g}\right) = \frac{pellet\ weight - dry\ weight}{dry\ weight}$$

2.6. Molecular weight (MW) determination

B-glucan MW distributions were determined by size-exclusion chromatography (SEC) with calcofluor detection, as described by Rieder, Ballance, & Knutsen (2015). From these distributions weight average molecular weights (Mw) were calculated as described earlier (Rieder, Knutsen, Ulset, et al., 2015).

2.7. Rheology

The rheological properties of the different β-glucan containing gums were determined as follows. The amount of gum appropriate to achieve 1% of β-glucan in whole solution was transferred to a beaker and 100ml of sodium phosphate buffer (20 mM, pH 6.5) was added. The solution was then heated to 80°C with constant stirring and the temperature was maintained for 10 min. After heating, the solution was cooled down to ambient temperature with constant stirring. Solutions for were stored at 4°C for 24 h prior to measurement

Viscosity measurements were conducted using a Thermo Haake Mars III rheometer with cylindrical rotor CC25 Din Ti (diameter: 25mm; length: 37.6mm and cone angle 120°) (Thermo Fisher Scientific Inc.; USA). Volume of sample was 16,1cm^3. The controlled temperature was 20°C. Viscosity was measured in the shear rate range of 0.01 to 1000 s^{-1}. Experimental data were fit to the power law equation:

$$\eta = K\gamma^{n-1}$$

where η is the apparent viscosity (Pa s^{-1}) , K is the consistency coefficient (Pa s^{-1}); γ is the shear rate (s^{-1}); n is the flow behavior indexes (dimensionless). Values $n < 1$ are applicable for shear-tinning fluids (Li, Zhu, Guo, Peng, & Zhou, 2016). A shear rate of 202s^{-1} was chosen for comparison of apparent viscosities of the different gum solutions.

2.8. DSC measurements

Differential scanning calorimetry was employed to determine the thermal properties of extracted β-glucan using Mettler Toledo DSC 1 equipment (Mettler Toledo, USA). The extracted β-glucan

was weighed (2mg) on an aluminum pan and hydrated by adding water in 50% w/w with microsyrinage. Then the pan was hermetically sealed. Samples were heated dynamically from 20-200°C with a heating rate 10°C/min. The reference was the empty aluminum pan. The peak temperature (T_p) and melting enthalpy (ΔH) were determined.

2.9. Statistical analysis

The means of obtained measurements were compared using one-way analysis of variance (ANOVA) followed by Tukey's tests and for the results, means were compared by the least significant difference (LSD) employing Statistica 12.5 (Statsoft, USA). P values ≤ 0.05 were considered as significant.

3. Results and discussion

3.1. Yield and purity of extracted β-glucan

The yield of total gum and β-glucan specific extraction yield are shown in Fig. 1, while the chemical composition including the β-glucan content of the gums is presented in table 1. The yield of total gum was highest for the control samples OCN and BCN (7.91 and 9.07 %), while the extractions performed with gelatin as a flocculant resulted in the lowest yield (4.9-6.3%). In oat samples, increasing concentrations of chitosan slightly decreased the yield, but this was not statistically significant. However, there were significant differences ($p \leq 0.05$) between OGG 0.2 and OGG 0.6 samples, where increasing guar gum concentrations led to a higher extraction yield. The yield of the process, which was between 4.94 and 9.40 %, can be considered as high compared to other studies, such as Hematian Sourki et al. (2017). This could be explained by the fact that, in our study, barley and oat flour were produced on a laboratory scale from kernels and more dietary fiber was present in the raw material (12.5% for barley with 4.58% β -glucanand 11.15% for oat flour with 3.56% β -glucan).

Even though all three flocculants decreased the total yield of gum, the β-glucan specific extraction yield increased with both chitosan and guar gum, but decreased with gelatin. Gums extracted with

0.6% chitosan had the highest β-glucan content (69.01±.97 and 39.64 ± 1.14 for barley and oat, respectively). Increasing the chitosan concentration from 0.2 to 0.6% increased the β-glucan content (barley and oat) and β-glucan extraction yield (only barley). For guar gum and gelatin, increased concentrations resulted in lower purity of β-glucan extracts (Table 1). Specific extraction yield was highest in the samples with OCT 0.6 where the values were 96.9% and the lowest with OGT 0.6 with 44.4%. The achieved results could be perceived as very high extraction yield of the samples with chitosan comparing to other authors who used thermostable alpha-amylase were specific extraction yield values were around 82% from freeze-dried samples (Rieder, Knutsen, & Ballance, 2017) and around 76% from oat bran cereals (Gamel, Abdel-Aal, Ames, Duss, & Tosh, 2014). The application of chitosan could be a sufficient tool in extraction protocols of β-glucan with high purity. The extractability of β-glucan from barley sources has been shown to be nearly 100%, but this particular study lacks information about the precise enzymatic protocol (Mikkelsen et al., 2017). Gelatin was not considered as an effective tool to remove impurities during β-glucan extraction since β-glucan content in the resulting gums was even lower than for control samples. The content of β-glucan in control samples from barley was quite similar to the results of Limberger-Bayer et al. (2014), where the optimized highest β -glucan content was 53.4%. The higher purity of samples obtained with the aid of chitosan may be related to the charge of chitosan. Chitosan has a cationic charge and a high molecular weight, which implies that it flocculates via a charge neutralization mechanism (Rojas-Reyna et al., 2010). During the first step of β-glucan extraction, water soluble constituents were present. Guar gum is used in water treatment plants to remove impurities and to reduce the application of synthetic flocculants (Banerjee et al., 2013). However, there is an particular dosage to achieve optimal results and guar gum is not commonly used as the only flocculant (Gupta & Ako, 2005). Gelatin was the only none polysaccharide based flocculant used in this study Gelatin is used as successful fining agent during wine production. The key factor that influenced the effect of wine fining with gelatin is that it is positively charged. The substances that were to be removed during β -glucan extraction could have been positively charged as well so this could also be an explanation for its

low performance in experiment (González-Neves, Favre, & Gil, 2014). Gelatin has been used as a flocculant in kaolin samples to good effect. However, kaolin is composed of minerals and gelatin might not be so successful in applications where more complex molecules are used (Piazza & Garcia, 2010).

3.2. Chemical analysis

The values of insoluble dietary fiber (IDF), soluble dietary fiber (SDF), and total dietary fiber (TDF) are presented in Table 1. The aim of extraction is to clean the sample to achieve only soluble fractions of dietary fiber. However, some insoluble dietary fiber could still be determined in gum extracts. Only with gelatinthe content of IDF in gums was lower than in the control samples. The use of guar gum and chitosan as flocculants resulted in increased IDF content in all samples. The highest differences between the two different flocculant concentrations were observed in samples with chitosan. The values of SDF were directly correlated to IDF in almost all samples. Gelatin as a flocculant caused adverse effects because the SDF content was lowest in gums extracted with gelatin. The IDF and SDF were summed to obtain the TDF. The highest TDF was observed in samples from barley (BCT 0.6 – 96.36%) and lowest in BGT 0.6 (73.15). Increasing concentrations of chitosan increased TDF in oat and barley extracts. β-glucan is mainly perceived as a soluble fraction of dietary fiber. In BCN and OCN, β-glucan constitutes 63 and 60% of SDF (data not presented). The highest values were observed in chitosan samples because there nearly 96% of SDF was β-glucan.

The protein content was assessed because protease treatment was not included in the extraction process. The control sample had a significantly higher protein content when compared to samples with chitosan and guar gum, which implies that these flocculants were successful in cleaning the solution during β-glucan extraction. However, the highest values were observed in samples with gelatin, which could be because gelatin is a protein as well. This high amount of protein could be explained by the fact that gelatin could form coacervates spontaneously during mixing with the solution during the extraction processes. This might have caused encapsulation of IDF and SDF,

impairing their removal during centrifugation (Thimma & Tammishetti, 2003). The application of all types of flocculants was efficient in removing mineral impurities during β-glucan extraction.

3.3. Physical properties

The physical properties of β-glucan extracts - WHC and color, are presented in Table 2. The WHC values ranged from 4.56 up to 7.42 g/g.. However, all samples indicated quite high WHC so the extracts could be industrially applicable thickeners. The range of results is similar to other research (Ahmad, Anjum, Zahoor, Nawaz, & Ahmed, 2010; Liu et al., 2015b). The higher WHC values could be explained by the low solubility of β-glucan in room temperature. Moreover, it could be explained by the fact that β -glucan possibly formed complexes with other materials that were present in extracted gums. . This parameter could be also correlated with the TDF and IDF/SDF amount in samples because the samples that were higher in SDF could not reach high WHC values as the SDF was probably discarded in the centrifuging step during measurement.

The color of gums was assessed as lightness (L*) and was higher in oat extracts than in barley. Flocculants generally decreased the L* parameter compared to control samples. In BCT increasing chitosan concentrations decreased sample lightness. All barley samples were more brownish as indicated by the highest values of a*. The differences between control samples and extracts with flocculants were presented as ΔE. The differences between the control sample and flocculants were visible to the observer apart from BCT 0.2 and OCT 0.6 sample. Highest differences were observed in OCT 0.2 and BCT 0.6. The brighter the β-glucan gum is, the more universal its application in industry will be. Barley samples could be used as thickeners in foods with more yellow or brown colors like sauces or soups. Oat gums were white. The color is also affected by impurities or substances other than β-glucan, which might have been washed out with chitosan and guar gum.

3.4. Molecular weight

The molecular weight (MW) of β-glucan is a vital parameter connected to its physiological properties. High molecular weight β-glucan plays important role in cholesterol and blood glucose

lowering with various mechanism (Brummer et al., 2012; Y. Wang et al., 2016) The molecular weight of β-glucan depends on many different factors, such as cereal variety, agronomy, and extraction conditions. The molecular weight of obtained extracts is presented in the Table 3. In barley samples, the highest weight average MW (Mw) was in control samples (64 873 g/mol) and the use of flocculants resulted in lower Mw. This might be related to a longer extraction process due to the extra step of flocculation during which β-glucanases present in the barley flour can degrade β-glucan. The lowest Mw was observed in GG samples (34 648 g/mol). The tendency of an increasing Mw caused by increasing concentrations of flocculant was observed in GG and GT samples. The samples obtained with the aid of gelatin were not significantly different than those obtained with chitosan or guar gum. A different tendency was observed in oat samples in which the β-glucan Mw was higher for extractions with flocculants. The highest Mw was observed in OCT 0.6 (551 000 g/mol) and the lowest in the control sample (280 515 g/mol). Generally, the Mw in oat samples was higher than in barley ones (10^5 and 10^4, respectively) which is an evidence of the presence of β-glucanases in barley, which were still active during extraction processes. Compared to the other flocculants, the use of chitosan resulted in a significantly higher β-glucan molecular weight in the oat samples. Similar to the barley samples, β-glucan molecular weight increased with increasing concentrations of guar gum and gelatin also for the oat samples. .

3.5. Rheology

The viscosity of β-glucan is an important parameter because it is linked to functional properties during food production and related to the physiological benefits of β-glucan. The viscosity of β-glucan containing food products during in vitro digestion could be linked to their potential of lowering postprandial glucose levels (Rieder et al., 2017) The ability to reduce LDL-cholesterol levels was also linked to β-glucan viscosity (Wang et al., 2016; Wolever et al., 2010). . Apparent viscosity is presented in Table 3. The lowest viscosity was observed in the samples with gelatin as flocculant and highest in the sample with OCT 0.2 (21.02 Pas). The increasing concentration of chitosan in barley samples caused a slight decrease in viscosity. This tendency was different in oat

307 samples, meaning that chitosan interacted with some components that were only present in oats.
308 The OGT and BGT samples had significantly lower viscosity because these samples contained
309 more non-β-glucan substances (lowest β-glucan content and highest protein content), which may
310 have disturbed molecular entanglement and gel formation (Ahmad, Anjum, Zahoor, Nawaz, &
311 Ahmed, 2010). . Increasing the concentration of chitosan decreased the values of the flow behavior
312 index, meaning that the pseudoplasticity increased at the same time (Hematian Sourki et al.,
313 2017).

314 **3.6. Thermal characteristics**

315 The melting temperature was assessed with DSC. The highest values of T_p were observed in GG
316 (143.2-144.2°C) samples and the lowest in GT (102.7-110.0°C). Differences of T_p indicated that
317 there is no homogeneity in the structure of extracted β-glucan (De Souza et al., 2015). There were
318 significant differences in the enthalpy of reactions, showing that the highest energy was required
319 to melt the GG samples and the least energy consumption samples were GT, which is consistent
320 with the T_p values achieved. The increasing melting temperature suggests a higher percentage of
321 organized arrangements (Ahmad, Gani, Shah, Gani, & Masoodi, 2016).

322 **4. Conclusions**

323 Chitosan and guar gum used as flocculants during extraction of β-glucan increased the purity of
324 extracts obtained from oat and barley flour, while gelatin was not successful. Specifically, the use
325 of flocculants decreased protein (chitosan, guar gum) and mineral (all three) impurities. Despite
326 considerable differences in β-glucan content and Mw, all extracts showed high water-holding
327 capacity, which makes them applicable to the industry where thickeners based on β-glucan are
328 being sought. The color of extracts was mainly affected by the origin, not the extraction method.
329 The use of biodegradable and food grade polymers like chitosan or guar gum could be a renewable
330 and ecological friendly improvement in the extraction of β-glucan.

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