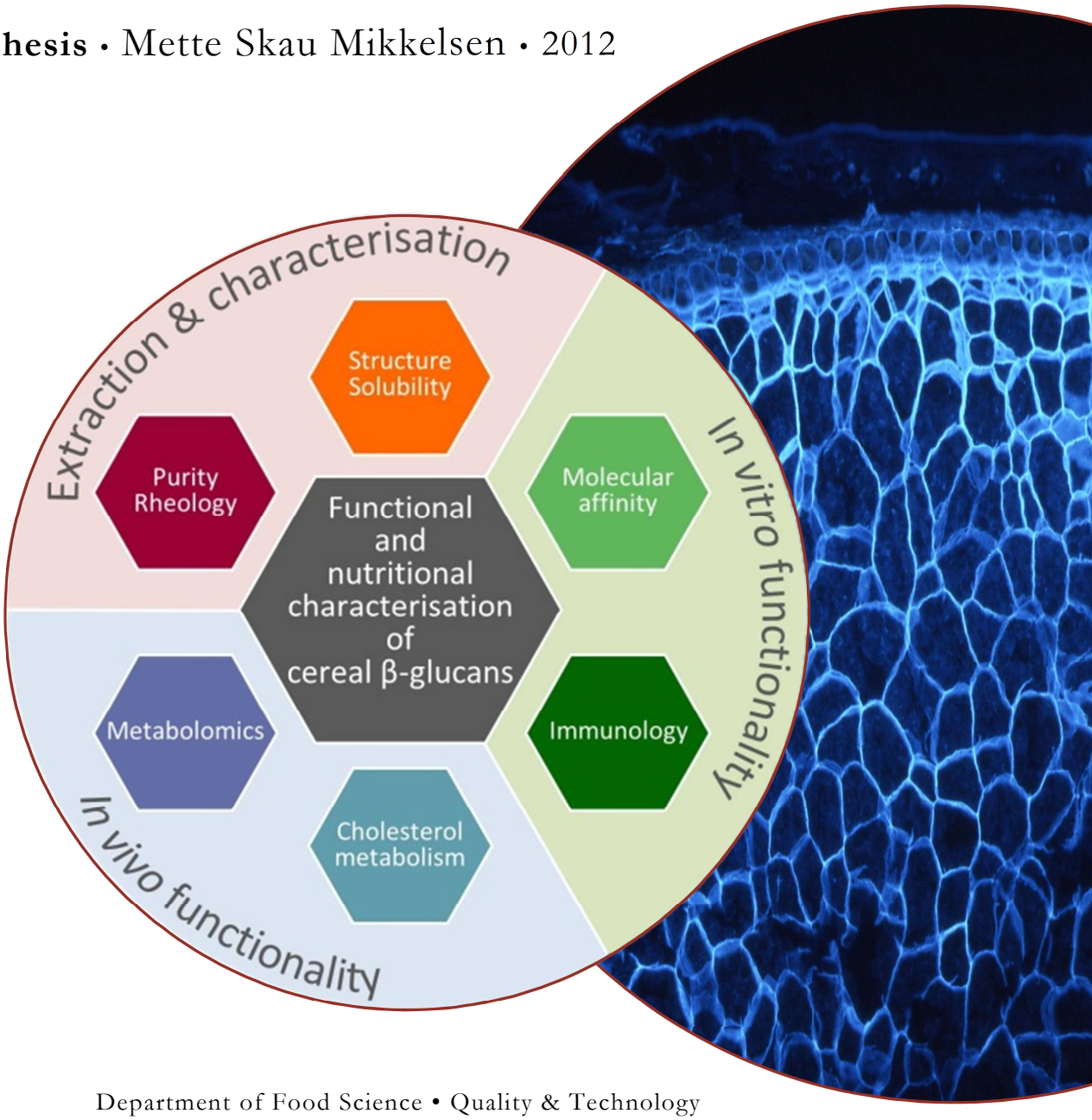
DEPARTMENT OF FOOD SCIENCE
FACULTY OF SCIENCE
UNIVERSITY OF COPENHAGEN

Comparison of functional and nutritional characteristics of barley and oat mixed linkage β-glucans

PhD Thesis · Mette Skau Mikkelsen · 2012



Department of Food Science • Quality & Technology
Rolighedsvej 30 • 1958 Frederiksberg C • Denmark

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Supervisors
Professor Søren Balling Engelsen
&
Associate Professor Birthe Møller Jespersen
Department of Food Science
Faculty of Science, University of Copenhagen

Assistant Professor Mette Kristensen
Department of Nutrition, Exercise and Sports
Faculty of Science, University of Copenhagen

Opponents
Senior Scientist Baljit Singh Ghotra
Mondelez International
East Hanover, NJ, USA

Adjunct Professor Peter Olesen
Actifoods ApS
Hillerød, Denmark

Associate Professor Thomas Skov (Chairman)
Department of Food Science
Faculty of Science, University of Copenhagen, Denmark

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Preface

The present PhD thesis is based on work carried out in the research group Quality & Technology (Q&T) at the Department of Food Science, Faculty of Science, University of Copenhagen. The project was granted by the Faculty of Science, University of Copenhagen with support from the strategic research initiative BEST.

The project has been supervised by Professor Søren Balling Engelsen, Associate Professor Birthe Møller Jespersen and Assistant Professor Mette Kristensen from University of Copenhagen.

A large number of people have contributed to the completion of this thesis. First of all, I am grateful to my three supervisors. Søren, for giving me the opportunity to conduct this thesis, for great freedom in choosing focus and direction of the work and for your valuable knowledge and inspiration. Birthe, for all the hard work we did together, for always taking time to discuss problems and ideas and for an always positive attitude at the office. Mette, for great company on journeys, for facilitating the possibility to conduct a human intervention study and for sharing valuable knowledge in this field.

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Mette Skau Mikkelsen

Frederiksberg, November 2012

Summary

It is well accepted that dietary fibres, especially mixed linkage (1→3, 1→4)-β-D-glucans (β-glucan) from barley and oat, have beneficial effects on health and prevent modern lifestyle diseases. Even though recent research has shed some light on the mechanisms of action and structure-functionality relationship of β-glucans, the exact functional principle remain elusive. The overall aim of this project was to provide new knowledge into the relation between β-glucan and health at a molecular level. For the first time two barley and one oat fractions of well-defined and structurally different β-glucans were compared in a human intervention study. The work first focussed on large-scale extraction and physico-chemical characterisation of barley and oat β-glucans. The second step was to investigate the *in vitro* health effects of barley and oat β-glucans in relation to their physico-chemical properties. Finally, health effects from barley and oat β-glucans were studied *in vivo* in humans using traditional biomarkers and plasma metabolomics.

Results from Paper I demonstrated that structural characteristics and viscous properties of barley and oat β-glucans dominate the functional traits over the presence of α-glucan impurities. Paper II identified a structurally unique barley high β-glucan variety with an extraordinary high amount of cellotriosyl units and showed that the oligomer block structure of barley and oat β-glucans influence their solubility. The main findings from Papers III and IV *in vitro* studies were that small molecule interaction with barley and oat β-glucans is influenced by degree of polymerisation and β-glucan fine structure whereas β-glucan solubility and aggregation are key elements for understanding their immune modulating capacity. Results from the Paper V *in vivo* human study showed that consumption of 3.3 g/day extracted barley and oat β-glucan for 3 weeks does not significantly lower total and LDL cholesterol levels in young healthy adults. However, an indicated potential effect of oat suggests the importance of solubility for β-glucan interference with the cholesterol metabolism. The findings from Paper VI confirmed the general absence of barley and oat β-glucan effect on blood metabolites but showed the existence of subject unique lipoprotein profiles depended on gender, BMI and diet.

In conclusion, the results show that barley and oat β-glucan fine structures are of great importance for their functionality. β-Glucan solubility and polymer aggregation in solution is dependent on the block structural pattern and differently structured β-glucans may exert various functional and bioactive properties in our body. It is important to account for these diverse effects in the future evaluation of β-glucan effectiveness in functional foods and health.

Sammendrag

Det er almindeligt accepteret, at kostfibre, særligt (1→3, 1→4)-β-D-glukan (β-glukan) fra byg og havre, har gavnlig effekt på vores sundhed og forebygger moderne livsstilssygdomme. Selvom nyere forskning til dels har belyst virkningsmekanismerne og struktur-funktionalitet forholdet hos β-glukaner, forbliver det nøjagtige funktionelle princip fortsat uafklaret. Det overordnede formål med dette projekt var at skabe ny viden om β-glukaner og sundhed på et molekylært niveau. For første gang sammenlignes to veldefinerede og strukturelt forskellige byg og en havre β-glukan i et humant kostforsøg. Første del af arbejdet fokuserede på stor-skala ekstraktion og fysisk-kemisk karakterisering af byg og havre β-glukaner. Næste del af projektet undersøgte *in vitro* sundhedsvirkninger af byg og havre β-glukaner i forhold til deres fysisk-kemiske egenskaber. Endelig blev sundhedsmæssige virkninger af byg og havre β-glukaner undersøgt *in vivo* i mennesker ved brug af traditionelle biomarkører og plasma metabolomics.

Resultaterne fra Artikel I viste, at de strukturelle karakteristika og viskøse egenskaber hos byg og havre β-glukaner dominerer de funktionelle egenskaber over α-glukan urenheder. Artikel II identificerede en strukturelt unik byg β-glukan med en ekstraordinær stor mængde cellotriosyl enheder og viste, at oligomer blokstrukturen af byg og havre β-glukaner påvirker deres opløselighed. De vigtigste resultater fra Artikel III og IV *in vitro* studier var, at små molekylers interaktion med byg og havre β-glukaner påvirkes af graden af polymerisering samt β-glukaners blok struktur, mens β-glukan opløselighed og aggregering er centrale elementer for at forstå deres immunmodulerende kapacitet. Resultater fra det *in vivo* humane studie i Artikel V viste, at indtag af 3,3 g/dag ekstraheret byg og havre β-glukan i 3 uger ikke sænker total og LDL kolesterol signifikant hos unge sunde voksne. En indikeret potentiel virkning af havre antyder dog vigtigheden af opløselighed for β-glukan effekt på kolesterolmetabolismen. Resultaterne fra Artikel VI bekræftede den generelle mangel på byg og havre β-glukan effekt på blodets metabolitter, men viste, at der findes individ unikke lipoprotein profiler som afhænger af køn, BMI og kost.

Sammenfattende viser resultaterne, at byg og havre β-glukan blok struktur er af stor betydning for deres funktionalitet. β-Glukan opløselighed og aggregering afhænger af blok strukturelle mønstre og β-glukaner med forskellige strukturer kan udøve varierende funktionelle og bioaktive egenskaber i vores krop. Det er vigtigt at redegøre for disse egenskaber i den fremtidige evaluering af β-glukaners effektivitet i funktionelle fødevarer og sundhed.

List of publications

Paper I
Mikkelsen MS, Jespersen BM, Møller BL, Lærke HN, Larsen FH, Engelsen SB. Comparative spectroscopic and rheological studies on crude and purified soluble barley and oat β-glucan preparations. Food Research International, 43: 2417-2424, 2010.

Paper II
Mikkelsen MS, Jespersen BM, Larsen FH, Blennow A, Engelsen SB. Molecular structure of large-scale extracted β-glucan from barley and oat. Identification of a significantly changed block structure in a high β-glucan barley mutant. Food Chemistry, 136:130-138, 2013.

Paper III
Simonsen HT, **Nielsen MS**, Christensen NJ, Christensen U, La Cour TV, Motawia MS, Jespersen BM, Engelsen SB, Møller BL. Molecular interactions between barley and oat β-glucans and phenolic derivatives. Journal of Agricultural and Food Chemistry, 57: 2056-2064, 2009.

Paper IV
Mikkelsen MS, Jespersen BM, Mehlsen A, Engelsen SB, Frøkjær H. Cereal β-glucan immune modulating activity depends on polymer fine structure and in turn the polymer solubility and aggregation in solution. Molecular Nutrition & Food Research, *submitted November 2012*.

Paper V
Ibrügger S, Kristensen M, Poulsen MW, **Mikkelsen MS**, Ejsing J, Knudsen KEB, Jespersen BM, Dragsted LO, Engelsen SE, Bügel S. Effect of oat and barley β-glucans on cholesterol metabolism in young healthy adults: a randomized controlled trial. Journal of Nutrition, *submitted November 2012*.

Paper VI
Mikkelsen MS, Rasmussen MA, Savorani F, Jespersen BM, Kristensen M, Engelsen SB. Observations from a non-confirmatory dietary fibre intervention study with barley and oat β-glucans using NMR metabolomics. *Manuscript in preparation*.

Additional publications

Paper VII
Savorani F, Rasmussen MA, **Mikkelsen MS**, Engelsen SB. A primer to nutritional metabolomics by NMR spectroscopy and chemometrics. Food Research International, *accepted December 2012*.

Paper IIX
Kristensen M, Jensen MG, **Mikkelsen MS**, Astrup A. Flaxseed dietary fibers lower cholesterol and increase fecal fat excretion, but magnitude of effect depend on food type. Nutrition & Metabolism, 9:8, 2012.

Paper IX
Ibrügger S, Kristensen M, **Mikkelsen MS**, Astrup A. Flaxseed dietary fiber supplements for suppression of appetite and food intake. Appetite, 58:490-495, 2012.

Paper X
Nielsen MS, Karlsson AO, Engelsen SB. Cereal (1→3, 1→4)-β-D-glucans – functional properties and molecular interactions. Annual Transactions of the Nordic Rheology Society, 16: 179-183, 2008.

List of abbreviations

AOAC	Association of Official Analytical Chemists
BMI	Body Mass Index
CVD	Cardiovascular Disease
DC	Dendritic Cell
DP	Degree of Polymerisation
DP3/DP4	Ratio of cellotriosyl to cellotetraosyl units
EFSA	European Food Safety Authority
FDA	US Food and Drug Administration
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A
IDF	Insoluble Dietary Fibre
IL-10	Interleukin 10
IL-12	Interleukin 12
LDL	Low Density Lipoprotein
NMR	Nuclear Magnetic Resonance
PCA	Principal Component Analysis
PLS	Partial Least Square
RMSE	Root Mean Square Error
SCFA	Short Chain Fatty Acid
SDF	Soluble Dietary Fibre

Contents

Chapter 1

Introduction

In the Western world, healthy diets have become a major quest in our endeavour of living a good and long life, and bioactive components in food may constitute a key factor in the prevention of contemporary lifestyle related diseases.

Dietary fibres, particularly soluble fibres like mixed linkage (1→3, 1→4)-β-D-glucan (β-glucan) from oat and barley have been recognised for a long time for their positive effects on health. The dietary fibre hypothesis implies that a high intake of fibre-containing foods is directly related to or is associated with a low incidence of many disorders related to a Western lifestyle (e.g. chronic bowel disease, diabetes, coronary heart disease and colon cancer)[1-4]. Establishing a definition for dietary fibre has historically been a balance between nutritional knowledge and analytical capabilities[5]. After years of discussion the Codex[6] finally agreed in 2009 on the following definition of dietary fibre:

Dietary fibre means carbohydrate polymers with ten or more monomeric units which are not hydrolysed by the endogenous enzymes in the small intestine of humans and belong to one of the following categories*:

- Edible carbohydrate polymers naturally occurring in the food as consumed.
- Carbohydrate polymers, which have been obtained from food raw material by physiological, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health.
- Synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health.

Especially, the association between β-glucan intake and a risk reduction of cardiovascular diseases (CVD), which represent the number one death cause globally[7], is being continuously documented[2,3]. As a consequence, the US Food and Drug Administration (FDA)[8] in 1997 permitted a health claim on oat β-glucan stating that: "A diet rich in β-glucans may reduce the cholesterol level and reduce the risk of heart disease". Likewise, the European Food Safety Authority (EFSA)[9] in 2009 reacted favourable to the

*Note 1: Includes also lignin and other compounds if quantified by AOAC 991.43.
Note 2: Decision on whether to include carbohydrates with monomeric units from DP 3-9 should be left to national authorities. The European Union has decided to include DP 3-9.

application of a health claim for oat and barley products providing at least 3 g β-glucan/day which states, that: "Regular consumption of β-glucans contributes to maintenance of normal blood cholesterol concentrations".

Extracted β-glucan can be utilised as texture promoting additives in food production due to their viscous hydrocolloid properties[10] and considerable work has been conducted to develop β-glucan enriched functional food products such as breads and beverages in relation to the food labelling claims of FDA and EFSA[11-21]. In Denmark, the Nordic recommendations[22], which dates from 2004, calls for a dietary fibre intake of 25-35 g/day for optimal benefits. However, the actual consumption of dietary fibre in Denmark has been estimated to be about 20-23 g/day[23]. Altogether, this leaves a potential of benefit from β-glucan for the consumer, manufacturer as well as for society as a whole, as functional and healthy foods are expected to play a major role in preventing lifestyle related problems in the future[24].

In contrast to the numerous studies proclaiming a significant cholesterol lowering response to β-glucan, other studies have failed to prove this effect. Such variability in response is speculated to derive from structural/functional differences of various β-glucans or a reduction in the efficacy of the β-glucan e.g. following extraction and processing[1,2,25]. It is thus of great importance for the utilisation of β-glucan fibres to gain thorough knowledge on their physico-chemical characteristics prior to consumption. β-Glucan viscosity is suggested as the key molecular mechanism governing health effects, but other β-glucan physico-chemical properties mediated by the same, partly overlapping or different parameters such as molecular structure contributing to viscosity may also be important factors.

The aim of the present thesis study is to provide new insight into the relation between β-glucan and health at a molecular level using large-scale extraction, physico-chemical characterisation as well as *in vitro* and *in vivo* investigations.

Chapter 2

Objectives and hypothesis

The objective of this project was to investigate the structure-functionality properties of different barley and oat β-glucan fibres, and the *in vitro* and the *in vivo* effects of the β-glucans. It was hypothesised that due to structural differences, β-glucans possess diverse *in vitro* and *in vivo* functionalities.

As illustrated in Fig. 1, the studies presented in the six included papers are categorised into three thematic work packages covering: β-glucan extraction and characterisation, β-glucan *in vitro* functionality and β-glucan *in vivo* functionality. This thesis work constitutes the first comparison of barley and oat β-glucans all the way from plant raw materials to health effects.

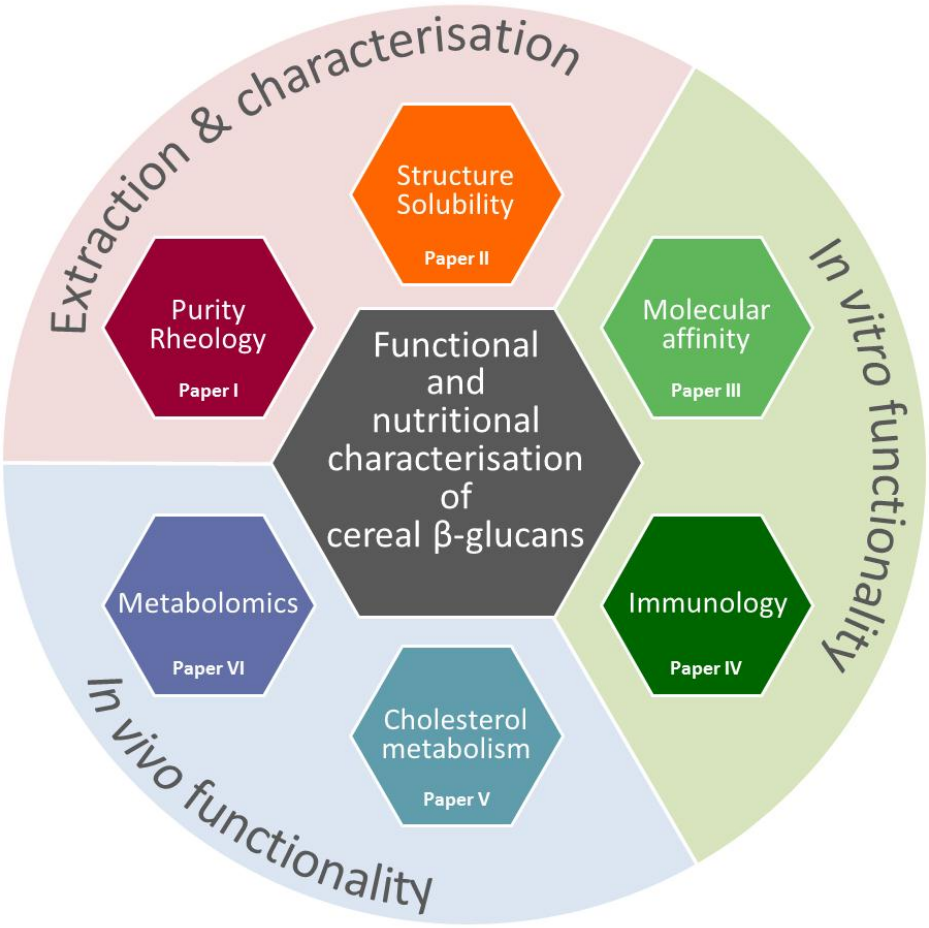

Fig. 1 Schematic overview of the six included studies.

2.1 Subaims of the work packages

For the comparison of the functional and nutritional characteristics of barley and oat mixed linkage β-glucans, the specific objectives for the three work packages were:

β-Glucan extraction and characterisation

To study the structure-functionality relationship of barley and oat β-glucans.

β-Glucan in vitro functionality

To study the *in vitro* effects of barley and oat β-glucans in relation to their physico-chemical properties.

β-Glucan in vivo functionality

To study the health effects of barley and oat β-glucans by comparative studies.

Based on the hypothesis that β-glucan structure is a key factor for the functional and nutritional properties, unique samplesets for the in-depth comparison of barley and oat β-glucan structure and functionality were generated on the basis of:

- Identical large-scale extraction of barley and oat β-glucan
- A new mutant barley high in β-glucan content and of unexplored structure
- Significant differences in barley and oat β-glucan fine structures
- Thorough physico-chemical characterisation of β-glucan products
- Side-by-side comparison of equally processed barley and oat β-glucans in a human intervention study

Chapter 3

Background

3.1 Cereal mixed linkage (1→3, 1→4)-β-D-glucans

The physico-chemical properties of barley and oat β-glucans are outlined in the following section with special emphasis on β-glucan block structural characteristics, solubility and aggregation capability. When comparing equally sized and concentrated β-glucan polymers, these molecular features are expected to determine the β-glucan viscous functionality.

Origin and structure

Within the plant cell wall, which provides the physical strength to the plant tissue, cellulose (pure β-(1→4)-glucan) microfribrils are embedded in cross linking networks of pectins and hemicelluloses as illustrated in Fig. 2. The hemicellulose glycans with linear backbones of e.g. β-(1→4)-glucan either contain short side chains or different linkage types, which limits their intermolecular aggregation and provide flexibility to the cell wall.

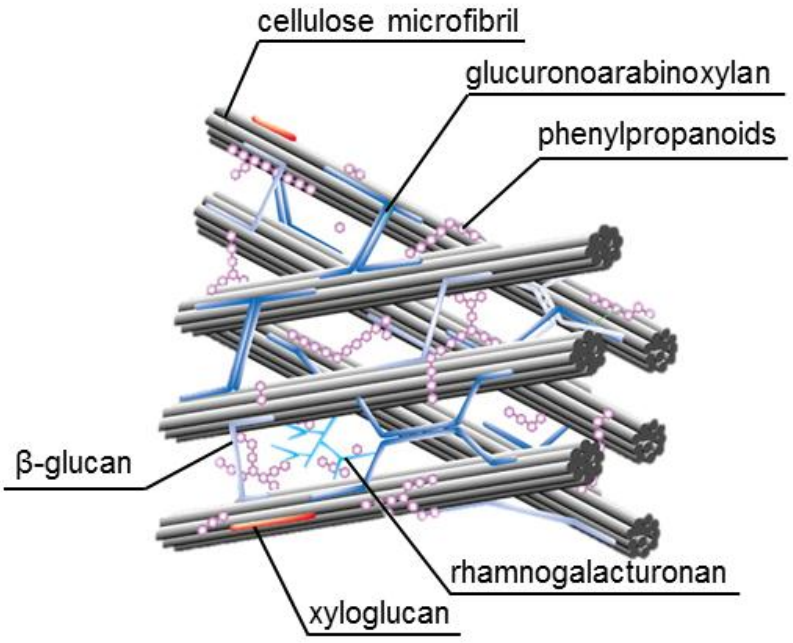

Fig. 2 Schematic structural model of type II plant cell wall from monocotyledonous plants as represented by rice (*Oryza sativa*). The microfibrillar phase (cellulose microfibrils) is embedded in the matrix phase consisting of a variety of pectins (rhamnogalacturonan) and hemicelluloses (β-glucan, xyloglucan, glucuronoarabinoxylan) along with phenylpropanoids. Modified after Yokoyama and Nishitani[26].

Mixed linkage (1→3, 1→4)-β-D-glucan (Fig. 3) is particularly a hemicellulose found within the cereal grasses with oat and barley containing significant amounts (3-5%, dry weight basis), and some oat cultivars holding as much as 6-7% and some barleys even 12% or more. As the other hemicellulose components of the cereal grains, β-glucan is mainly located in the aleurone layer (oat) and endospermic cell walls (barley)[27-29], but β-glucan has also been reported to be present in non-endospermic tissues of cereal grasses such as elongating meristematic cell walls[30,31].

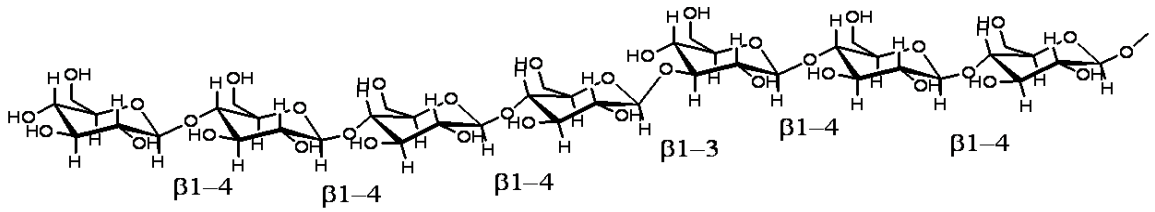

Fig. 3 Primary structure of mixed linkage (1→3, 1→4)-β-D-glucan (β-glucan) from cereals.

β-Glucans are linear homopolymers that contain about 70% (1→4)-linked and 30% (1→3)-linked β-D-glucopyranosyl residues. Blocks of (1→4)-linkage sequences, with cellotriosyl and cellotetraosyl units constituting 85-90% of the total, are separated by single (1→3)-linkages, which break up the regularity making the molecule more soluble and flexible[14,32]. The molar ratio of the cellotriosyl to cellotetraosyl units with degree of polymerisation (DP) of 3 and 4, respectively, is referred to as the DP3/DP4 ratio and considered as a "fingerprint" of the individual β-glucan. Generally, β-glucans derived from oat have been reported to exhibit smaller DP3/DP4 ratios (1.5-2.3) than those of barley (1.8-3.5). Smaller amounts of cellulosic oligosaccharides with degree of DP 5-15 are also present and may constitute up to 10% of the polymer[33-35].

Cereal β-glucan polymers are often more than 1000 units long. Reported molecular mass values range between 44-3,000 kDa for oat and 130-2,500 kDa for barley[14,34,36,37]. In contrast to the structural features, substantial differences has been observed in the molecular mass of β-glucans isolated from various oat and barley genotypes[38]. The large variations reflect the diversity of botanic origin, but might also result from the methodology of extraction and molecular mass determination. β-Glucanase activity and mechanical damage during extraction have been reported to cause β-glucan depolymerisation[38,39].

Solubility and rheological properties

Solubility of β-glucan depends on how the glucose building blocks are interlinked by different and altering β-glucosidic bonds (1→3 and 1→4). Pure structures of either β-(1→4)-glucose as in cellulose or β-(1→3)-glucans as found in curdlan (microbial origin) form helical structures, which will aggregate and precipitate. In case of the mixed linkage (1→3, 1→4)-β-glucans, increasing the ratio of β-(1→3)-linkages will introduce more

kinks, which provide a more open conformation and water soluble polymer[40,41]. In comparison to water soluble mixed linkage β-glucans, the insoluble β-glucans are characterised by high ratios of cellotriosyl to cellotetraosyl units and/or larger amounts of long contiguously linked β-(1→4) segments[34]. If either cellotriosyl or cellotetraosyl units predominates a β-glucan polymer, the overall chain becomes more regular in shape and hence less soluble because of chain alignment (Fig. 4, upper examples). In contrast, random arrangement of a mixture of the cellotriosyl and cellotetraosyl units means that the β-glucan chains will not aggregate over extended regions and can remain in aqueous solutions, as illustrated in Fig. 4 (lower example). Such differences in β-glucan fine structure could be used to the plant's advantage in meeting different functional requirements in different cells or at different stages of development[28], for which reason the existence of a great variety of β-glucan sequential patterns can be assumed.

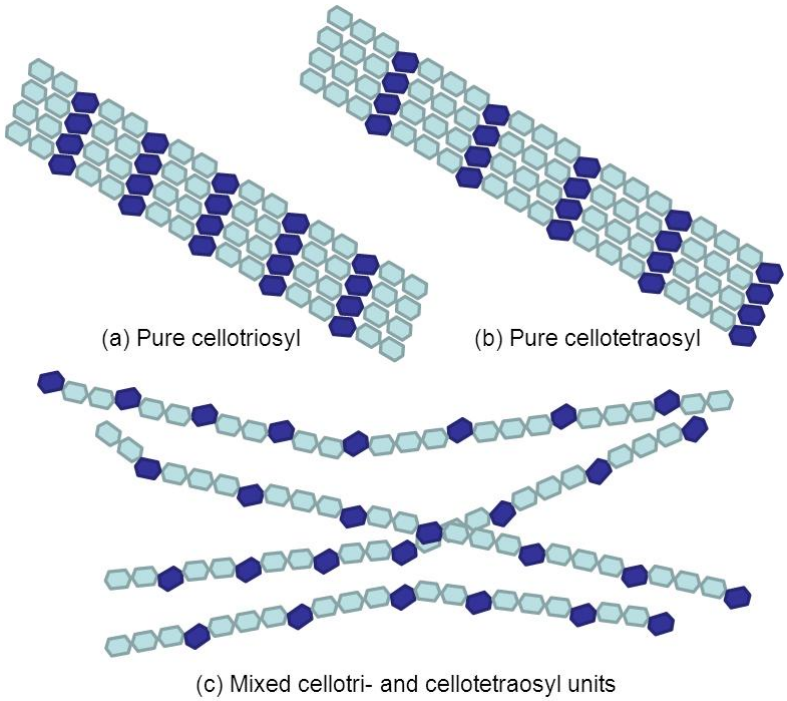

Fig. 4 The effect of β-glucan cellotriosyl and cellotetraosyl block structures on aggregation and solubility. (a) Aggregated pure cellotriosyl polymers, (b) aggregated pure cellotretraosyl polymers and (c) mixed cellotri- and cellotetraosyl polymers in solution. β-(1→3)-Linked residues are shown in dark blue whereas β-(1→4)-linked residues are depicted in light blue. Modified after Burton et al. [28].

Both, linear regions of repeated units of the same length as well as long cellulosic oligomer blocks has been suggested to affect β-glucans aggregation, as illustrated in Fig. 5. Woodward et al.[42] showed that longer sequences of (1→4)-linkages give less soluble β-glucans because of intermolecular associations. However, Izawa and co-workers[43] suggested that even if there are long blocks of β-(1→4)-linkages, their influence on insolubility would be insignificant compared to that of long blocks of contiguous cellotriosyl residues. More recent data support this latter conclusion that structural

regularity, arising from increasing proportions of β-(1→3)-linked cellotriosyl units, reduces solubility and also increases the tendency to gel[32,44,45].

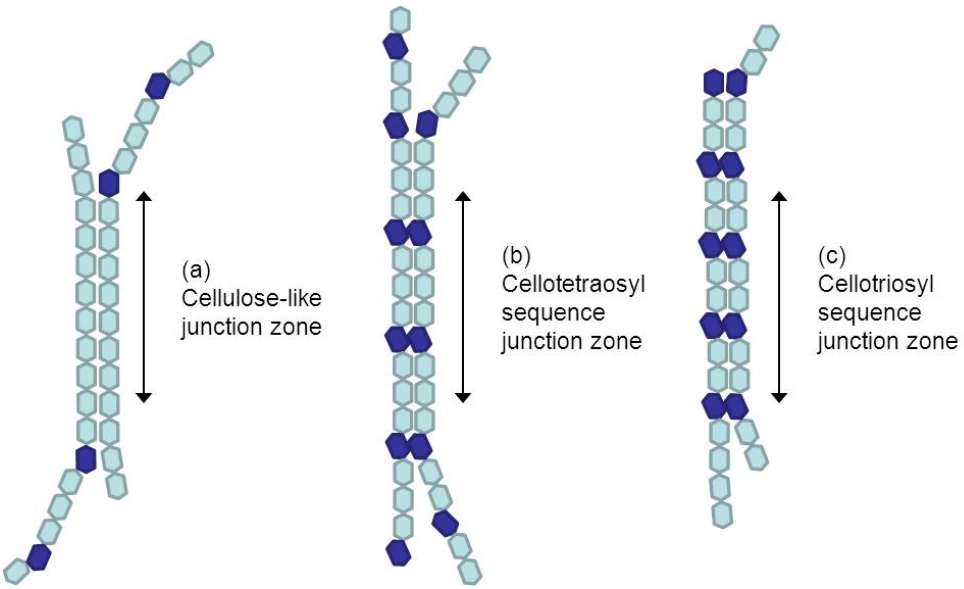

Fig. 5 Schematic diagrams of interactions that may form junction zones between (1→3, 1→4)-β-glucan polymers. (a) Interaction between cellulose-like regions of the polymer, (b) interaction between sequential cellotetraosyl units and (c) interaction between sequential cellotriosyl units. β-(1→3)-Linked residues are shown in dark blue whereas β-(1→4)-linked residues are depicted in light blue.

The solubility of β-glucan is not only influenced by the fine structure of the polymer but also by properties of the solute. Wood et al.[46] stated that the amount of β-glucan dissolved depends on temperature, ionic strength and pH of the solvent. β-Glucan solubility is also affected by the microstructure, which the polysaccharides receive during various extraction and drying processes. Depending on the methods, which are most frequently freeze-drying and spray-drying of aqueous dispersions and solvent exchange from aqueous media at various values of pH, polymer chain to chain interactions of different strength are formed. These might resist redissolution of the polysaccharide and some of them also resist treatment with aprotic solvents. As the aggregated polymers are not fully solvated, supramolecular aggregated species might remain present even in very dilute solutions[46,47].

Viscosity is a physico-chemical property associated with most soluble dietary fibres[48]. Native cereal β-glucans are generally of very high viscosity. However, the actual viscosity of a specific β-glucan solution is a function of extraction method, block structural characteristics, solubility, molecular mass, concentration and temperature[14,49,50]. There is a positive non-linear relationship between the molecular mass of β-glucans in solution, at equal concentrations (w/w), and viscosity[51]. Many studies suggest that the viscous properties of β-glucans are responsible for the health benefits associated with consumption of these fibres[2]. Thus, from the perspective of physiological effects most

studies have focussed on the flow viscosity of β-glucans. However, the properties of aqueous solutions of polysaccharides are controlled by both volume occupancy in solution (hence viscosity) and propensity of the molecules to self-associate or aggregate, which refer to the mechanism of gel formation. These two properties are not the same, and β-glucans can exhibit both[2,52-54]. Vaikousi et al.[36] reported a detailed study on the flow behaviour and gelling properties of water soluble barley β-glucans varying in molecular mass. The results indicated that during storage low molecular mass β-glucans showed unusual shear-thinning behaviour, which was explained by aggregation tendency. Such aggregates were proposed to be fringed micelles formed by side-to-side aggregation of chains of molecular mass ~175 kDa[55,56]. Burkus and Temelli[52] found that network formation in solutions of β-glucans is highly time- and concentration dependent. This mechanism makes gelation of β-glucan unlikely to occur in the human intestine, where constant shear and mixing with other food constituents will hinder intermolecular β-glucan association.

3.2 In vitro and in vivo functionalities of β-glucans

Physiological effects and proposed mechanism of action by oat and barley β-glucans are reviewed in the following section. Both *in vitro* and *in vivo* functionalities are outlined to present the diverse field of β-glucan health investigations.

Absorption and metabolism

General proposed beneficial effects of dietary fibres are presented in Table 1. Dietary fibres pass undigested through the human small intestine and are completely or partially fermented in the large intestine by the intestinal microflora yielding short-chain fatty acids (SCFA), which are protective to the colon mucosa. Major end-products of the β-glucan fermentation are the SCFAs; acetate, propionate and butyrate. β-Glucans can be fermented to a degree between 70 and 100%[57-59]. SCFAs are efficiently absorbed in caecum and colon where they serve as fuel for a large variety of cells e.g. colonocytes[60]. Consumption of dietary fibres and hence β-glucans is basically associated with increased faecal weight[61] and accelerated transit time[62].

Table 1 Proposed effects on human health and physico-chemical properties of dietary fibre.

Physico-chemical property	Proposed mechanism	Effect on health
Soluble dietary fibre		
Viscosity	Delays gastric emptying, prolonging intestinal phase[63,64]	Contributes to satiety
	Prevents or delays nutrient uptake in the small intestine[65]	Lowers glucose, insulin and lipid levels after a meal
	Prevents the reabsorption of bile acids[3,66,67]	Lowers blood cholesterol levels
	Prevents the reabsorption of oestrogen[68]	Protects against breast cancer
Interaction/"binding"	Binding to bile acids (only demonstrated *in vitro*)[69-71]	Lowers blood cholesterol levels
	Interaction with digestive enzymes (only demonstrated *in vitro*)[72]	Lowers glucose, insulin and lipid levels after a meal
Fermentation	Growth of health-promoting bacteria[73]	Protects against inflammation and colorectal cancer
	Production of short-chain fatty acids[3]	Lowers blood cholesterol levels and protects against colorectal cancer
Insoluble dietary fibre		
Intact particles	Increase stool weight[61,74]	Reduces the incidence of colorectal cancer and intestinal diseases
	Accelerate transit time[62]	Reduces time for nutrients to be absorbed; lowers glucose, insulin and lipid levels after a meal
Water-holding capacity	Reduces or delays nutrient uptake (animal study)[75]	Lowers glucose, insulin and lipid levels after a meal

Mechanism of action for health effects

The far most documented nutritional benefits of oat and barley β-glucans is the lowering of the serum cholesterol levels and in turn a reduced risk for the development of cardiovascular diseases[2,3,66,76-78]. Many attempts have been made to clarify the mechanisms by which β-glucans exert this effect, and there are typically three suggested mechanisms whereby β-glucans may exhibit the hypocholesterolemic property.

One proposed mechanism concerning water soluble fibres in general involves interference with the lipid and/or bile acid metabolism. The viscous properties of β-glucans may potentially prevent the reabsorption of bile acids plus cholesterol in the small intestine either via the formation of a barrier layer upon the intestinal absorptive cells (Fig. 6a) or by entrapment of mixed bile salt-cholesterol micelles in a β-glucan network (Fig. 6b)[66,67,79]. This leads to increased excretion of bile salt via faeces. As a consequence, hepatic conversion of cholesterol into bile acids will increase, hepatic pools of free cholesterol will decrease and endogenous cholesterol synthesis will increase. This is thought to increase activity of 7-α-hydroxylase and HMG-CoA reductase to compensate for the loss of bile acids and cholesterol from the liver stores. Furthermore, hepatic Low Density Lipoprotein (LDL) cholesterol receptors become up regulated to restore the hepatic cholesterol pool, and this will lead to decreased serum LDL cholesterol concentrations[3].

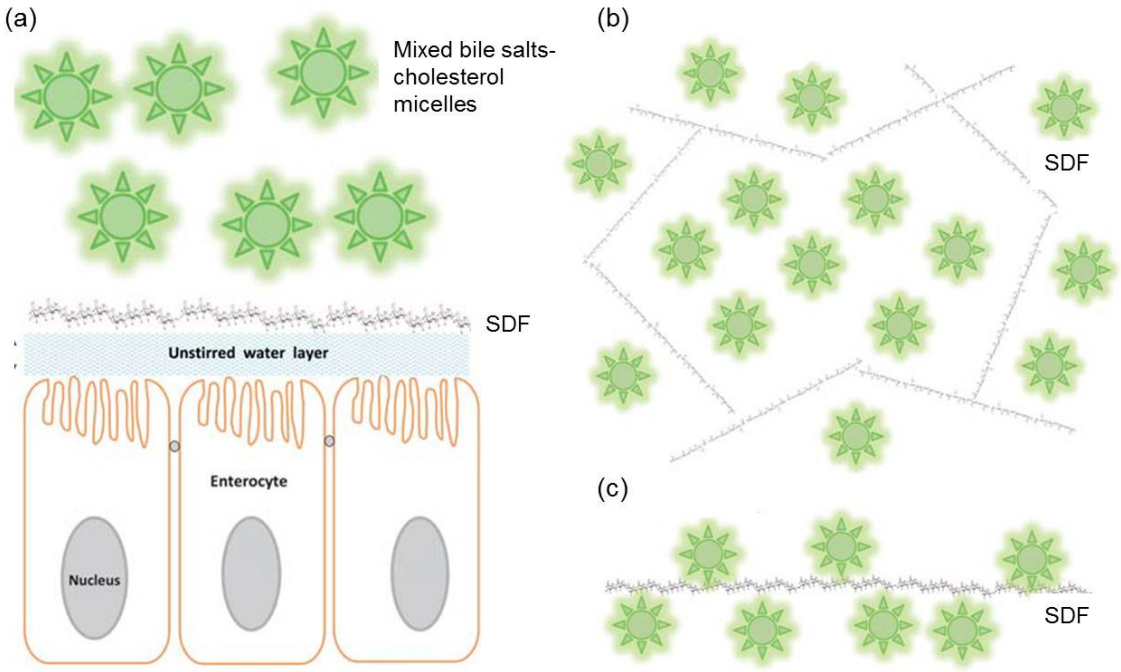

Fig. 6 Proposed hypocholesterolemic actions of soluble dietary fibres (SDF) like β-glucan in the intestinal lumen. The soluble fibres increase the intestinal viscosity whereby reabsorption of cholesterol and bile salts may be prevented by a potential β-glucan barrier layer (a) or entrapment of mixed bile salt-cholesterol micelles in a β-glucan network (b). Alternatively, mixed bile salt-cholesterol micelles may bind to β-glucan polymers in the intestinal lumen (c). All three hypotheses (a-c) lead to increased excretion of bile salt via faeces. A cascade of reaction patterns following this will eventually lead to decreased serum low LDL cholesterol concentration. Modified after Gunness and Gidley[66].

Besides a higher bile acid excretion due to an increased intestinal viscosity, it is also suggested that β-glucans may directly bind to bile acids leading to excess faecal bile acid excretion (Fig. 6c). Kritchevsky and Story[69] conducted pioneer work within *in vitro* studies on dietary fibre binding of bile salts. In 1974 they measured bile salt binding by non-

nutritive fibre using radioactively labelled bile salts. Since then, many approaches have been used to investigate the molecular interactions between fibre and small molecules like bile salts[70,80-85], however, there has been very little data reported on the nature of any molecular interaction between bile salt micelles and dietary fibre. One of the few examples is the study by Bowles and coworkers[86] who investigated specific interaction between barley β-glucan and glycocholic acid using solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy. However, no evidence for specific binding (e.g. hydrogen bonding or hydrophobic interactions) was found. Using the same method, Gunness et al.[71] reported interaction between β-glucan and a model bile salt (taurochenodeoxycholate) and proposed a model for interaction where bile salt micelles have frequent close molecular contact with β-glucan chains causing changes in the bile salt micelle conformation. This suggests that β-glucan interacts directly on a molecular length scale with the micelles.

Another suggested mechanism of action links to the flattering of the postprandial blood glucose and insulin rises following β-glucan intake[87-89], which again has been related to: the amount and quality of fibre[90], increased intrinsic viscosity and hence the gastrointestinal environment[91] and physical integrity of the food[92]. Viscous dietary fibres may reduce the rate of intestinal absorption of glucose with subsequent decrease in insulin production by the pancreas[93]. As insulin is an activator of HMG-CoA reductase, it is possible that reduced insulin levels could lead to a decrease in cholesterol synthesis[94].

Thirdly the cholesterol reducing effect from soluble dietary fibres like oat and barley β-glucans has been associated with the SCFA produced by fermentation of microbiota in the colon. Propionate has been reported to inhibit cholesterol synthesis in the liver[95], and this is the main argument in the SCFA-cholesterol lowering theory. On the other hand, propionate is found as a substrate for hepatic gluconeogenesis, and in this way it seems to have two opposite and competing effects on the gluconeogenesis[60]. Acetate is hypothesised as a primary substrate for cholesterol metabolism, and a dependence on the ratio of propionate and acetate in the possible utilisation of SCFA in lipid synthesis has been suggested[96,97]. However, the effect of SCFA in cholesterol metabolism may still be regarded as unclear.

In summary, the properties that could contribute to the cholesterol lowering effect of oat and barley β-glucans in animal and humans are the prevention of bile salt reabsorption (with subsequent excess faecal excretion) and decreased hepatic cholesterol synthesis modulated by insulin and/or by fermentation products such as propionate.

The immune modulatory capacity of mixed linkage cereal β-glucans has been confirmed in several studies though showing less efficiency than β-glucans of microbial origin [98-101]. The immunological potency of fungal and yeast β-glucans was found to vary with the

molecular mass, solution conformation, structural characteristics and the cell type that is targeted[102-105]. Cereal β-glucans have shown *in vitro* activity on cytokine secretion[106], phagocytic activity and cytotoxicity of isolated immune cells[107] and activation of the complement system[108]. In an *in vitro* test system favouring complement activation via the alternative pathway, aggregated barley β-glucan significantly inhibited haemolysis indicating complement activation, whereas high molecular mass soluble β-glucan showed no activity[109]. In addition, cereal β-glucans have shown an effect on cytokine secretion by isolated dendritic cells (DCs) as they increased the interleukin-10 (IL-10) and reduce the IL-12 levels[106]. This effect has been linked to the binding of β-glucan to dectin-1 receptors that initiates the immune response of dendritic cells[102,110]. Clinical trials on either athletes or hypercholesterolemic individuals, ingesting up to 6 g oat β-glucan daily, did not change immune parameters[111,112]. Thus, so far, cereal β-glucans have not shown immune modulation in the few conducted human studies.

Chapter 4

Overview of studies and main outcomes

An overview of the six included studies (Fig. 1) and the main outcomes is presented in the following section. A full presentation of the results can be found in the respective papers.

4.1 β-Glucan extraction and characterisation

Paper I

Objective: Crude and purified barley and oat β-glucan samples were characterised in terms of composition, structure and rheological features to investigate the effect of α-glucan impurities on the β-glucan viscous properties.

Results: Oat β-glucan was approximately three times the size of barley β-glucan and no degradation was seen throughout the purification process. Considerable amounts of α-glucan impurities were found in crude and purified oat samples as compared to the purer barley samples. No significant structural differences were seen between oat and barley β-(1→3) to β-(1→4) linkage ratios. A direct viscosity dependence on β-glucan content regardless of amount and composition of α-glucan impurities was found for both barley and oat samples.

Conclusion: The study demonstrates that structural characteristic of the β-glucan polymers including the molecular mass dominates the functional traits over the presence of larger amount of starch/α-dextrins.

Paper II

Objective: A high β-glucan content mutant barley, mother barley and oat β-glucan were large-scale extracted, characterised and compared in terms of physico-chemical properties to elucidate structure-functionality differences.

Results: Large-scale extraction by comparable protocols lead to high purity and similar molecular mass barley and oat β-glucans. The main variance among sample compositional, structural and functional features was primarily explained by differences in the β-glucan block structures i.e. the ratio of cellotri- to cellotetraosyl units (mutant barley>mother barley>oat) and by differences in the ratio of β-(1→3) to β-(1→4)

glycosidic linkages. The differences corresponded to a lower solubility of barley samples as compared to oat. Mutant barley β-glucan demonstrated a unique block structure with an extraordinary high number of cellotriosyl units as captured by both chromatographic and spectroscopic methods. Hypothetical illustrations of the varying barley and oat β-glucan block structures were presented. Low solubility of the barley samples was prospectively a consequence of repetitive cellotriosyl substructures that are prone to aggregate.

Conclusion: The study stresses the importance of β-glucan fine structure analysis for the relation to solubility and in turn to health effects.

4.2 β-Glucan in vitro functionality

Paper III

Objective: The molecular affinity between barley and oat β-glucans and phenolic compounds was studied in order to elucidate the mechanisms behind small molecule (e.g. bile acid) retention by β-glucan fibres.

Results: It was shown that phenolic aglycones in contrast to their corresponding glycosides were retained by both barley and oat β-glucans, which indicate the possible importance of the β-glucan hydrophobic properties for small molecule absorption. The different phenolics were retained at varying levels, but the nature of the molecular interaction could not be explained by simple correlation to any specific physico-chemical feature of the phenolic compounds. However, increased β-glucan viscosity showed to increase the small molecule retention and degradation of the β-glucan tertiary structure by enzymatic hydrolysis released some but not all of the phenolics.

Conclusion: The results suggest that at least two independent β-glucan properties are involved in determining the binding of small molecules to fibres: the degree of polymerisation and the fine structure of the polymers.

Paper IV

Objective: This study addressed the importance of the physico-chemical properties of barley and oat β-glucans for their capacity to affect immune responses.

Results: All the 23 β-glucan samples characterised in Paper II were found to modulate a microbial lipopolysaccharide induced dendritic cell cytokine pattern for which IL-10 levels increased and IL-12 levels decreased. Likewise, the β-glucans modulated a *Lactobacillus acidophilus* induced cell system giving rise to up-regulation of IL-10 but only modest changes of IL-12 cytokine levels. The extent to which β-glucan modulated the immune responses was found to depend on the β-glucan solubility, block structure as expressed by the ratio of cellotri- to cellotetraosyl units and especially the polymer aggregation in

solution. Hypothetical speculations on β-glucan fringed micelle interaction with dendritic cells were presented.

Conclusion: This comparative study reveals that the β-glucan fine structure and in turn the polymer solubility and aggregation tendency are key elements for understanding their immune modulatory capacity.

4.3 β-Glucan in vivo functionality

Paper V

Objective: Barley and oat β-glucans which had been processed and dried by the same method were compared in a human study to investigate possible differences in the effectiveness and relate β-glucan functionality to specific physico-chemical properties.

Results: The mutant barley, mother barley and oat β-glucans from Paper II were consumed by 14 healthy adults in a blinded 4-arm cross-over intervention study as a daily dosage of 3.3 g/day fibre for a 3-week period. As compared to control (no β-glucan added) it was found that 3.3 g of extracted barley and oat β-glucan was not enough to significantly lower total and LDL cholesterol levels within the 3-week period. However, in contrast to barley oat β-glucan appeared to be more potent in lowering cholesterol when compared to baseline, presumably due to higher solubility and viscosity as compared to barley.

Conclusion: The results indicate that β-glucan solubility and in turn the spatial appearance in the intestine is of prime importance for the fibre interference with the endogenous lipid and/or bile acid metabolism.

Paper VI

Objective: In order to further clarify the *in vivo* β-glucan fibre functionality, as investigated in Paper V, the plasma metabolic patterns after β-glucan intake was studied using NMR metabolomics.

Results: It was shown that the plasma metabolic data correlated well with reference plasma glucose and serum triglyceride measurements. Subject variance was found to significantly dominate data although participant age and BMI exhibited little variance. The second most influential variation could be assigned to gender and characteristic lipoprotein landscapes were found for male and female samples. No significant difference between 3-week β-glucan treatments and control was found in agreement with the findings in Paper V. However, two sets of measurements (0, 2 and 4hour), one male and one female, appeared to represent fat boosted systems prior to β-glucan exposure, and these systems might prove more sensitive to β-glucan fibre exposure compared to average metabolomes of the young healthy adults.

Conclusion: The results of this study reveal the existence of subject unique lipoprotein profiles, which are strongly dependent on gender, BMI and diet. It is hypothesised that β-glucan driven health effects from 3.3 g/day may only be detectable in young healthy adults after a diet high in fat.

Chapter 5

Methodological considerations

In the following section selected methods applied in the experimental part of the project are presented and discussed: 1) β-glucan extraction, 2) human study and 3) metabolomics and multivariate data analysis. The approach of using these methods in combination makes the present thesis work unique compared to other studies investigating cereal β-glucan functionality. The reader is referred to Paper I and II for a detailed survey of the physical/chemical principles, advantages and disadvantages of rheological, chromatographic and spectroscopic analytical techniques in relation to β-glucan characterisation. Reflections upon *in vitro* methods are presented later in the general discussion and in the respective papers.

5.1 β-Glucan extraction

Intervention product characteristics and study design are key elements in the cereal β-glucan nutritional understanding[25] and β-glucan extraction methods largely affect the product characteristics. Thus, choice of an appropriate extraction technique is important as it may affect quantity[113], purity[114,115], structure[33], molecular mass[116], microstructure[47] and in turn the rheological properties of the extracted β-glucan[117,118].

Traditionally, β-glucan extraction methods involve several steps: 1) inactivation of endogenous enzymes in the grain, 2) extraction with water or alkali solutions, 3) removal of contaminating protein and starch using hydrolytic enzymes and/or selective absorption, 4) precipitation of β-glucan from the purified solution with alcohol and 5) freeze, drum or spray drying of the extract[119]. The extractability or solubility of β-glucan is dependent on the extraction parameters (temperature, ionic strength, pH, duration of extraction and liquid-solids ratio), pretreatment (heating, drying and fineness of grind) and presence of enzymes (endogenous or from contaminating microorganisms). In general, the extractability of cereal β-glucans increases with elevated temperatures and pH[39]. Higher temperature extracts larger β-glucan molecules and the extractability of oat β-glucan seems to be higher than that from barley[46,120]. Wood et al.[37] found that extraction under alkaline conditions increases the starch solubilisation and the proportion of co-extracted arabinoxylan from barley. Adjustment of the pH below the isoelectric point of proteins (pH 6) has been used for removing additional amounts of protein[121]. For complete extraction of β-glucan from cereal grains and high purity, rather drastic conditions are needed[122], often inappropriate for potential food uses of the isolate[119,120].

For food applications, cost effective production of β-glucan concentrate in terms of yield, purity, viscosity stability and in turn preservation of the functional properties is the main concern. In the present thesis work (Paper II) a method of extracting soluble oat and barley β-glucan in large-scale for food applications using hot water (95ºC), heat-stable α-amylase and centrifugally separation was adapted from Kvist and Lawther[123] with some modifications. In addition to starch dextrification by α-amylase and protein removal by centrifugation, solution was protease treated and ethanol precipitated to purify β-glucan from additional proteins and starch derivatives. The extraction conditions were held at high temperatures (85-95 ºC) for minimum endogenous enzyme activity, and pH 6 was chosen to balance α-amylase and protease enzyme optimum with starch solubilisation and the isoelectric point of barley proteins.

The final β-glucan purity largely depended on the starch content in the raw material in spite of adjustment of α-amylase amount to the crude material starch content. This indicate lower extractability of β-glucan from solutions highly dominated by starch and suggest a greater extraction potential in turn of purity and yield from more β-glucan rich grain fractions like the high β-glucan barley mutant or oat bran. Since processing has been shown to cause depolymerisation and loss of viscosity of β-glucan polymers[1] and considering the small effect from starch on β-glucan viscosity (Paper I) no further purification of the large-scale extracted β-glucans was performed. Optimisation experiments prior to the large-scale extraction showed some degradation of barley β-glucan molecular mass during the extraction steps (500-350 kDa), but the magnitude of depolymerisation may be considered as small and the final molecular masses of the three extracted barley and oat β-glucans ranged 250-300 kDa, which is medium as compared to other extracted β-glucans[116].

In summary, the extraction conditions used for the large-scale extraction of barley mutant, barley and oat β-glucan was optimised to balance product quality and yield. Using the same procedure large batches of pure, comparable and reproducible β-glucans with similar molecular masses and specific genotypic structural characteristics were extracted for the use in the human intervention study.

5.2 Human study

The human gastrointestinal tract can be considered as a multivalent sink capable of converting biological material to energy. However, on the detailed chemical and kinetic level there is a great intra- and inter-individual variation in efficiency and specificity. In order to deal with this, three key issues are particularly important to consider when planning an intervention study: 1) a full cross-over design should be preferred to parallel studies because of the high inter-individual variation, 2) controlled diets should be preferred to habitual diets due to the large diversity of compounds present in different

foods and seasonal changes in habitual diets and 3) different metabolomes display different kinetics upon a given challenge[124]. Because the human homeostasis will efficiently restore the status quo, sampling dynamics is an important issue when analysing blood metabolic changes to a nutritional exposure. Recently, the response kinetics of the metabolome upon a nutritional challenge, e.g. postprandial sampling, has been favoured from the previous more static approaches based on single end-point or few samples along the time line of the intervention[125].

The intervention study of this thesis work was designed as a blinded randomised cross-over trial examining the hypocholesterolemic effects from two barley and one oat β-glucan as compared to a non-fibre control during four 3-week periods separated by 2-3 weeks washout. Fourteen normocholesterolemic young adults, 8 females and 6 males, aged 19-25 years completed the study. Recently, we investigated traditional exposure and effect markers from flaxseed dietary fibre intake in a similar study design and found a relation between fibre viscosity and hypocholesterolemic effect[126]. Hence, the study design, fibre effect in young healthy people and statistical power had been validated prior to the present β-glucan intervention study.

The food platform used may influence the trial outcome by increasing the variability of individual subject response and in turn reduce the power and precision of a trial. Study subjects of the present trial maintained their habitual diet due to lack of external project funding for setting up controlled diets and because the FDA and EFSA health claims on 3 g β-glucan/day are based on individuals eating their habitual diet. The full cross-over design compensated some statistical power, but enrolment of a different cohort that habitually consumed a low-fibre diet presumably would have been advantageous. Extreme fibre intake up to 50 g/day was observed for some subjects.

Before and after each period fasting and postprandial (0, 2 and 4 hour) blood samples were drawn. Fasting samples potentially show the long term changes in metabolically cleared systems whereas postprandial samples monitor fast changes following a nutritional exposure. Postprandial effects from intervention were strongly declined at 2 hour. Hence, for investigation of postprandial blood glucose and triglyceride small kinetic changes upon β-glucan treatment more frequent sampling between 0 and 4 hours would have been favourable. Again, funding was a restricting factor along with considerations of participant safety in terms of total amount of blood drawn.

Altogether the present human trial enclosed a highly refined study design including repeated measures and cross-over for handling of different sources of variation, which inevitably makes the interpretation of both confirmatory and non-confirmatory results relevant. The general lack of effect of β-glucan treatment as compared to control is further considered later in the general discussion.

Conclusively, human trials have to be carefully designed in the future if they are to yield clear evidence of the value of β-glucan inclusion in diets at sensible levels. Most often, however, choice of an appropriate study design is a balance between cost and statistical power at the lowest tolerated level, which obviously decline the study robustness to mistakes.

5.3 Metabolomics and multivariate data analysis

Both exploratory and targeted metabolomics approaches using NMR spectroscopy and multivariate data analysis were employed in Paper VI to investigate plasma exposure and marker effects from treatment with the large-scale extracted barley and oat β-glucans.

In metabolomics the profile of metabolites in a biological system e.g. bodyfluids, tissues and cellular extracts is identified and quantified[127] often with the purpose of linking a specific dietary intake to a metabolic fingerprint. The overall metabolome of an organism is a complex signature depending on: 1) the metabolites from cells and tissues in the host, 2) foreign metabolites derived from e.g. drugs, 3) metabolites derived from the digestion of food and 4) metabolites produced by the gut microbiota. The human metabolome consists of a large number of small metabolites (~20,000) belonging to a variety of different compound classes, such as amino acids, peptides, organic acids, lipids, nucleotides[128]. Both nuclear magnetic resonance (NMR) spectroscopic and mass spectrometry (MS) based technologies have been widely used to measure the effect of dietary components in the nutri-metabolomics approach[129,130].

NMR spectroscopy functions by the application of a strong magnetic field and resonance frequencies pulses to the nuclei of atoms in a sample. Nuclei in different chemical environments absorb energy at slightly different resonance frequencies, which is referred to as the chemical shift of the atom[131]. The majority of NMR metabolomics studies have been using ^{1}H NMR spectroscopy since it is the most informative and sensitive NMR method and the most rapid method suited for high throughput studies[124]. The larger magnetic field the better is the resolution and the sensitivity[132]. NMR spectroscopy is a non-destructive technique with the ability to simultaneously quantify multiple classes of metabolites. Moreover, NMR metabolomics studies of biofluids have shown high reproducibility and in most cases only one analytical replicate is sufficient per sampling point[133].

Numerous factors have to be carefully considered to gain reliable results from metabolomics studies. A general challenge in metabolomics is the relative low number of samples compared to the number of variables, which means that a full cross-over design should be preferred to parallel studies due to the possible high inter-individual variation in human studies[134]. Likewise, dynamic/kinetic studies should be preferred to static studies

only including end-point measures. The most commonly used biological samples for nutri-metabolomics studies are easy accessible samples such as urine, salvia and blood/plasma/serum. Great care must be taken to sample collection because biological samples is highly susceptible to degradation[135]. Both urine and plasma samples should be handled quickly and stored preferably at -80ºC[133].

The metabolomics work included in this thesis explored plasma samples analysed by 600 MHz ^{1}H NMR spectroscopy, and the best possible preservation procedures for the samples was carried out[133]. Choices of acquisition conditions and type of experiments were based on previous experience[136] and generally spectra of very high quality were achieved. Metabolite identification is an essential part of a metabolomics study[134] and approximate full assignment of metabolite signals was possible. Especially, the lipoprotein region showed well resolved profiles of interest, and here the favourable quantitative nature of NMR spectroscopy was successfully used to study lipoprotein fluctuations in relation to diets.

The complexity and richness, which are key qualities of metabolomics data, generally makes data interpretation very complicated. Multivariate data analysis are here an obvious choice due to their ability to decompose complex multivariate data into simpler and potentially interpretable structures[137].

Data preprocessing such as normalisation (scaling between samples), centering and scaling between variables is typically applied to remove unwanted systematic bias in spectra while retaining the interesting biological information. Before multivariate data analysis each sample is usually normalised to the global or to a reference signal area which adjusts for the variance that may arise from sample inhomogeneity or minor differences in sample preparation. Furthermore, the data matrix is normally mean centered in order to focus on the differences between samples rather than the direction of the overall variance. Centering converts all the concentrations to fluctuations around zero instead of around the mean of the metabolite concentration and hereby adjusts for offset variation between the high and low abundant metabolites. The most common scaling method for metabolomics data is autoscaling which employs the standard deviation as a scaling factor. This adjusts for the fold difference between the detected metabolites[138].

Principal component analysis (PCA) summarises the major variation in a data set and capture systematic variation in a model that can be used to quickly visualise which samples are similar or dissimilar to each other. Mathematically a PCA model can be written as:

$$X = T \cdot P' + E$$

X is the data matrix representing samples and variables decomposed into a score matrix (T) and a transposed loading matrix (P'). The E matrix contains the residuals, the part of the data not explained by the principal component model[137]. From this, possible spectral loadings causing any treatment-related separation may be identified.

Partial least square (PLS) regression is a multivariate calibration/regression method by which two sets of data X (e.g. spectra) and Y (e.g. a response variable/biomarker) are related by means of regression. This enables establishment of a linear model that can predict Y from the measured spectra in X[139]. Like PCA, PLS regression generates a model of the data, but where PCA models the main variance in the data itself, PLS derives a model that describes the correlation between the X variables and a feature (Y variable) of interest. In addition, refined PLS methods like the interval partial least square (iPLS) regression[140] used for variable selection and the discriminant analysis (PLS-DA) approach[141] applied in supervised classification using the study design as *a priori* knowledge were used in the metabolomics data analysis.

Validation of PLS models is a very central issue to ensure construction of reliable models and estimates of e.g. prediction error and to determine the optimal number of components. Cross validation, where the X data matrix is divided into a number of segments containing one or more samples (full or segmented cross validation), may be applied when the number of samples is limited. As a stronger validation method, test set validation can be used when a study contains enough samples to be divided into a calibration set and a validation test set. Here, the calibration set is used to build the model, and the test set is subsequently applied to estimate the prediction error[139]. Test set validation was used in the metabolomics study whereas full and segmented cross validation was used in subsequent studies due to limited number of samples. The often used estimate of prediction error is the root mean square error (RMSE), which mimics the traditional standard deviation.

PCA and PLS represent the basic data analytical approaches broadly applied throughout this thesis work and they were successful used to obtain overview of data, detect 'outliers', select variables, classify samples into groups and relate data to reference values for construction of prediction models. For examples on usage of multivariate data analysis in the investigation of non-metabolomics data matrices the reader is referred to Papers II, III and IV.

In the metabolomics study, the initially used exploratory metabolomics approach did not provide new insight to potential β-glucan exposure and effect markers. The major disadvantage of NMR spectroscopy is the low sensitivity and resolution why NMR application may not be the first choice for identification of new biomarkers. Instead, the nature of NMR as a quantitative technique makes a targeted metabolomics approach a

more evident option[124] in accordance with the investigation of β-glucan action on blood lipids, which revealed the potential existence of subject unique lipoprotein profiles dependent on gender, BMI and diet. This exemplifies how metabolomics may promote new and unexpected findings from hypothesis based human intervention studies.

Chapter 6

General discussion and perspectives

The main objective of this thesis study was to investigate the structure-functionality properties of different barley and oat β-glucan fibres, and the *in vitro* and the *in vivo* effects of these β-glucans. It was hypothesised that delicate structural differences of β-glucans provide different *in vitro* and *in vivo* functionalities.

Even though recent research has shed some light on the mechanisms of action and structure-functionality relationship of β-glucans, the exact functional principle remain elusive. A number of expert reviews suggest that β-glucan viscosity is the main parameter governing health effects, on the other hand, there is no clear evidence in clinical studies of blood lipids to demonstrate an effect of viscosity[142]. In 2010 Wood[142] stated the following: "Repeated clinical studies that provide no physico-chemical information about the β-glucan in the foods used lend little additional information to the very large body of literature on this subject and may simply add further confusion because of different responses." Similar considerations lead to the ambitious intention of this thesis; to conduct an in-depth comparison of barley and oat β-glucan structure and functionality all the way from raw plant material to health effects. Until the present thesis work, β-glucan fine structure characteristics have not yet been ascribed a direct role in the health related effects of dietary fibres. However, the fact that barley β-glucan have not proved the same hypocholesterolemic capability as oat β-glucan lead to speculations whether differences in the β-glucan oligomer block structural patterns is of crucial importance. Knowledge in this field would add valuable information to the discussion of the effectiveness of β-glucans in food systems and human health, and also be of great marketing value for the functional food industry. From 1995 to 2010 the medicine and doctor expenses per Danish household increased with almost 100%[143]. This underlines the request for health promoting bioactive food products in approaching a healthy population in the future.

β-Glucan block structure and solubility

Results from Paper I demonstrated that barley and oat β-glucan structural characteristics, especially the molecular mass, compared at equal β-glucan concentration, dominate the viscous properties over the presence of α-dextrin impurities. Hence, the presence of starch derivatives in the barley and oat β-glucan preparations was not considered as a source of variation disturbing the β-glucan functionality in the further *in vitro* and *in vivo* investigations. Paper II showed that β-glucan block structural differences explain the main variance among identically extracted mutant barley, mother barley and oat β-glucans. In addition, the higher DP3/DP4 ratio of barley samples corresponded to a lower solubility

of these as compared to oat. This finding is in good agreement with the hypothesis that structural regularity, arising from repeated sequences of cellotriosyl or cellotetraosyl units, reduces solubility and increases the tendency of the molecules to self-associate and form aggregates[32,36,44,45,55,56] of fringed micelle structures as illustrated in Fig. 7a. Such fringed β-glucan micelles is speculated to contain an inner part with chain junction zones resulting in a rather hard gel, a gelling mid-zone, and a rand-zone with thickened water. Most probably these micro aggregated species can also associate in extended net formations by crosslinking, using the same principle exemplified in Fig. 7b.

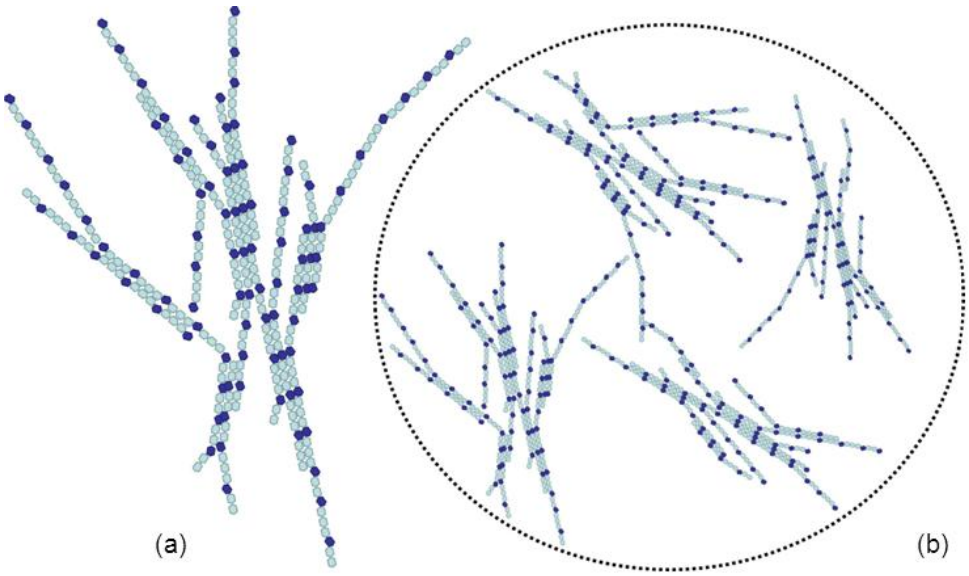

Fig. 7 Fringed β-glucan micelle (a) and micelle net formation (b) in aqaues solution. β-(1→3)-Linked residues are shown in dark blue whereas β-(1→4)-linked residues are depicted in light blue.

This model of β-glucan aggregation seems to be supported by a recent study using asymmetrical flow field-flow fractionation showing that boiling of cereal β-glucan samples may disrupt aggregates and thereby increase solubility[144]. The same technique applied on a barley β-glucan sample of defined average molecular mass revealed that the sample solubilised in water consisted of low molecular mass species with elongated or rod-like conformation, intermediate size species with random coil conformation and high molar mass species consisting of aggregates[145]. Hence, β-glucan molecular mass, fine structure, solubility and polymer aggregation are parameters that are highly connected features and may vary even within the same β-glucan sample making the procedure of good solubilisation difficult. The problem of dispersibility and stability of various polysaccharides in dilute aqueous solutions is persistent. Pressure-cell and microwave solubilisation methods were developed for complete β-glucans solubilisation, however, time and temperature parameters important for polymer degradation was difficult to control in these methods[10]. Classical heating and stirring (30 min at 80°C) was applied for standardised and stable hydration of β-glucans by Burkus and Temelli[49] and in Papers I-VII and no degradation was observed. Nevertheless, in the context of the above it must

be assumed that β-glucan solutions prepared for the various experiments of this thesis work consisted of a rather heterogeneous group of polysaccharide conformations even if they were prepared identically and from the exact same β-glucan sample.

In vitro functionalities

It is believed that an increased viscosity of the upper gastrointestinal tract content upon β-glucan consumption may entrap or encapsulate mixed bile acid micelles leading to a greater bile acid excretion[3,66,67,79,146-148]. Also a direct binding of bile acids by β-glucan has been suggested, although less studied[70,80-85]. In Paper III, both types of interaction were studied experimentally using an equilibrium dialysis *in vitro* system. The dialysis system was set to mimic physiological conditions (37°C and constant shear) although far from being representative of the human intestinal environment. The retention of small molecules (phenolic compounds and bile acids) by both barley and oat β-glucan could not be ascribed specific physico-chemical properties of the small molecules but rather it was suggested that β-glucan properties such as the degree of polymerisation and the fine structure of the polymers were important features determining the molecular interaction. Conclusively, this study indicated that the multiplicity of parameters determining the binding of small molecules to fibres obscures a direct correlation of the observed binding to specific descriptors. Nevertheless, as the dialysis approach in combination with molecular modelling offers a fast and cheap method for screening large series of small molecules and β-glucan fibres it has a great potential in the further investigation of the underlying mechanism of β-glucan functionality.

The use of different β-glucan preparations in various immunological test systems complicates attempts to draw conclusions on structure-functional relationships of the observed effects. Among fungal β-glucans, fine structure, molecular mass, conformation and solubility have been shown to influence immunological activity[105], and it is likely that these parameters also affect the activity of cereal β-glucan. Paper IV addressed the importance of barley and oat β-glucan physico-chemical properties for their capacity to affect immune responses and the results showed that the immune modulatory capacity of equally sized β-glucans is dependent on the DP3/DP4 ratio, solubility and especially polymer conformation in solution. In context with the above presented hypothesis of β-glucan supramolecular aggregate formation, the high immune response of the rather insoluble mutant barley β-glucan can be speculated to arise from fringed micelle formation of this polymer favoured by the dendritic cell receptors as compared to receptor interaction with single chain polysaccharides. This would be in agreement with a reduced immune modulatory effect from soluble β-glucans as compared to aggregated β-glucans seen elsewhere[109]. The evidence for an immune potentiating activity for cereal β-glucans is primarily based on *in vivo* animal and *in vitro* cell culture studies and it remains to be seen whether such observations in animal and *in vitro* studies have consequences for human physiology and health.

The choice of using *in vitro* methods to investigate the action of β-glucans has several reasons. As shown earlier in this thesis, the complexity of β-glucan functionality is extensive for which reason it becomes relevant to study β-glucan in less complex systems (*in vitro*) where covariant factors can be limited and sampling is much easier. Secondly, it is difficult to provide more mechanistic knowledge about the effects of β-glucan through epidemiological studies – such detailed information could to a certain degree be obtained from *in vitro* models. The drawback to this, however, is the fact that physiological conditions can never be exactly mimicked *in vitro* and that possible inconsistence between *in vitro* and *in vivo* results may be difficult to explain. In conclusion, the *in vitro* models applied in the experimental work added valuable knowledge to the general understanding of β-glucan solution properties as well as the interaction of β-glucan with small molecules and immune cells.

In vivo functionalities

The FDA-approved health claim concerning the intake of soluble fibre from oat and a reduced risk of heart disease was based on a review of 37 scientific studies, 17 of which demonstrated positive effects mostly in hypercholesterolemic subjects. Four studies did not meet the inclusion criteria and the remaining 16 studies showed insignificant or no effects on blood cholesterol levels, with explanations such as low β-glucan content, processing or compliance[8]. The report comments on the critical effects of processing, but no requirements on molecular mass or viscosity measurements were outlined in conjunction with the use of the claim. The EFSA claim on barley and oat β-glucan recommends a daily intake of at least 3 g of minimal processed β-glucan for LDL-cholesterol lowering activity in both normocholesterolemic and hypercholesterolemic subjects[9], but there is still no definition of what minimally processed β-glucans means, nor any guidelines on how the functionality of dietary fibre can be guaranteed.

In two reviews by Wood[2,25] on the relationship between the solution properties of cereal β-glucans and observed physiological effects it was concluded, that the efficacy of various soluble β-glucan preparations could not solely be related to the amount, size and viscosity of the β-glucans as the included studies represented a heterogeneous group of trials in terms of both study design and intervention product characteristics. The investigation of the health effects of the physico-chemical characterised barley and oat β-glucans of Paper II in a human intervention study is presented in Paper V. Although it was demonstrated that 3.3 g/day barley and oat β-glucan was not sufficient to significantly lower the LDL-cholesterol levels of young and healthy male and females a greater potential observed for oat β-glucan was suggested to derive from a higher solubility and viscosity of this as compared to mutant and mother barley β-glucans. Hence, differences in the fine structure remain a likely reason for the successful cholesterol lowering in some human intervention studies and the lack of effect in other studies on barley and oat β-glucan. The lack of β-

glucan efficiency in the human study could also be speculated to be due to: 1) too low active β-glucan dose, 2) loss of β-glucan functionality through processing, 3) choice of study subjects or 4) too short intervention period. In fact, it might be that dietary fibre nutritional functionality is predominately preventative in contrast to curative for which reason effects would sparsely be detectable in short intervention studies on healthy subjects.

The large number of factors influencing the effectiveness of β-glucans calls for larger intervention studies with sufficient statistical power. The human study reported in Paper V used a full cross-over design whereby the exact individual response for each treatment could be evaluated. However, inclusion of more subjects (n=14) in the study would have strengthened the data analysis and interpretation of the study. Using nutri-metabolomics techniques as in Paper VI, it may be possible to detect additional responses to those presumably found with the traditional used biomarkers. The initially exploratory nature of the metabolomics data analysis was alternatively replaced by a more targeted approach where the *a priori* knowledge on how the β-glucan fibres might affect the lipid metabolism was used. Nevertheless, in Paper VI, the absence of β-glucan treatment effect on cholesterol metabolism found in Paper V was confirmed and no additional responses were identified between 3-week baseline samples. This may be due to the above outlined influential factors or additionally lack of NMR sensitivity as discussed earlier.

The recommended β-glucan dose in the FDA- and EU-approved health claims are the same for men/women, young/old and obese/slim although there potentially is a large difference in health effects between individuals. The recommended intake in the health claims should perhaps be based on energy intake, and hence differ for men/women, young/old and obese/slim. Also, it might be that *in vivo* cereal β-glucan activity is determined by the subject health situation. Therefore, addition of extracted soluble β-glucans to food may be useful for certain indications and risk groups, but for the majority of those who want to increase their dietary fibre intake, it should be sufficient to eat a diet containing naturally fibre-rich food products. Food is eaten as a whole and therefore also the combined effect of components has to be taken into account when assessing the health effects of fibre-rich foods.

In this thesis work we used the state-of the-art technological platforms for large-scale extraction of barley and oat β-glucans, physico-chemical characterisation and *in vitro* and *in vivo* investigations. However, it seems obvious that we in future work need:

- improved analytical methods for studying the oligomer block structural patterns of β-glucans
- tools for understanding how and why the plants modify the fine structure of β-glucans
- improved models for understanding β-glucan aggregation
- improved models for understanding β-glucan affinity to small molecules such as bile acids
- to set up new intervention studies with many more subjects, higher doses and longer monitoring by metabolomics with additional urine and faces samples

There is at present no standardised methodology for characterisation of β-glucans that would be accessible to most laboratories. However, with the introduction of more general regulations for health claims, it is necessary to verify that the fibre tested in the lab is also functional *in vivo*. This involves the measurement of several fibre physico-chemical properties as outlined throughout this thesis work, but for future studies, it might also be relevant to use *in vitro* digestion methods to evaluate how β-glucans behave in combination with a food product and how the β-glucan-rich product behaves in the gastrointestinal environment. By measuring the releasability of β-glucans, it would be possible to estimate the "active" dose required in the intestine, and by optimising the releasability, less fibre should be required for retained physiological effects.

It is important for the food industry that the health promoting properties of soluble fibre, as well as the factors that may influence these properties, can be modelled or evaluated *in vitro* before studies are carried out in humans, or even to eliminate the need for *in vivo* studies for every single product.

Chapter 7

Conclusions

In summary, the highlights from this thesis work are:

β-Glucan extraction and characterisation

- Barley and oat β-glucan preparations vary significantly in purity
- β-Glucan dominates the viscous properties over the presence of α-glucan impurities in water dispersions
- The fine structure of barley and oat β-glucans influence their solubility
- Repeated cellotriosyl or cellotetraosyl substructures in β-glucan are prone to aggregate
- High β-glucan barley mutant showed a significantly changes and unique oligomer block structure as compared to the mother barley variety

β-Glucan in vitro functionality

- Small molecules (phenolics and bile acids) interact with β-glucan *in vitro*
- Degree of polymerisation and β-glucan fine structure influence the retention of small molecules
- Barley β-glucan of low solubility as compared to soluble oat β-glucan has a greater immune modulatory potential *in vitro*
- β-Glucan fine structure and in turn solubility and aggregation are key elements for understanding their immune modulatory capacity

β-Glucan in vivo functionality

- 3.3g/day extracted barley and oat β-glucan does not significantly lower total and LDL cholesterol levels in young and healthy adults
- An indicated potential effect of oat in contrast to barley suggests the importance of solubility for β-glucan interference with the cholesterol metabolism
- Plasma metabolic patterns from 3-week 3.3 g β-glucan/day treatment does not differ significantly from control treatment
- β-Glucan health effects on subject unique lipoprotein profiles of young healthy adults may only be detectable after a diet high in fat

In conclusion, the results show that barley and oat β-glucan fine structures are of great importance for their functionality. Significant differences in β-glucan block structures were found between barley and oat and even between two related barley varieties. The results demonstrate that β-glucan solubility and the polymer aggregation in solution is dependent on the block structural pattern. Barley β-glucans with high DP3/DP4 ratios as compared to oat show lower solubility and are suggested to form heterogeneous mixtures of free polymers, fringed micelles and networks of aggregated species in solution.

Small molecule retention by β-glucan is influenced by the degree of polymerisation and the fine structure of the polymer. The immune response to β-glucans is especially connected to the presence of aggregated species from insoluble β-glucans. In contrast, the *in vivo* metabolic response to β-glucan is related to high solubility of the polymers. Altogether, the results demonstrate that the health effects form barley and oat β-glucans are largely influenced by the polymer fine structure, and that different structures may affect various mechanisms in our body differently. It is very important to account for these multiple functional and bioactive properties of β-glucans in the future evaluations of β-glucan effectiveness in functional foods and health.

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Paper I

Mikkelsen MS, Jespersen BM, Møller BL, Lærke HN, Larsen FH, Engelsen SB

Comparative spectroscopic and rheological studies on crude and purified soluble barley and oat β-glucan preparations

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Comparative spectroscopic and rheological studies on crude and purified soluble barley and oat β-glucan preparations

Mette S. Mikkelsen [a,*], Birthe M. Jespersen [a], Birger L. Møller [b], Helle N. Lærke [c], Flemming H. Larsen [a], Søren B. Engelsen [a]

[a] Quality & Technology, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark
[b] Plant Biochemistry Laboratory and Laboratory for Molecular Plant Biology, Centre for Molecular Plant Physiology, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark
[c] Molecular Nutrition and Cell Biology, Department of Animal Health and Bioscience, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark

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ABSTRACT

The rheology of crude and purified barley (BBG) and oat (OBG) β-glucan samples were characterized. Sample content and major impurities was characterized by Fourier-transform near infrared Raman and infrared (FT-IR) spectroscopy revealing substantial differences between the β-glucan samples. The purification procedure increased the β-glucan content from 66.7 to 82.4% and from 30.1 to 68.4% for BBG and OBG, respectively. Proton nuclear magnetic resonance (^{1}H NMR) analysis was applied to estimate the β-(1→3) to β-(1→4) linkage ratio of the β-glucans. The molar mass of BBG and OBG was determined by high performance size-exclusion chromatography (HPSEC) using β-glucan standards and was found to be 126 and 355 kDa, respectively.
The viscosity of crude and purified β-glucans was studied at various concentrations (2.5, 5% w/v), temperatures (10–80 °C) and shear rates (1–100 s^{-1}). BBG was characterized as a low-viscosity β-glucan with Newtonian flow behavior while OBG was characterized as a high-viscosity β-glucan with shear thinning flow behavior. At equivalent β-glucan concentration in solutions the viscosity for OBG was found to be ~100 fold higher than for BBG. A direct viscosity dependence on exact β-glucan content regardless of amount and composition of α-glucan impurities was found for both OBG and BBG. This study suggests that the structural characteristics of the β-glucan polymers such as molar mass are of greater functional importance than the presence of lager amounts of starch/α-dextrins as long as the β-glucan samples are compared at equivalent β-glucan doses.

1. Introduction

Health benefits and hydrocolloid functionality of mixed linkage (1→3, 1→4)-β-D-glucans from cereals make them interesting food constituents and they have been suggested as ingredients in health-promoting functional foods (Brennan & Cleary, 2005; Ebringerova, Hromadkova, & Heinze, 2005; Inglett, Carriere, Maneepun, & Tungtrakul, 2004; Lazaridou & Biliaderis, 2007; Lee, Warner, & Inglett, 2005; Temelli, Bansema, & Stobbe, 2004; Volikakis, Biliaderis, Vamvakas, & Zerfiridis, 2004). However, β-glucan sample composition and β-glucan structure are crucial characteristics affecting the functionality in food systems and human health. In complex food systems β-glucan functionality may be affected by interactions or incompatibilities between β-glucan and other food components (Lee, Schwarz, & D'Appolonia, 1995; Satrapai & Suphantharika, 2007) and detailed knowledge about structure/function relationships of β-glucan containing systems is required in order to exploit the full health beneficial properties of this important dietary fiber.

Traditionally plant cell wall structure has been analyzed using chemical agents that gently extract and purify specific components. The cell walls are typically analyzed for sugars and sugar linkages using chemical (methylation analysis) and physical techniques (^{1}H and ^{13}C NMR). Additional information on the repetitive units in the polymers can be gained from the use of glycanases that cleave specific glycosidic bonds and subsequent analysis of the oligosaccharides by chromatographic and/or mass analysis (Guillon, Saulnier, Robert, Thibault, & Champ, 2007). Cereal β-glucan polymers generally contain ~70% (1→4)-β-glucosyl residues and ~30% (1→3)-β-glucosyl residues. The (1→3)-linkages occur singly, linking together three (cellotriosyl unit) or four (cellotetraosyl unit) (1→4)-β-linked sequences and to a lesser extend longer cellulose-like fragments (Ebringerova et al., 2005). Structurally related β-glucans differ in the ratio of tri- and tetraosyl units which is 2.8–3.3 for β-glucan of barley and 2.1–2.4 for oat (Lazaridou, Biliaderis, Micha-Screttas, & Steele, 2004; Wood, Weisz, & Blackwell, 1994).

Nuclear magnetic resonance (NMR) spectroscopy has been widely used for the overall structure and linkage sequence analysis of cereal β-glucans (Cui, Wood, Blackwell, & Nikiforuk, 2000; Johansson, Tuomainen, Ylinen, Ekholm, & Virkki, 2004; Lazaridou et al., 2004;

* Corresponding author. Tel.: +45 35 33 35 07; fax: +45 35 33 32 45.
E-mail address: skau@life.ku.dk (M.S. Mikkelsen).

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Morgan, Roberts, Tendler, Davies, & Williams, 1999; Petersen, Krah, Duus, & Thomsen, 2000; Roubroeks, Mastromauro, Andersson, Christensen, & Aman, 2000; Seefeldt, Larsen, Viereck, Wollenweber, & Engelsen, 2008; Wood et al., 1994). Liquid state ^{1}H NMR allows estimation of the ratio of β-(1→3) to β-(1→4) linkages in the β-glucan polymers as well as the residual constituents present in the β-glucan extract. Fourier-transform infrared (FT-IR) spectroscopy has also been successfully used for the analysis of plant cell wall polysaccharides including β-glucans (Johansson et al., 2004; Kacurakova & Wilson, 2001; Seefeldt, Blennow, Jespersen, Wollenweber, & Engelsen, 2009). Raman spectroscopy has a great potential in analyzing plant cell wall polysaccharides (Engelsen & Norgaard, 1996; Goral & Zichy, 1990; Mohacek-Grosev, Bozac, & Puppels, 2001; Salomonsen, Jensen, Stenbaek, & Engelsen, 2008; Sene, Mccann, Wilson, & Grinter, 1994) but to the best of our knowledge, structural and compositional cereal β-glucan analysis by Raman spectroscopy has not been reported before. Both IR and Raman spectroscopic methods are rapid, sensitive and able to distinguish the α- and β-conformations of saccharides (Cael, Koenig, & Blackwel, 1974; Engelsen & Norgaard, 1996; Kacurakova, Capek, Sasinkova, Wellner, & Ebringerova, 2000). In contrast to chemical, chromatographic, or mass spectrometric methods, spectroscopic methods require no chemical extraction as the intact sample matrix is examined which is an advantage when analyzing delicate and complex biological samples (Seefeldt et al., 2008; Seefeldt et al., 2009).

The molar mass of a β-glucan polysaccharide is an important characteristic as it determines the physicochemical properties such as viscosity (Ebringerova et al., 2005). Molar mass values for oat and barley β-glucans have been reported to range between 44–3000 and 126–2500 kDa, respectively (Beer, Wood, & Weisz, 1997; Ebringerova et al., 2005; Lazaridou et al., 2004; Papageorgiou, Lakhdara, Lazaridou, Biliaderis, & Izydorczyk, 2005; Vaikousi, Biliaderis, & Izydorczyk, 2004; Wood, Weisz, & Mahn, 1991). The values strongly depend on the method of extraction and analysis which makes comparisons difficult. Increasing the extraction temperature leads to an increase in molar mass of the extracted β-glucan (Izydorczyk, Macri, & MacGregor, 1998; Zhang, Doehlert, & Moore, 1998; Temelli, 1997). Zhang et al. (1998) extracted oat β-glucan at 40, 65 and 100 °C and reported molar mass values of 118–1024 kDa at 40 °C, 985–1919 kDa at 65 °C and 2300 kDa at 100 °C. Size-exclusion chromatography (SEC) is often used in β-glucan size determination (Johansson et al., 2004; Morgan & Ofman, 1998). SEC require calibration standards for the estimation of the molar masses unless it use a molar mass sensitive detection system such as LALLS, MALLS or RALLS (Christensen et al., 2001). Various studies have discussed the use of β-glucan, pullulan or dextran standards as references in molar mass determination of β-glucans (Beer et al., 1997; Christensen et al., 2001; Roubroeks et al., 2000; Varum, Martinsen, & Smidsrod, 1991; Zhang et al., 1998). It is well-known that calibration with pullulan standards leads to overestimation of the molar mass, however, pullulan standards offer a greater range of molar masses compared to β-glucan standards, especially for large molecules (Christensen et al., 2001; Varum et al., 1991).

Rheological methods such as viscometry have been widely used in studies of β-glucan flow behavior (Burkus & Temelli, 2005; Dawkins & Nnanna, 1995; Doublier & Wood, 1995; Lazaridou, Biliaderis, & Izydorczyk, 2003). Increasing the concentration or the molar mass of a β-glucan polymer generally results in increased viscosity (Anttila, Sontag-Strohm, & Salovaara, 2004; Wood, 2004). Often, β-glucan from oat exhibits somewhat higher viscosity compared to barley β-glucan due to longer polymer chains (Beer et al., 1997). Johansson, Karesoja, Ekholma, Virkki, and Tenhu (2008) compared viscosities of equal size oat and barley β-glucans and the higher viscosity of the oat β-glucan was suggested to stem from differences in the fine structure i.e. the ratio of tri- and tetraosyl units. Shear thinning flow behavior of β-glucan solutions is an established fact (Burkus & Temelli, 2005). In addition, low-viscosity β-glucan extracts and high-viscosity β-glucan extracts at low concentrations often show Newtonian flow behavior (Autio, Myllymaki, & Malkki, 1987; Burkus & Temelli, 2005; Doublier & Wood, 1995). Since starch is one of the major components of foods, understanding the mechanism of interaction of β-glucan with native starch and its hydrolytic products and its implication for rheological properties is of interest (Faraj, Vasanthan, & Hoover, 2006; Grimm, Krüger, & Burchard, 1995; Lee et al., 1995; Satrapai & Suphantharika, 2007). Grimm et al. (1995) studied the aggregation of β-glucan molecules in aqueous maltose solutions and found a minimum of aggregation near 5% maltose. This was attributed to a preferential binding of maltose to β-glucan, which partly breaks up the aggregated β-glucan clusters. Faraj et al. (2006) investigated the influence of hydrolysed starch fractions (low, medium and high molar mass) on the solution viscosities of low and high purity barley β-glucans. None of the hydrolysates affected the high purity β-glucan viscosity whereas the viscosity of the low purity β-glucan increased in the presence of the medium molar mass starch fractions. It was concluded that some non-β-glucan components in the low purity β-glucan may influence the solution viscosity of β-glucan-hydrolysed starch blends.

The aim of the present study was to conduct comparative spectroscopic, chromatographic and rheological studies on β-glucan concentrate samples. Special interest in these samples was the detection and characterization of α-glucans and to study the effect of α-glucans on the viscous properties of the sample solutions. Two of the β-glucan samples were recently investigated in a dialysis study targeted at studying the β-glucan affinity with small molecules (Simonsen et al., 2009).

2. Materials and methods

The crude materials were soluble barley β-glucan (BBG) Glucagel™ (GraceLinc Ltd., Christchurch, New Zealand), with molar mass 120–180 kDa and purity ~75% according to the supplier, and soluble oat β-glucan (OBG) Promoat™ (Biovelop, Kimstad, Sweden), with molar mass >1000 kDa and purity ~35% according to the supplier. Purification of the crude samples was performed as follows: the β-glucan powders were dissolved in distilled water (5%, w/v) at 90–95 °C, pH 6.2 and starch was hydrolyzed using Termamyl 120 L thermostable α-amylase (0.1%, v/v) (Novo Nordisk A/S, Bagsværd, Denmark) and continuous stirring for 45 min. Alpha amylase activity was stopped by autoclaving solution (120 °C) for 10 min. The polysaccharides were precipitated using 80% ethanol at 4 °C for 24 h. The precipitate was washed in diethyl ether and the resulting β-glucan gum was air dried and grinded into particle sizes <500 μm. The yield from the purification procedure was 86.4% for BBG and 39.2% for OBG. Pure (>97%) high-viscosity barley (BBG_{Mz}) and high-viscosity oat (OBG_{Mz}) β-glucans from Megazyme International Ltd. (Bray, Ireland) were used as references in the spectroscopic analyses along with wheat starch from Sigma-Aldrich (Brøndby, Denmark).

2.1. Chemical analyses

The crude and purified β-glucan samples were characterized for their β-glucan, starch and protein contents. Each sample was analyzed in duplicates. Standard reference samples were included in all chemical analysis and the methods proved to be accurate. Total β-glucan content was determined by the method of McCleary and Mugford (1997) using the Megazyme (Megazyme International Ltd., Bray, Ireland) mixed linkage β-glucan assay kit with a sample amount of 10 mg instead of 80–120 mg and 3 h of lichenase hydrolysis instead of 1 h. The extended hydrolysis time was suggested by Johansson et al. (2006) for concentrated β-glucan samples and the smaller sample amount was based on an internally pre-study. Total starch was determined according to the Megazyme procedure provided with their test kit (AACC, 2000). Protein was analyzed using the Kjeldahl Method (AACC, 2000).

2.2. Spectroscopic measurements

Spectroscopic measurements were preformed in triplicates. The samples were homogenous. Since spectra were used for visual inspection and not for data analyses one representative spectrum for each sample was selected for paper presentation. Raman spectra were collected on a Perkin Elmer System NIR FT-Raman interferometer (Perkin Elmer Instruments, Waltham, USA) equipped with a Nd:YAG laser emitting at 1064 nm with a laser power of 400 mW. Data were collected using an InGaAs detector and stored as Raman shifts in the range of 3600–200 cm^{-1}. A 180° back-scattering arrangement was used and no correction for the spectra response was applied. A total of 256 scans were averaged for each sample and the resolution was 32 cm^{-1}. Data are presented as raw spectra as well as extended inverted signal corrected (EISC) (Pedersen, Martens, Nielsen, & Engelsen, 2002) spectra in the range of 1200–800 cm^{-1}.

IR spectra were collected on an Arid-Zone MB100 FT-IR spectrometer (Bomen, Quebec, Canada). Spectra were acquired using an attenuated total reflectance (ATR) device with a triple-bounce diamond crystal. A total of 64 scans were averaged for each sample and the resolution was 4 cm^{-1}. The spectra were ratioed against a corresponding single-beam spectrum of the clean ATR crystal and converted into absorbance units in the range of 4400–550 cm^{-1}. Data are presented as raw spectra as well as extended inverted signal corrected (EISC) (Pedersen et al., 2002) spectra in the range 1200–800 cm^{-1}.

^{1}H NMR spectra were acquired on a Bruker Avance 400 (9.4 T) spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at a Larmor frequency of 400.13 MHz for protons, using a High-Resolution Magic-Angle Spinning (HR-MAS) probe equipped with a 4 mm (o.d.) rotor. Samples (20 mg) were dissolved in 2 ml D_2O (99%) for 30 min at 80 °C and added 200 µl D_2O containing 5.8 mM TSP-d4, prior to loading of 50 µl in the rotor. Spectra were acquired using a recycle delay of 4 s, 64 scans, a spin-rate of 7 kHz, and a dwell time of 60.4 µs for acquisition of 32 k data points, resulting in a total acquisition time of 1.979 s. All experiments were performed at 85 °C using a single-pulse experiment. Spectra were referenced to TSP-d4 (0.0 ppm) and processed using the software package Topsin 1.3 (Bruker Biospin, Rheinstetten, Germany). Intensities normalized to the TSP-d4 signal are presented as full spectra as well as spectra in the anomeric region 4.4–5.6 ppm.

2.3. Chromatographic analysis

The molar masses were determined by high performance size-exclusion chromatography (HPSEC) using a Water HPLC Module 1 (Waters Corporation, Milford, USA) fitted with 3 columns in series; TSKgel GMPWxl (Tosoh Bioscience LLC, Montgomeryville, USA, calibration range 0.5–8 × 10^6 Da with PEG and oxides), Shodex B-806 HQ, and SB-806 M HQ (Showa Denka K.K., Tokyo, Japan, exclusion limit 2 × 10^7 Da). As eluent 0.2 M acetate buffer containing 2 g oxalic acid/l at a flow rate of 0.5 ml/min was used and a Water 2410 Refractive Index (RI) detector (Waters Corporation, Milford, USA) was used for detection. Samples were dissolved in water containing 0.02% Sodium azide for 2 h at 70 °C after pre-wetting with 50% ethanol. Samples, injected at a volume of 100 µl, were calibrated against β-glucan standards (Megazyme International Ltd., Bray, Ireland) and pullulan standards (Shodex Standard P-82, Showa Denko K.K., Tokyo, Japan). The β-glucan standards had molar masses of 40, 82, 123, 183, and 245 kDa, respectively, as measured by the manufacturer in 50 mM sodium hydroxide on Hydrogel 2000, 500, and 200 columns at 70 °C, and dual angle light scattering detector fitted inside WATERS M411 RI detector. The molar masses of the pullulan standards were 5.9, 11.8, 22.8, 47.3, 112, 212, 404, 788 and 1660 kDa, respectively, measured by an ultracentrifugal sedimentation equilibrium method by the manufacturer. Molar mass values calculated relative to the β-glucan and pullulan standards using the Waters Millenium32 software are reported in number average (M_n), weight average (M_w), and maximum of the chromatographic peak (M_p). The polydispersity index = M_w/M_n (PDI) expresses the broadness of the peak. If more than one component was identified, either as multiple peaks or shoulders, the chromatogram was manually divided and molar mass distributions calculated for each fraction separately along with values obtained for the total sample.

2.4. Viscosity

Viscosity measurements were conducted using a Stresstech rheometer (Reologica Instruments AB, Lund, Sweden) equipped with a circulating water bath for temperature regulation, and a single gap-measuring cylinder CC 25 (dimensions of cup and bob, 26.0 and 25.0 mm, respectively). Samples were dissolved in distilled water (2.5% and 5%, w/v) with continuous stirring for 30 min at 80 °C. On each of the fresh aqueous β-glucan solutions (80 °C) flow curves were measured at shear rates of 0.01–100 s^{-1}. Measurement duration was 300 s and repeating measurements were performed for every 10 °C (±0.1%) descending from 80 to 10 °C. When changing the temperature, a time gap of 300 s was held for the sample to reach constant temperature before the next measurement. To prevent evaporation during high temperature testing, samples were covered with a few drops of low-viscosity paraffin oil. Data were processed using the supporting rheometer software program, RheoExploer V5. Data are presented in logarithmic plots in the range of 1–100 s^{-1}.

3. Results and discussion

3.1. Chemical analyses

Extraction protocols for cereal β-glucans (Burkus & Temelli, 1998; Dawkins & Nnanna, 1993; Faraj et al., 2006; Lazaridou et al., 2003; Vaikousi et al., 2004; Westerlund, Andersson, & Aman, 1993; Wood, Siddiqui, & Paton, 1978) often start with grain flour or bran and employ treatments with thermostable α-amylase and protease, followed by centrifugation, and dialysis or alcohol precipitation. In this study, α-amylase treatment and ethanol precipitation was employed to purify two β-glucan concentrates and the method showed to be efficient in increasing the β-glucan content of both concentrates.

The crude and purified samples were characterized for their β-glucan, starch, and protein contents (Table 1). The concentration of β-glucan was much higher in crude BBG (66.7%) than in crude OBG (30.1%), hereafter referred to as BBG_{67} and OBG_{30}, respectively. As a consequence, the starch content was much lower in BBG_{67} (9.8%) than in OBG_{30} (46.9%). After purification the β-glucan content in BBG and OBG up concentrated samples increased to 82.4 and 68.4%, hereafter referred to as BBG_{82} and OBG_{68}, respectively. The corresponding starch contents were lowered to 0.9 and 8.8%, respectively. Although no protease treatment was employed, the concentration of protein in samples was also reduced by the purification procedure, from 4.15% in BBG_{67} to 1.52% in BBG_{82} and from 4.36% in OBG_{30} to 2.08% in OBG_{68}. Thus, the simple two-step purification procedure proved to be very efficient in increasing the β-glucan content and removing starch and protein from the samples. Faraj et al. (2006) found that amyloglucosidase treatment of their low

Table 1
Composition (% dwb) of crude and purified barley (BBG) and oat (OBG) β-glucan samples.

Sample	β-glucan	Starch	Protein
BBG_{67}	66.7 ± 3.2	9.8 ± 0.1	4.15 ± 0.00
BBG_{82}	82.4 ± 1.3	0.9 ± 0.3	1.52 ± 0.11
OBG_{30}	30.1 ± 0.5	46.9 ± 0.3	4.36 ± 0.02
OBG_{68}	68.4 ± 2.6	8.8 ± 0.5	2.08 ± 0.00

Values are means of duplicate determinations ± standard deviation.

purity β-glucan (50.7%) concentrate decreased the residual starch content more efficiently (<1%) than termamyl (~5%). As seen in our study, Termamyl 120 L was capable of lowering the starch content in BBG_{82} to less than1%. However, due to the much higher starch content in OBG_{30} the same amount of termamyl during the 45 min of hydrolysis time showed to be insufficient for lowering the starch content of OBG_{68} to the same level as BBG_{82}. By way of comparison, this means that the polysaccharide composition of purified OBG_{68} is more equivalent to crude BBG_{67}.

3.2. Spectroscopic analyses

The raw and pre-processed Raman and FT-IR spectra of crude and purified β-glucan samples along with a reference sample of wheat starch are shown in Figs. 1 and 2, respectively. In the FT-IR spectra, the intensity of the spectra depends on the samples contact with the ATR crystal. In the Raman spectra, artefacts are observed due to particle scatter and fluorescence. These differences in the spectra are a result of physical differences between the samples which are not in focus in this work. Therefore, these effects were filtered away by applying a spectral pre-processing technique (EISC). The results are seen as the stacked spectra (bottom of figures) in the 1200–800 cm^{-1} region which provide information about the main polysaccharides present in a complicated system of polysaccharide mixtures (Kacurakova et al., 2000). Both Raman and FT-IR spectra show characteristic absorption bands of α- and β-anomeric configuration at ~850 and ~890 cm^{-1}, respectively (Cael et al., 1974; Engelsen & Norgaard, 1996; Kacurakova et al., 2000) and typical bands of starch are found at 930, 1047 and 1078 cm^{-1} of which the shoulder at 1047 cm^{-1} in IR spectroscopy is sensitive to the crystallinity of starch (Shingel, 2002; Wilson et al., 1991).

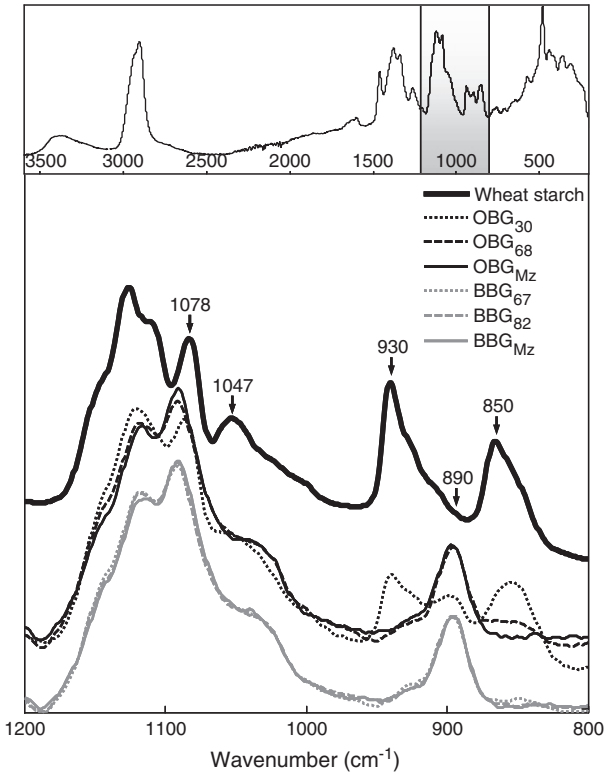

Fig. 1. Full raw (top) as well as extended inverted signal corrected (bottom) Fourier-transformed near infrared Raman spectra (1200–800 cm^{-1}) of wheat starch, crude and purified barley (BBG) and oat (OBG) β-glucan samples. BG_{no} refer to the percentual purity of the sample. BG_{MZ} corresponds to pure samples from Megazyme. Arrows indicate locations of major dissimilarities between the spectra.

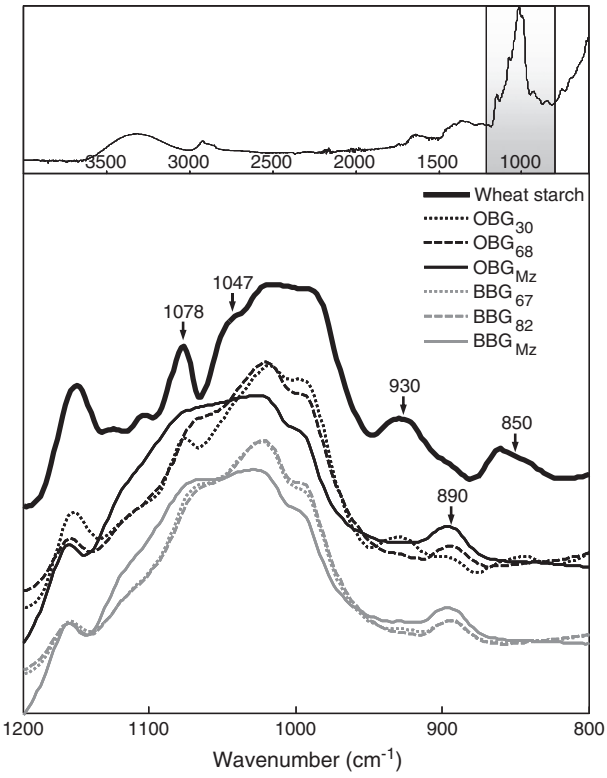

Fig. 2. Full raw (top) as well as extended inverted signal corrected (bottom) Fourier-transformed infrared spectra (1200–800 cm^{-1}) of wheat starch, crude and purified barley (BBG) and oat (OBG) β-glucan products. BG_{no} refer to the percentual purity of the sample. BG_{MZ} corresponds to pure samples from Megazyme. Arrows indicate locations of major dissimilarities between the spectra.

As seen from Fig. 1, the Raman spectra of BBG_{67}, BBG_{82} and OBG_{68} are very similar to the >97% pure Megazyme oat and barley β-glucan spectra. All spectra except the spectrum of wheat starch display the characteristic β-anomeric band at 890 cm^{-1} but it is evident that OBG_{30} is a dilute β-glucan preparation with a high content of starch or starch derivatives. The oat β-glucan samples are clearly distinguished by the α-anomeric band (850 cm^{-1}) where OBG_{30} has the strongest band and OBG_{Mz} has the weakest band. The spectra of BBG_{67}, BBG_{82} and BBG_{Mz} are very similar. Only a tendency of a more intense α-anomeric band at 850 cm^{-1} is found in BBG_{67}.

As apparent from the FT-IR spectra (Fig. 2), the starch associated bands at 930 and 1078 cm^{-1} are strong in the OBG_{30} spectrum and absent in the other spectra. None of the β-glucan samples contain significant amounts of crystalline starch as evidenced by the absence of the 1047 cm^{-1} band. As in Fig. 1, all spectra except wheat starch display the β-anomeric band at 890 cm^{-1}. Here the β-glucan purity of the samples, and especially the oat samples, is distinguished so that the >97% pure OBG_{Mz} sample has the strongest band and OBG_{30} has the weakest band. However, by simple inspection the FT-IR method was not able to distinguish between BBG_{67} and BBG_{82} at the β-anomeric band (890 cm^{-1}). This means that by simple visual inspection of the spectra we were capable of identifying quantitative β-glucan differences above 15% between our 6 samples and it indicates that more absolute β-glucan content calculations would be possible by setting up a calibration experiment. This is, however, outside the scope of this paper. Johansson et al. (2004) reported similar IR spectra (4000–650 cm^{-1}) for their soluble and insoluble oat and barley β-glucans. As observed in Fig. 2, the BBG_{Mz} and OBG_{Mz} spectra, internally similar, showed to be different from the other β-glucans in the region of 1150–1000 cm^{-1}. These differences are preliminarily assigned to differences in the physical form (morphology) between Megazyme β-glucans and

the other samples due primarily to extraction and drying. Conclusively, Raman spectroscopy showed to be very effective in discriminating the samples according to the presence of α-glucans using the α-anomeric band (850 cm^{-1}) whereas FT-IR spectroscopy proved to be more successful in discriminating the samples according to the content of β-glucans using the β-anomeric band (890 cm^{-1}).

The ^{1}H HR-MAS NMR spectra of crude and purified β-glucan samples are presented in Fig. 3. The resonance at 4.75 ppm is assigned to anomeric β-(1→3) and the resonance at 4.55 ppm is assigned to β-(1→4) in accordance with Petersen et al. (2000). The OBG_{Mz} and BBG_{Mz} samples were confirmed to be very pure with no additional anomeric carbohydrate signals, besides those of β-(1→3) and β-(1→4)-linkages. The differences between OBG and BBG sample composition observed in the chemical determinations and the Raman and FT-IR analyses were confirmed by the ^{1}H NMR analysis. Apart from the signals of β-(1→3) and β-(1→4) linkages, only minor signals originating from starch/α-dextrin (5.38 ppm), α-(5.25 ppm) and β-glucose (4.65 ppm) were observed in the spectra of BBG_{67}, BBG_{82} and OBG_{68}. The α- and β-glucose signals may also partly originate from end-groups in the β-glucans or the starch/α-dextrins. From the ^{1}H NMR spectra the ratio of β-(1→3) to β-(1→4)-linkages was calculated to; OBG_{30} 33:67, OBG_{68} 32:68, OBG_{Mz} 33:67, BBG_{67} 32:67, BBG_{82} 32:67 and BBG_{Mz} 35:65 which is in good agreement with the ratio of 30:70 generally reported (Wood et al., 1994).

Presented spectral details (FT-IR, Raman and NMR) are all convertible with what one would expect to see in β-glucan solutions and in raw and processed β-glucan foods although certain features relating to their 3D crystalline arrangements will be absent. As an example, Seefeldt et al. (2008) classified barley flours with regard to β-glucan content by FT-IR measurements.

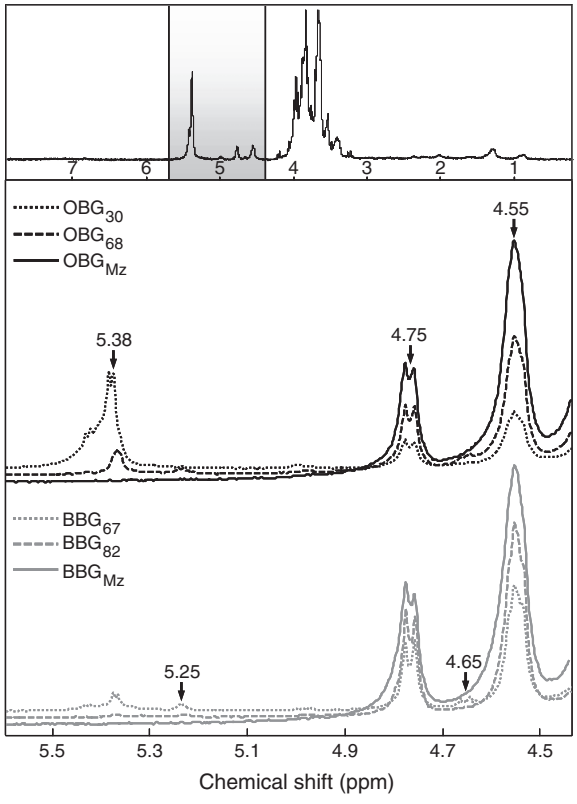

Fig. 3. Full ^{1}H high-resolution magic-angle spinning nuclear magnetic resonance spectra (top) as well as the anomeric regions (bottom) of crude and purified barley (BBG) and oat (OBG) β-glucan samples. BG_{no} refer to the percentual purity of the sample. BG_{MZ} corresponds to pure samples from Megazyme. Arrows indicate locations of major dissimilarities between the spectra.

Table 2
Molar mass (kDa) of crude and purified barley (BBG) and oat (OBG) β-glucan samples as equivalents to β-glucan and pullulan standards. M_n = Number average molar mass, M_w = Weight average molar mass, M_p = Peak molar mass. M_w/M_n expresses the broadness of the peak (=the polydispersity index PDI).

Sample		β-glucan standards				Pullulan standards				
		M_n	M_w	M_p	M_w/M_n	M_n	M_w	M_p	M_w/M_n	Area %
BBG_{67}	Total sample	89	126	123	1.4	95	268	226	2.8	100
BBG_{82}	Total sample	87	125	122	1.4	75	263	222	3.5	100
OBG_{30}	Total sample	67	241	379	3.6	21	1129	1387	54.8	100
	Main peak	253	355	379	1.4	610	1752	1387	2.9	64
	2nd peak	35	41	35	1.2	16	38	24	2.3	31
	3rd peak	14	14	14	1.0	2	2	2	1.1	5
OBG_{68}	Total sample	158	339	362	2.1	58	1633	1271	28.2	100
	Main peak	206	347	362	1.7	238	1670	1271	7.0	98
	3rd peak	14	14	13	1.0	2	2	1	1.1	2

3.3. Chromatographic analysis

The results from the HPSEC analysis are shown in Table 2. The molar mass average (M_w) of barley β-glucan expressed as equivalent to pullulan standards was 268 kDa for BBG_{67} and 263 kDa for BBG_{82}. In comparison, the molar masses of BBG_{67} and BBG_{82} obtained when using β-glucan standards were 126 kDa and 125 kDa, respectively. This confirms that the purification procedure (including α-amylase and heat treatment at 80–120 °C) did not lead to a significant degradation of the molecules. As observed in Fig. 4, the OBG_{30} chromatogram display three well resolved peaks (Main peak, 2nd peak and 3rd peak) with a PDI value of 54.8 for the total sample. The main peak was determined to be 1752 kDa as equivalent to pullulan standards and 355 kDa when using β-glucan standards. After purification, the relatively large 2nd peak (31% of the chromatographic area) of OBG_{30} was eliminated which indicate the presence of high DP dextrins in the sample and OBG_{68} showed an almost pure component with main peak values of 1670 kDa and 347 kDa when calculated from pullulan or β-glucan standards, respectively. Both OBG_{30} and OBG_{68} showed to contain small amounts of very low DP dextrins, seen as 3rd peak in Fig. 4. Usage of pullulan standards in β-glucan size determination has been suggested to induce overestimation due to differences of the hydrodynamic volumes (Beer et al., 1997; Roubroeks et al., 2000; Varum et al., 1991). However, the β-glucan standards currently available, and used in this study, were of lower molar masses than

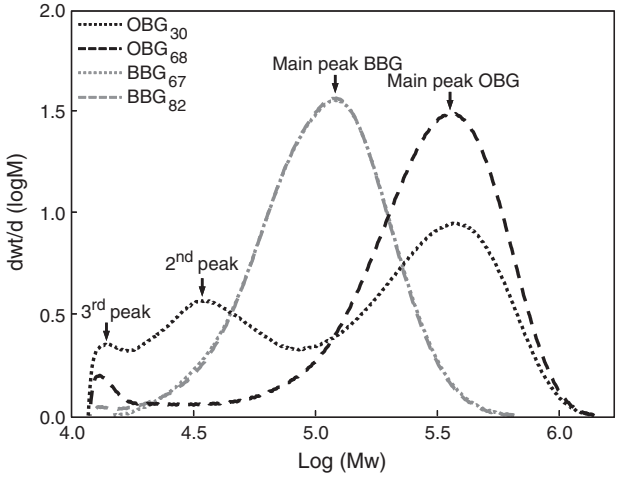

Fig. 4. High performance size-exclusion chromatograms of crude and purified barley (BBG) and oat (OBG) β-glucan samples. BG_{no} refer to the percentual purity of the sample. Dwt/d(logM) expresses the different weight fraction with the area under the curve expressing 100% of the sample.

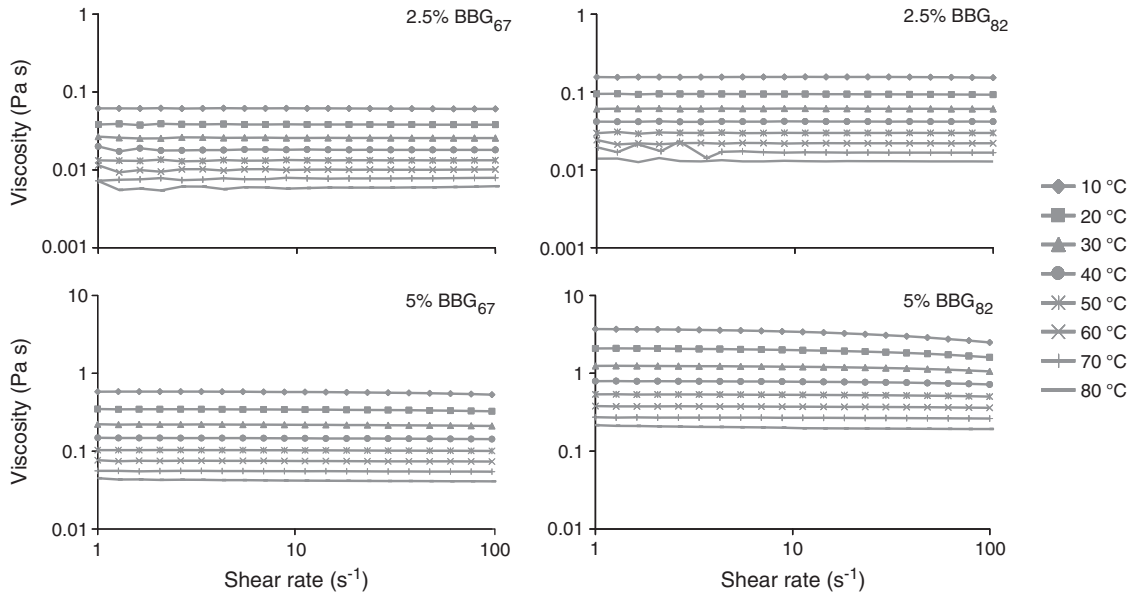

Fig. 5. Apparent viscosity of crude and purified barley (BBG) β-glucan aqueous solutions at concentrations 2.5% and 5% (w/v).

the largest β-glucan analyzed. Compared to the supplier's product information, the molar mass of OBG_{30} largely corresponds to our pullulan standard calculations whereas the provided molar mass of BBG_{67} corresponds to our β-glucan standard calculations. Despite the two different calculation methods, OBG_{30} is in general characterized as a high and BBG_{67} as a relative low molar mass β-glucan compared to molar masses reported elsewhere for barley and oat β-glucans (Beer et al., 1997; Lazaridou et al., 2004; Papageorgiou et al., 2005; Vaikousi et al., 2004; Wood et al., 1991). The large differences observed between OBG_{30} and BBG_{67} molar masses were expected since OBG_{30} is extracted at ~90 °C (Kvist & Lawther, 2005) whereas BBG_{67} is extracted at ~50 °C (Morgan & Ofman, 1998).

3.4. Viscosity

The apparent viscosities as a function of shear rate of crude and purified β-glucan samples at concentrations, 2.5 and 5% (w/v) and temperatures, 10–80 °C are shown in Figs. 5 (barley) and 6 (oat). The viscosities for oat β-glucan aqueous solutions are significant higher than for barley β-glucan solutions at all shear rates, temperatures and concentrations. Barley β-glucans generally show Newtonian flow behavior at shear rates of 1–100 s^{-1} whereas oat β-glucans show increasingly shear thinning behavior when concentration increases and temperature decreases. The overall viscous properties of BBG_{67} and OBG_{30} correspond to the low- and high-viscosity barley β-glucans examined by Burkus and Temelli (2005), respectively. They found their low-viscosity β-glucan to be strictly Newtonian and their shear thinning high-viscosity β-glucan to alter into Newtonian flow behavior at low concentrations and elevated temperatures. Vaikousi et al. (2004) found the onset of shear thinning behavior to be depended on molar mass which agree with low-viscosity found for BBG_{67}, molar mass of 126 kDa, compared with high-viscosity found for OBG_{68}, molar mass of 355 kDa. As expected, viscosity decreases as a function of temperature for all β-glucan samples. In general, the temperature–viscosity profiles of β-glucans are important functional as well as process parameters in e.g. the food industry. Most recently,

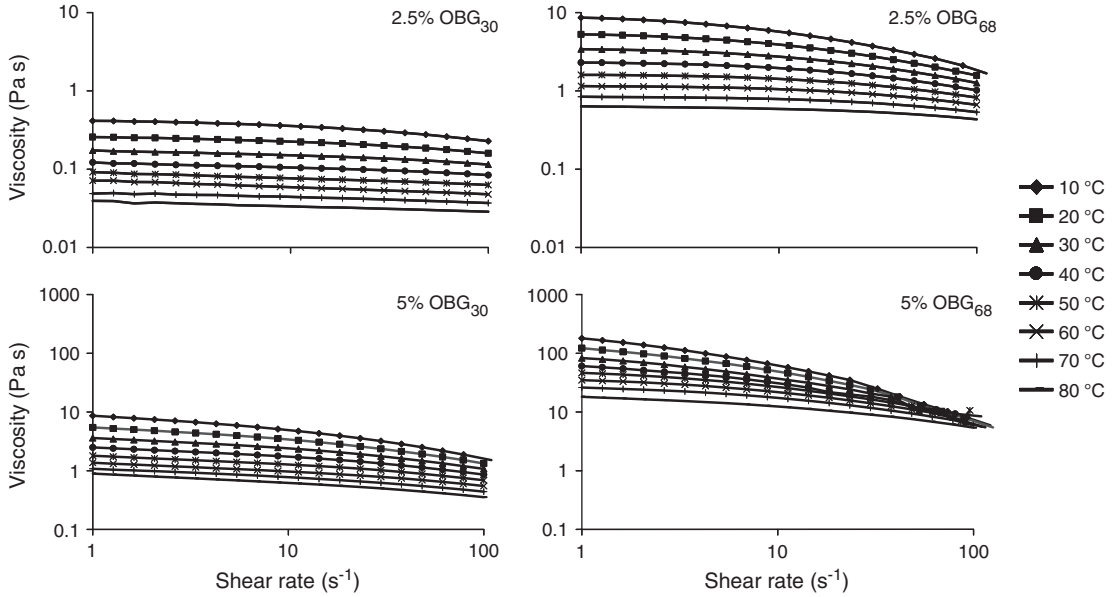

Fig. 6. Apparent viscosity of crude and purified oat (OBG) β-glucan aqueous solutions at concentrations 2.5% and 5% (w/v).

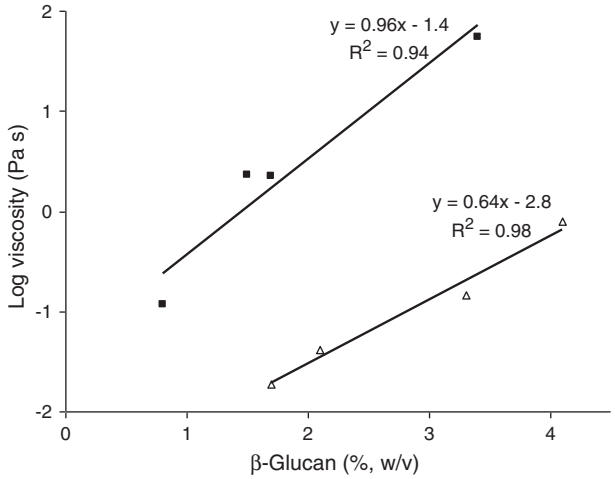

Fig. 7. Viscosity dependence on exact β-glucan content in oat (squares) and barley (triangles) solutions at 40 °C and 1.6 s^{-1}. Concentration of β-glucan samples shown for 2.5% (top) and 5% (bottom).

we employed the present temperature–viscosity profiles of BBG_{67} and OBG_{30} in the preliminary work on their interactions with phenolic derivatives to monitor the mobility of the small molecules in solution throughout sample handling (Simonsen et al., 2009).

The purification of BBG_{67} and OBG_{30} resulted in increased solution viscosities. Both Lee et al. (1995) and Satrapai and Suphantharika (2007) suggested that β-glucan competes with starch for water. Lee et al. (2005) even observed a synergistic effect between wheat starch and β-glucan, since the combined viscosity was greater than the sum of the individual viscosities. These findings are not verified by our study since viscosity increases in the following order: 5% BBG_{82}>5% BBG_{67}>2.5% BBG_{82}>2.5% BBG_{67} (Fig. 5) and 5% OBG_{68}>2.5% OBG_{68}>5% OBG_{30}>2.5% OBG_{30} (Fig. 6). A direct linear dependence between log viscosity and exact β-glucan solution content at physiological temperature (40 °C) and 1.6 s^{-1} is observed for both barley and oat (Fig. 7) which implies that the contribution of α-glucans to the overall viscosity is negligible. Faraj et al. (2006) found that the viscosity of their β-glucan-hydrolysed starch blends were dependent on the purity of the β-glucan. Hence, a critical β-glucan level in solution was suggested. In contrast, our study shows that the viscous solution properties of β-glucan/dextrin blends are primarily dominated by the β-glucan molecules even at low concentration and relative low molar mass. Grimm et al. (1995) found that maltose affected β-glucan viscosity. Since the impurities of our β-glucan samples were a wide distribution of dextrin molar masses we cannot conclude whether solution dominance of either large or small starch fractions would have an effect on β-glucan viscosity. At equivalent β-glucan concentration in solutions (2.5% $BBG_{67} \approx$ 2.5% OBG_{68} and 5% $BBG_{67} \approx$ 5% OBG_{68}) the viscosity for OBG_{68} is approximately 100 fold higher than for BBG_{67}. This dramatic increase in viscosity rely partially on the changed block structure of the β-glucan polymer (Christensen et al., 2010) and is in good agreement with measurements reported elsewhere, where high molar mass β-glucans have significant higher viscosities compared to low molar mass β-glucans (Varum et al., 1991; Wood, Beer, & Butler, 2000).

4. Conclusions

The investigated barley (BBG) and oat (OBG) β-glucan samples demonstrated large differences in composition, molar mass and rheological properties. The compositional and structural features of the β-glucan were characterized by Raman, FT-IR and ^{1}H NMR spectroscopic methods. The purification procedure efficiently increased the β-glucan content by removing starch and protein from the samples. The purified β-glucan samples contained ~70–80% β-glucan with unchanged molar masses compared to the crude samples. At equivalent β-glucan concentration in solutions OBG was approximately 100 times more viscous than BBG. Higher viscosity of OBG showing shear thinning behavior compared to BBG showing Newtonian behavior was found to be primarily caused by structural characteristics such as molar mass of the β-glucan polymers since we found a direct viscosity dependence on β-glucan content in both OBG and BBG solutions. The study indicates that the viscous characteristics of the β-glucan solutions are not caused by a cooperative effect between α-glucan and β-glucan. In conclusion, our study shows that the functional properties of oat and barley β-glucans are very different and that a variety of analytical techniques are needed for the full characterization. Spectroscopic methods such as Raman, FT-IR and ^{1}H HR-MAS NMR showed to complement each other very efficiently in this endeavor.

Acknowledgements

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Paper II

Mikkelsen MS, Jespersen BM, Larsen FH, Blennow A, Engelsen SB

Molecular structure of large-scale extracted β-glucan from barley and oat: Identification of a significantly changed block structure in a high β-glucan barley mutant

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Molecular structure of large-scale extracted β-glucan from barley and oat: Identification of a significantly changed block structure in a high β-glucan barley mutant

Mette S. Mikkelsen [a,*], Birthe M. Jespersen [a], Flemming H. Larsen [a], Andreas Blennow [b], Søren B. Engelsen [a]

[a] Quality & Technology, Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark
[b] VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Science, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark

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ABSTRACT

Health effects of β-glucan are typically related to dose, size and viscosity without taking the specific molecular structure into account. High β-glucan mutant barley, mother barley and oat β-glucans were large-scale extracted by comparable protocols using hot water, enzyme assisted hydrolysis and ethanol precipitation leading to similar molecular masses (200–300 kDa). Multivariate data analysis on all compositional, structural and functional features demonstrated that the main variance among the samples was primarily explained by block structural differences as determined by HPSEC–PAD. In particular the barley high β-glucan mutant proved to exhibit a unique block structure with DP3 and DP4 contributions of: 78.9% and 16.7% as compared to the barley mother (72.1% and 21.4%) and oat (66.1% and 29.1%). This unique block structure was further confirmed by the ^{1}H NMR determination of the β-1,4 to β-1,3 linkage ratio. Low solubility of the barley samples was potentially an effect of substructures consisting of longer repetitive cellotriosyl sequences. FT-Raman and NMR spectroscopy were useful in measuring sample impurities of α-glucans and prediction of β-linkage characteristics.

1. Introduction

Viscous dietary fibres, such as mixed-linkage (1 → 3, 1 → 4)-β-D-glucan, a polysaccharide particularly occurring in oats and barley, account for the majority of the clinical benefits observed with dietary fibres (Kendall, Esfahani, & Jenkins, 2010). The most widely documented nutritional benefits of β-glucan in food are the flattering of the postprandial blood glucose and insulin rises as well as the reduction of serum cholesterol levels. Both barley and oat β-glucans give rise to these responses and effectiveness is strongly related to dose, size and viscosity (Lazaridou & Biliaderis, 2007; Wood, 2007). However, the bare amount of β-glucan is insufficient to determine the health effects of the fibre as also the molecular structure and physical properties directly related to these parameters are potentially important for the health promoting effects. Normally, these more detailed data are not taken into account in human intervention studies which is partially the reason why the results often remain controversial (Wood, 2004). In addition, there is sparse evidence for parallel studies comparing the health effects of barley and oat β-glucans (Biorklund, van Rees, Mensink, & Onning, 2005; Delaney et al., 2003).

For this reason we intended to conduct a thorough comparison of barley and oat β-glucan structure and functionality all the way from raw plant material to health effects. The present work includes large-scale extraction of barley and oat β-glucans, molecular structure and physico-chemical functionality elucidation and multivariate data analysis for exploitation of significant differences and inner relations between β-glucan properties.

Due to the complexity of the raw plant material, β-glucan extraction and purification typically involve several steps: (i) inactivation of endogenous enzymes in the grain, (ii) extraction with water or alkali solutions, (iii) removal of protein and starch using hydrolytic enzymes and/or selective adsorption, (iv) precipitation of β-glucan from the purified solution with alcohol and freeze-, drum- or spray drying of the extract (Izydorczyk & Dexter, 2008). The extraction method affects the purity of the product, the fundamental molecular structure and the molecular mass of the β-glucan polymer (Beer, Wood, & Weisz, 1997; Burkus & Temelli, 1998; Roubroeks, Mastromauro, Andersson, Christensen, & Aman, 2000; Temelli, 1997). Kvist and Lawther (2005) previously extracted soluble barley and oat β-glucan in large-scale for food applications

* Corresponding author. Tel.: +45 35 33 35 07; fax: +45 35 33 32 45.
E-mail address: skau@life.ku.dk (M.S. Mikkelsen).

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using hot (95 °C) water, heat-stable α-amylase and centrifugal separation of the solution to provide a soluble fibre complex and an aqueous pellet mostly comprising of the protein and oil together with the insoluble fibre material from the milled grain. This method was modified for the large-scale extraction of β-glucans investigated in the present study.

Total β-glucan content of barley grain generally ranges between 2.5% and 11.3% by weight, whereas the range for oat is 2.2–7.8%. Munck, Moller, Jacobsen, and Sondergaard (2004) reported β-glucan levels as high as 15–20% in a barley low starch mutant line *lys5f* and explained the overall constant production of polysaccharides as a pleitropic effect of the mutation (Patron et al., 2004). This high β-glucan mutant barley was included in the present study along with its barley mother line. β-Glucan from different genera of cereals share the same general linear molecular structure, but exhibit variations in molecular mass, linkage pattern (ratio of β-1,4 to β-1,3 linkages), block structure (ratio of cellotriosyl/cellotetraosyl units; DP3/DP4) and amount of longer cellulose-like fragments (DP $\geqslant$ 5) (Izydorczyk, Biliaderis, & Lazaridou, 2006). The oligomer block structure of β-glucan can be analysed using digestion with endo-1,3-β-D-glucanase (lichenase) that releases the β-1,4-linked segments of DP3-DP_n (Blennow, Bay-Smidt, Wischmann, Olsen, & Moller, 1998). The differences in the molar ratio of DP3/DP4 units can be regarded as a fingerprint of the structure of cereal β-glucans and generally follows the order of wheat (3.0–4.5), barley (1.8–3.5), rye (1.9–3.0) and oat (1.5–2.3) (Fincher, 2009; Izydorczyk et al., 2006; Lazaridou, Biliaderis, Micha-Screttas, & Steele, 2004; Wood, Weisz, & Blackwell, 1991, 1994).

Raman and ^{1}H liquid-state NMR spectroscopy are widely applied methods in compositional and structural investigations of polysaccharides (Mikkelsen et al., 2010; Salomonsen, Jensen, Stenbaek, & Engelsen, 2008; Synytsya, Copikova, Matejka, & Machovic, 2003). The advantage of using spectroscopic techniques is the high throughput and exploratory character of the measurements that enables fast and simultaneous detection of several different components, and in combination with multivariate data analysis spectroscopy makes a powerful approach to screen for variation in large sample sets (Munck et al., 2010). Multivariate data analysis can also be used to predict structural and compositional features of polysaccharides and cereal grains from spectral data (Jacobsen, Sondergaard, Moller, Desler, & Munck, 2005; Salomonsen et al., 2008). Earlier we have found β-glucan specific structural information in Raman (the anomeric β-configuration adsorption band at ~890 cm^{-1}) and ^{1}H NMR spectra (the anomeric β-1,3 and β-1,4 resonances at 4.75 and 4.55 ppm, respectively) (Mikkelsen et al., 2010). In this work we demonstrate how Raman and ^{1}H NMR spectroscopy can be used to predict β-glucan compositional and structural features. These features were further linked to variation in physical characteristics important for determining health related assets of β-glucans.

2. Materials and methods

Barley materials for large-scale β-glucan extraction were high β-glucan-low starch mutant line in BOMI *lys5f* (β-glucan: 16.5–19.8%, starch: 30.0%, protein: 16.0%) and mother line BOMI (β-glucan: 6.0%, starch: 52.3%, protein: 12.8%) (Munck et al., 2004). Extracted barley mutant samples were denoted: Bm1, Bm2, Bm3, Bm4 and barley mother samples were denoted: B1, B2, B3, B4. Oat β-glucan (O1, O2) was purified from oat β-glucan concentrate (Promoat™, β-glucan: ~35%, starch: ~45%, protein: ~4.5%) obtained from Biovelop (Kimstad, Sweden) using the same large-scale procedure as for the barley raw materials. For the structural and functional comparison of β-glucan isolates, pure low, medium and high viscosity barley (BL, BM, BH) and medium and high viscosity oat (OM, OH) β-glucans from Megazyme International Ltd. (Bray, Ireland) were used as references along with crude and purified barley (BC, BP) and oat (OC, OP) β-glucan extracts earlier studied by Mikkelsen et al. (2010). Lichenan$_{1,3/1,4\text{-BG}}$ (Lich), cellulose$_{1,4\text{-BG}}$ (Cell) and curdlan$_{1,3\text{-BG}}$ (Curd) (Sigma–Aldrich, Brøndby, Denmark) were included as structural reference samples (Table 1).

2.1. Large-scale extraction of β-glucans

β-Glucans were isolated from barley grains by hot water and enzymatic hydrolysis treatment using a modified procedure of Kvist and Lawther (2005). A flow diagram outlining the steps during β-glucan extraction is shown in Fig. 1. The Application Pilot Plant at Novozymes A/S (Bagsværd, Denmark) operating with 600 L tanks, pipe connections and mono pumps was used for the following extraction steps except for the ethanol precipitation which was conducted at the University of Copenhagen. Barley grains were ground on a Brabender disc mill twice before dispersion using different concentrations of the raw materials (10% w/v mother line, 4% w/v mutant line, 5% w/v oat β-glucan concentrate) into 450 L, 95 °C, tap water. Termamyl SC (EC 3.2.1.1, Novozymes A/S, Bagsværd, Denmark) thermostable α-amylase (2% w/w, enzyme/starch) was added and the solution held with mixing for 30 min, pH 6. The mixture was then passed through a FrymaKoruma Toothed Colloid MZ 130 wetmill (600 L/h, Romaco FrymaKoruma, Neuenburg, Germany) and circulated back to the tank for a further 30 min of starch dextrification, 95 °C and pH 6. Large particles and insoluble fibres were decanted away using a Westfalia CA 225-110 separator (600 L/h, GEA Westfalia Separator Group GmbH, Oelde, Germany) and amylase activity was stopped in a Hydroheather M101MG (400 L/h, 2 bar, Hydro-Thermal Corporation, Waukesha, WI, USA) at 125 °C for 4 min. The solution was centrifuged using a Westfalia SB7 centrifuge (300 L/h, GEA Westfalia Separator Group GmbH, Oelde, Germany) thereby removing most of the denatured protein fraction prior to addition of Alcalase AF 2.4L (EC 3.4.21.62, Novozymes A/S, Bagsværd, Denmark) protease (2.5% w/w, enzyme/protein) at 60 °C for 30 min, pH 7. Protease activity was terminated at 85 °C for 15 min and the solution cooled to 25 °C before precipitation of β-glucan in 50–80% food grade ethanol. The gum material was filtered, freeze-dried and ground into fine β-glucan powder. Barley β-glucans were extracted in two true replicate processes corresponding to samples: B1, B2, Bm1, Bm2 in repetition 1 and B3, B4, Bm3, Bm4 in repetition 2, whereas oat β-glucan (O1, O2) was purified in one single process. Batches 1 and 3 are β-glucans immediately precipitating out in 80–50% ethanol whereas batches 2 and 4 are β-glucans precipitating out in the remaining 50:50% β-glucan-ethanol solution overnight. However, as no significant differences were found for isolates of the same origin, Bm1–4, B1–4 and O1–2 were treated as replicate samples, respectively.

2.2. Compositional analyses

Samples were analysed for their β-glucan, starch, and protein contents in duplicates. Dietary fibre analysis was performed on four replicate samples. Total β-glucan content was determined by the method of McCleary and Mugford (1997) using the Megazyme (Megazyme International, Wicklow, Ireland) mixed linkage β-glucan assay kit and same conditions as Mikkelsen et al. (2010). The content of total starch along with total (TDF), soluble (SDF) and insoluble dietary fibres (IDF) was determined according to the Megazyme procedures provided with the test kits (Approved Methods 76–13 and 32–07, AACC 2000). Protein was analysed using the Kjeldahl method (Approved Method 46–12, AACC 2000).

Table 1
Compositional, molecular, structural and rheological features of β-glucan samples.

	Sample name	Specification	β-Glucan (% dm)	Starch (% dm)	Protein (% dm)	TDF (% dm)	IDF (% dm)	SDF (% dm)	Molecular mass (kDa)	DP3 (molar ratio)	DP4 (molar ratio)	DP5 (molar ratio)	DP6 (molar ratio)	DP3/DP4 (molar ratio)	Linkage ratio (% β-1,4)	Linkage ratio (% β-1,3)	Consistency coefficient, *c* (Pa s)	Flow behaviour index, *n*
Barley	Bm1	Mutant line	59.8 ± 0.3	4.8 ± 0.0	3.8 ± 0.0	69.5 ± 3.3	29.3 ± 0.9	40.2 ± 2.9	270	78.8	17.2	2.8	1.2	4.6	69.6	30.4	0.18	0.43
	Bm2		56.0 ± 0.3	5.9 ± 0.1	3.7 ± 0.0	63.4 ± 7.7	17.3 ± 2.8	46.1 ± 7.1	280	78.6	16.7	3.0	1.7	4.7	69.8	30.2	0.23	0.47
	Bm3		61.8 ± 1.7	6.2 ± 0.3	4.0 ± 0.0	79.4 ± 8.1	34.7 ± 4.8	44.7 ± 7.2	170	79.4	16.3	2.8	1.5	4.9	69.1	30.9	0.08	0.61
	Bm4		54.0 ± 3.9	5.1 ± 0.0	3.8 ± 0.0	72.1 ± 11.8	23.8 ± 2.6	48.3 ± 9.9	290	78.9	16.6	3.0	1.6	4.8	69.5	30.5	0.35	0.31
	B1	Mother line	45.3 ± 2.0	18.3 ± 1.5	3.9 ± 0.0	39.9 ± 4.2	23.8 ± 1.0	18.5 ± 1.1	260	71.8	21.9	2.7	3.5	3.3	70.8	29.2	0.02	0.87
	B2		44.8 ± 0.6	21.1 ± 0.5	3.5 ± 0.0	44.3 ± 7.9	16.8 ± 1.0	27.6 ± 8.1	260	72.4	20.4	2.5	4.8	3.6	70.8	29.2	0.01	0.80
	B3		47.2 ± 1.5	16.6 ± 0.5	3.9 ± 0.1	48.8 ± 8.4	22.8 ± 0.4	26.0 ± 8.0	270	72.6	21.5	2.7	3.1	3.4	70.5	29.5	0.05	0.67
	B4		47.1 ± 0.6	18.2 ± 0.0	3.4 ± 0.0	41.4 ± 2.4	16.9 ± 1.3	24.5 ± 3.4	250	71.5	21.8	2.9	3.9	3.3	70.9	29.1	0.02	0.82
	BC	Crude	64.3 ± 0.5	10.0 ± 0.0	4.8 ± 0.1	41.6 ± 15.5	0.8 ± 0.9	40.9 ± 15.4	130	76.4	19.5	3.2	0.9	3.9	70.2	29.8	0.01	0.97
	BP	Pure	81.1 ± 8.9	1.8 ± 0.0	1.8 ± 0.0	38.5 ± 4.3	1.1 ± 1.4	37.7 ± 4.6	130	78.3	18.0	2.8	0.9	4.4	70.1	29.9	0.01	0.99
	BL	Low viscosity	81.9 ± 0.2	0.9 ± 0.0	0.2 ± 0.0	57.8 ± 27.2	1.7 ± 1.5	56.1 ± 28.7	140	77.3	19.3	2.6	0.8	4.0	70.1	29.9	0.01	0.98
	BM	Med. viscosity	88.2 ± 3.7	0.9 ± 0.0	0.3 ± 0.1	94.5 ± 3.7	1.2 ± 0.7	93.3 ± 3.0	200	75.7	19.0	4.5	0.9	4.0	69.5	30.5	0.03	0.98
	BH	High viscosity	84.8 ± 1.5	0.9 ± 0.0	0.2 ± 0.0	91.5 ± 14.7	1.4 ± 0.1	90.1 ± 14.8	390	79.8	16.8	2.5	0.9	4.8	68.7	31.3	0.48	0.86
Oats	O1		72.5 ± 0.8	5.7 ± 0.2	1.4 ± 0.0	81.5 ± 5.0	4.6 ± 3.4	76.9 ± 2.0	270	65.8	29.4	2.5	2.3	2.2	71.3	28.7	0.06	0.95
	O2		70.5 ± 1.2	7.9 ± 0.1	1.4 ± 0.0	85.2 ± 2.9	6.5 ± 2.1	78.7 ± 2.3	340	66.5	28.7	2.7	2.1	2.3	71.3	28.7	0.05	0.95
	OC	Crude	31.9 ± 1.7	43.1 ± 0.8	5.2 ± 0.1	25.4 ± 5.2	0.3 ± 0.3	25.3 ± 4.9	410	64.5	31.6	2.5	1.4	2.0	71.8	28.2	0.02	0.89
	OP	Pure	67.0 ± 1.0	7.3 ± 0.2	2.5 ± 0.0	66.2 ± 15.2	4.8 ± 3.1	63.0 ± 15.2	410	67.2	28.6	2.1	2.1	2.4	70.2	29.8	0.17	0.90
	OM	Med. viscosity	81.4 ± 1.1	0.8 ± 0.0	0.1 ± 0.0	96.5 ± 2.1	0.3 ± 0.5	96.5 ± 3.0	190	69.0	27.5	2.4	1.2	2.5	71.1	28.9	0.03	0.97
	OH	High viscosity	81.2 ± 2.8	0.8 ± 0.0	0.2 ± 0.0	91.6 ± 7.9	1.5 ± 2.2	90.4 ± 5.1	380	68.5	28.3	2.0	1.2	2.4	70.6	29.4	0.25	0.91
	Crd	$Curdlan_{1,3\text{-}BG}$	0.3 ± 0.3	0.8 ± 0.0	1.3 ± 0.0	94.7 ± 0.3	94.2 ± 2.3	1.2 ± 1.7	–	–	–	–	–	–	–	–	–	–
	Cell	$Cellulose_{1,4\text{-}BG}$	0.1 ± 0.0	0.8 ± 0.0	0.1 ± 0.0	74.2 ± 10.0	75.7 ± 8.1	0.0 ± 0.0	–	–	–	–	–	–	–	–	–	–
	Lich	$Lichenan_{1,3/1,4\text{-}BG}$	69.9 ± 1.7	0.9 ± 0.0	0.9 ± 0.1	43.0 ± 14.7	0.0 ± 0.0	44.7 ± 15.6	–	87.2	3.9	7.1	1.8	22.4	70.8	29.2	0.00	1.04
	Y	$Yeast_{1,3/1,6\text{-}BG}$	0.8 ± 0.1	0.8 ± 0.0	1.3 ± 0.2	87.8 ± 11.1	7.7 ± 0.5	80.1 ± 10.6	–	–	–	–	–	–	–	–	–	–

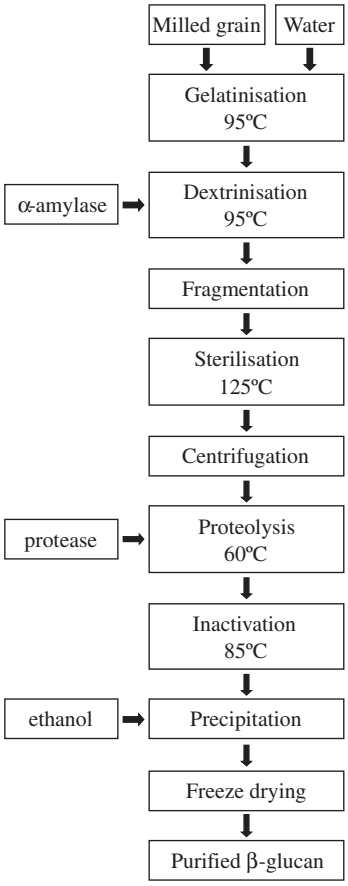

Fig. 1. Extraction and purification scheme of barley and oat β-glucan.

2.3. Rheology

Viscosity of the samples were determined with a AR-G2 rheometer (TA Instruments, New Castle, DE, USA) using a cone and plate geometry (60 mm, 1°) over a shear rate range of 0.1–1000 s^{-1} at 20 °C. Sample solutions (1% w/v) were prepared from 10 mg sample in 1 ml distilled water with continuous stirring for 30 min at 80 °C. Flow properties were approximated with a consistency coefficient *c* (related to viscosity, Pas) and flow behaviour index *n* from the Power Law model: $SS = c\ SR^n$, where SS (Pa) is shear stress and SR (s^{-1}) is shear rate.

2.4. Chromatographic analyses

2.4.1. High performance size-exclusion chromatography (HPSEC)

The HPSEC equipment for molar mass determination consisted of a Varian 9100 auto sampler and Varian 9012 pump module (Agilent Technologies, Santa Clara, CA, USA), 2 Waters columns in series (Ultrahydrogel 1000 and 2000, 7.8 × 300 mm, exclusion limit 12 μm, Waters Corporation, Milford, MA, USA) placed in a 60 °C column oven and a Varian Star 9040 Refractive Index (RI) detector (Agilent Technologies, Santa Clara, CA, USA). Prior to measurement samples (0.5% w/v) were dissolved in 0.05 M NaCl for 30 min at 80 °C and filtered through 45 μm filtrates. A 0.05 M NaCl eluent at a flow rate of 0.5 ml/min was used and sample injection volume was 100 μl. Identity of the β-glucan peak was confirmed by comparison to a chromatogram of a lichenase (EC 3.2.1.73) degraded β-glucan sample. Apparent molecular mass was calculated from a calibration curve for five authentic β-glucan molar mass standards (40, 123, 183, 245, 359 kDa, Megazyme International, Wicklow, Ireland) $r^2 > 0.99$.

2.4.2. High performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD)

For complete hydrolysis with lichenase 5 mg of β-glucan was dissolved in 100 μl of 1 M NaOH and incubated for 15 min at room temperature. To this 700 μl of milli-Q water was added, the sample mixed thoroughly and neutralised with addition of 100 μl of 1 M HCl and 100 μl of 100 mM potassium phosphate buffer pH 6.0. Lichenase (10 U, EC 3.2.1.73, Megazyme International, Wicklow, Ireland) was added to 500 μl of the dissolved β-glucan and incubated for 60 min at 40 °C. Following boiling for 3 min to inactivate the enzyme and centrifugation for 5 min at 20,000*g*, the reaction mixture was diluted 5-fold and 10 μL samples were injected for analysis. The HPAEC–PAD analysis of lichenase-generated β-glucan hydrolysis products was performed on a Dionex Dx-500 equipped with a CarboPac PA-200 column system (Dionex Corporation, Sunnyvale, CA, USA) operating with a 0.5 ml/min flow rate and the following elution profile: 0–25 min 150 mM isocratic NaOH overlaid with a 5–25 min 0–25 mM NaOAc linear gradient. Single peaks at Degree of Polymerisation (DP) 3–6 were integrated and relative molar ratios were calculated using PAD detector response factors calculated from molar detection of pure maltotriose–maltohexaose standards, where the ratios of the detector responses for two oligosaccharides with identical difference in DP are assumed to be identical (Blennow et al., 1998). Chromatograms were analysed and peaks integrated for the malto-oligosaccharides and lichenase generated fragments using the Chromeleon V.6.40 software.

2.5. Spectroscopic measurements

2.5.1. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR)

Sample solutions (1% w/v) for NMR analysis were prepared by adding 10 mg sample into 1 ml D_2O (99%) under continuous stirring at 80 °C for 30 min. Next 495 μl filtrate was transferred into 5 mm (o.d.) NMR tubes and mixed with 55 μl D_2O containing 5.8 mM TSP-d4. NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a Larmor frequency of 500.13 MHz for ^{1}H using a double-tuned BBI probe equipped for 5 mm sample tubes. All experiments were performed at 80 °C by a single-pulse experiment (90° pulse) using a recycle delay of 5 s, 64 scans, and a spectral width of 10,000 Hz for acquisition of 32 k data points, resulting in a total acquisition time of 1.639 s. Subsequently the FID's were apodised by Lorentzian line broadening of 0.3 Hz prior to Fourier Transformation. All spectra were referenced to TSP-d4 at 0.0 ppm and intensities were normalised to the TSP-d4 resonance. The relative populations of the anomeric protons of the β-1,3- and β-1,4-linkages were determined by integration of the spectral regions 4.85–4.70 ppm and 4.62–4.45 ppm, respectively.

2.5.2. Fourier transformed Raman spectroscopy (FT-Raman)

Spectra of dry samples were recorded on a Perkin Elmer System 2000 FT-NIR Raman interferometer (Perkin Elmer Instruments, Waltham, MA, USA) equipped with a Nd:YAG laser emitting 400 mW at 1064 nm and using an InGaAs detector. Signals were collected as 180° backscatter in the range 3500–500 cm^{-1} using a spectral resolution of 4 cm^{-1}. A total of 256 scans were averaged for each sample. Along with samples from Table 1 three cellulose:curdlan blends were measured; 75:25%, 50:50% and 25:75%.

2.6. Multivariate data analysis

Compositional, structural and functional data as well as Raman and NMR spectra were investigated by multivariate data analysis

using principal component analysis, PCA (Hotelling, 1933) and partial least squares regression, PLSR (Wold, Martens, & Wold, 1983). In PCA, the two-dimensional data matrix (samples × variables) is decomposed into systematic variation (and noise) which can be described by the principal components (PC1, PC2, etc.), each representing the outer product of scores and loadings. The scores contain information about the samples, while the loadings contain information about the variables. PLSR is a multivariate calibration method by which two sets of data, X (e.g. spectra) and y (e.g. DP3/DP4 ratio), are related by means of regression. The purpose of PLSR is to establish a linear model which enables the prediction of y from the measured spectrum X.

Prior to PCA and PLSR on NMR spectra (4.8–4.72 and 4.6–4.5 ppm) data were aligned (*i*coshifted) (Savorani, Tomasi, & Engelsen, 2010), normalised by standard normal variate transformation (SNV) (Barnes, Dhanoa, & Lister, 1989) and scaled (Pareto) (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006). PCA on Raman data (920–870 cm^{-1}) was performed on second derivative (Savitsky-Golay with 7 point gap size) (Rinnan, van den Berg, & Engelsen, 2009; Savitzky & Golay, 1964) and mean centered spectra, whereas PLSR was performed on MSC corrected and mean centered spectra (1800–800 cm^{-1}). The relative low number of samples available enforced random cross-validation. The PLSR results are presented as number of PLSR components (# PC), squared correlation coefficient (r^2) and root mean square error of cross-validation (RMSECV). The PCA and PLSR analyses were performed in MatLab v.7.10.0.499 (Mathworks, Natick, MA, USA) using the PLS-toolbox v.5.8.3 (Eigenvector Research Inc., Manson, WA, USA).

3. Results and discussion

3.1. β-Glucan extraction and chemical composition of samples

The average content of β-glucan in Bm, B and O samples was 58%, 46% and 72% dm, respectively, and larger amounts of starch were found in B samples (~19%) compared to Bm (~6%) and O samples (~7%) (Table 1). Hence, the final isolate purity largely depended on the β-glucan content in the raw material, even though the amounts of enzymes used under extraction were adjusted to the starch and protein contents of barley and oat crude materials. The content of TDF in Bm, B and O samples was 71%, 44% and 83% dm, of which 45%, 24% and 78% dm was SDF and 26%, 20% and 6% dm was IDF, respectively. Relatively large standard deviations were found for the fibre analysis due to problems with viscosity during the filtration (despite reduced sample amount; 100 mg instead of 1 g). Total dietary fibre and β-glucan content generally harmonised for B samples, whereas TDF values of Bm and O samples were approximately 10% higher than β-glucan. Since, β-glucan constituted the majority of the fibre population in Bm and O samples and considering the high standard deviations of the fibre analyses no further polysaccharide profiling was performed. The total sum of the major components in Bm, B and O samples, that is TDF, starch and protein, on average made up 81%, 67% and 91% dm, respectively. Lipids and minerals were not measured and could hence contribute to sample content. However, the main factor attributing to the low recovery of total sample through chemical analyses is assumed to be related to poor solubility of the β-glucan isolates. Bm and B samples were especially hard to dissolve in water using the standard method (mixing at 80 °C for 30 min), thus high shear mixing (colloid milling) prior to heating of the solution was introduced in all sample preparations. Kivela, Pitkanen, Laine, Aseyev, and Sontag-Strohm (2010) observed negative effects on β-glucan solution properties (drop in viscosity) due to homogenisation using a colloid mill. In this study it was not possible to observe any of these effects as long as high shear mixing was performed in cold solutions. In summary, the extraction conditions employed in the present study, which were optimised to balance product quality and yield provided 5 kg barley and oat β-glucan isolate batches of relatively high, but, varying purity.

3.2. Flow properties

Viscosity (expressed as consistency coefficient c) of extracted β-glucan samples decreased in the order: Bm > O > B (Table 1). Viscosity of Bm samples (r^2 = 0.86–0.97) varied considerably due to poor solubility. B (r^2 = 0.98–1) and O (r^2 = 1) samples showed stable viscosity measurements. Reference samples (low, medium and high viscosity barley and oat β-glucans) were readily dissolved and showed the expected pattern of viscosity along with Newtonian flow behaviour ($n \approx 1$). The flow behaviour of Bm, O and B samples showed shear thinning, Newtonian and slightly shear thinning characteristics, respectively. The results from the rheological analysis were included in the overall multivariate data analysis on relations between β-glucan properties as functional features.

3.3. Molecular and structural characterisation

3.3.1. Chromatography

Estimates of the apparent molecular mass from the peak fraction of the eluting polysaccharides, obtained using the standard curve of five β-glucan standards are given in Table 1. Reference β-glucan samples (BL, BM, BH) showed low, medium and high molecular mass, which accordingly verified the sensitivity of the method. Extracted β-glucan samples generally had similar medium molecular masses of 200–300 kDa. The relative smaller mass of Bm3 (170 kDa) is believed to derive from faulty preparation of this sample which resulted in bad instrumental reading. The molecular mass values reported elsewhere for barley and oat β-glucan varies in the ranges 150–2500 and 65–3000 kDa, respectively (Lazaridou et al., 2004). The large variation of the reported molecular mass values reflects the diversity of the biological origin, the methodology used for determination and the extraction protocol employed. We assume that the similarity in the molecular mass of the large-scale extracted β-glucan isolates originates from employing the same extraction method to all samples.

Complete hydrolysis of the β-glucans using lichenase produced oligosaccharides with main lengths of DP3 and DP4 (constituting 93–97% of DP3-6) and only small amounts of DP5 and DP6 (constituting 3–7% of DP3–6) (Table 1). Generally, barley samples had greater relative amounts of trisaccharides (72–80%) as compared to oats (65–69%). The molar ratio of tri- and tetrasaccharides (DP3/DP4) was calculated for all samples and found to follow the order of lichenan (22.4), barley (3.3–4.9) and oat (2.0–2.4), which is in good agreement with previous studies (Lazaridou et al., 2004; Wood et al., 1994). The DP3/DP4 ratio of Bm samples was 4.6–4.9 as compared to B samples, which were 3.3–3.6, indicating a greater relative amount of DP3 oligomers in Bm. Compared to the barley reference β-glucan samples, Bm represented the upper extreme and B the lower extreme of the DP3/DP4 ratio. The oligomer profile of oat isolates (O) was similar to the reference oat β-glucan samples. With regard to pentasaccharides and hexasaccharides B samples had a characteristic greater relative amount of DP6 as compared to Bm, whereas DP5 amounts were identical. Exaggerated structural profiles of extracted β-glucan samples with regard to relative content of cellotriose and cellotetraose units are illustrated in Fig. 2. Accordingly, the mutant barley (Bm) proved to have a significantly changed oligomer block structure characterised by increased number of DP3 units as compared to the mother barley (B) and other barley varieties studied elsewhere. Such features can affect physical characteristics as described below.

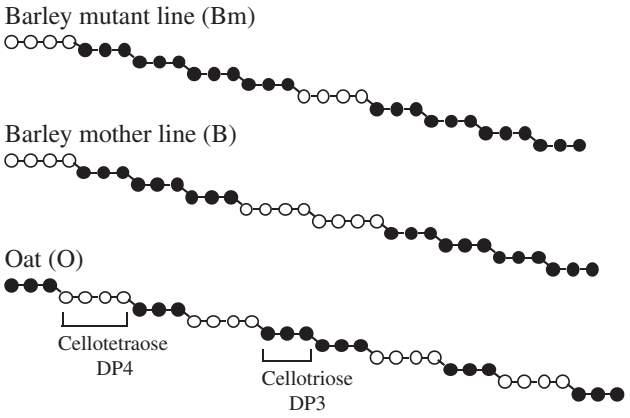

Fig. 2. Schematic molecular structure of extracted barley and oat β-glucans.

The SDF/IDF ratio of Bm, B and O was found to be significantly different, with oat samples mainly comprised of SDF and only minor amounts of IDF and Bm and B samples containing considerable amounts of IDF besides SDF (Table 1). Since water extractability or solubility (under comparable extraction conditions) of β-glucan from oats is generally greater than in barley (which is, in turn, greater than in wheat) β-glucan solubility appears to decrease with the increasing molar ratio of DP3/DP4 in the β-glucan chain (Izydorczyk & Dexter, 2008). However, as the SDF/IDF ratio of the barley mutant samples (Bm; DP3/DP4 ratio, 4.8) was higher than that of mother barley samples (B; DP3/DP4 ratio, 3.4) this relationship could not be confirmed. It can be assumed that the differences in solubility either stem from differences in the amounts of long cellulosic oligomer blocks (Izydorczyk et al., 2006) or alternatively that the DP3/DP4 arrangement in the polysaccharide chain has a solubility optimum. In either case, the sequence of repeated units of the same length would affect aggregation of the β-glucan segment and a high ratio of either DP3 or DP4 units would decrease solubility (Böhm & Kulicke, 1999; Fincher, 2009; Tosh, Brummer, Wood, Wang, & Weisz, 2004). Hence, the high content of DP3 for both barley β-glucans can possibly be linked to longer repetitive sequences of pure cellotriosyl units that are prone to aggregate in line with the low solubility of these β-glucans (Table 1 and Fig. 2).

The PCA score (A) and loading (B) plots based on the autoscaled compositional, structural and rheological data from Table 1 of the 19 β-glucan samples are displayed in Fig. 3. It was observed that the β-glucan samples split into two groups according to crop type: oat and barley (Fig. 3A), and that the main variance among samples (PC1, 40%) was primarily explained by differences in the DP3/DP4 block structure and the co-varying β-1,4 to β-1,3 linkage ratio, whereas the minor variance along PC2 (31%) explained by compositional features and DP6 was spanned by both barley and oat (Fig. 3B). Molecular mass, rheological features and DP5 contributed less to the systematic variation since they were located central in the loadings plot. Replicate (Bm1–4, B1–4, O1–2) samples were closely grouped in the score plot, indicating reasonable reproducibility in the preparations. This simple graphical inspection of the data in Table 1 thus confirmed the primary importance of the β-glucan structural features for sample classification rather than sample content of β-glucan, fibre, starch and protein. This was further validated in Fig. 4A which shows that the DP3/DP4 block structure and % β-1,3 linkage ratio were highly correlated features, whereas no apparent correlation could be found between β-glucan block structure (DP3/DP4 ratio) and viscosity as expressed by the consistency coefficient (Fig. 4B).

3.3.2. Spectroscopy

The compositional and structural features of β-glucan samples were further investigated by NMR and Raman spectroscopy. The full NMR spectra of the β-glucan isolates (Fig. 5A, top) showed small traces of lipids (2.9–0.5 and ~5.3 ppm). Since specific removal of lipids was not targeted in the β-glucan extraction procedure, this was expected. The β-glucan linkage patterns (ratio of β-1,4 to β-1,3 linkages) were established from the ^{1}H NMR spectra (Table 1): Bm (69.5:30.5%), B (70.8:29.2%) and O (71.3:28.7%). The barley mutant consistently proved to have the highest relative amount of kinks (β-1,3 linkages). Inspection of the PCA score plot (Fig. 5B) based on the anomeric β-1,3 and β-1,4 spectral regions confirmed the trend calculated from the peak integrals that barley and oat samples split into two groups (PC1, 40%). Fig. 5C shows the measured versus predicted plot from the model based on NMR spectral prediction of the log(DP3/DP4 ratio) (r^2 = 0.83). The model was based on 4 PLSR components and the prediction error was 0.06. Since DP3 and DP4 constitute only ~95% of the total oligomer sum (DP3–DP6) compared to the β-1,4 and β-1,3 linkage signals in NMR which represents all soluble β-linkages of DP2-DP$_{xx}$ a certain prediction error would be expected, and the method might be considered as a good alternative to the HPAEC oligomer profiling method.

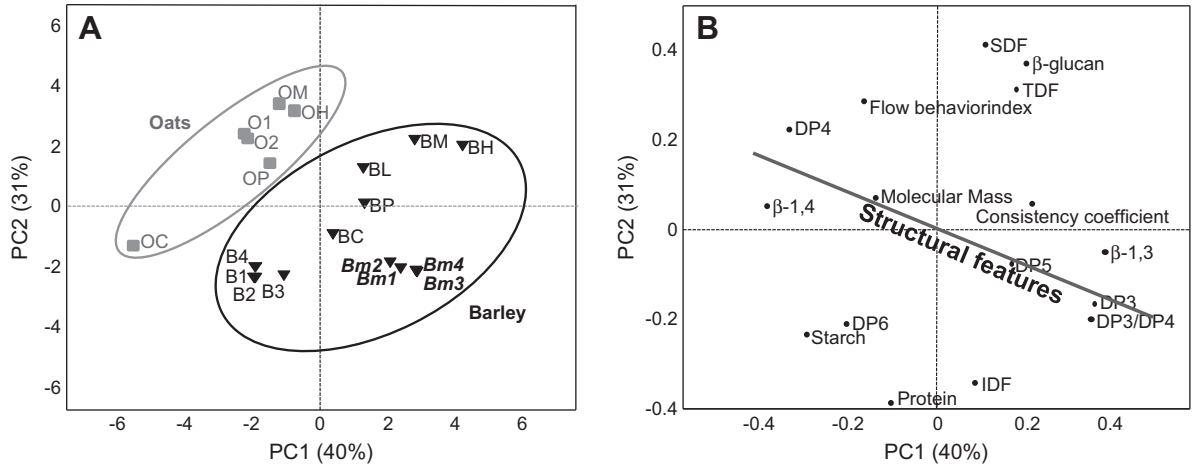

Fig. 3. PCA score (A) and loading (B) plot based on the compositional, structural and rheological properties of the 19 β-glucan samples. The first two principal components explain 71% of the data variation.

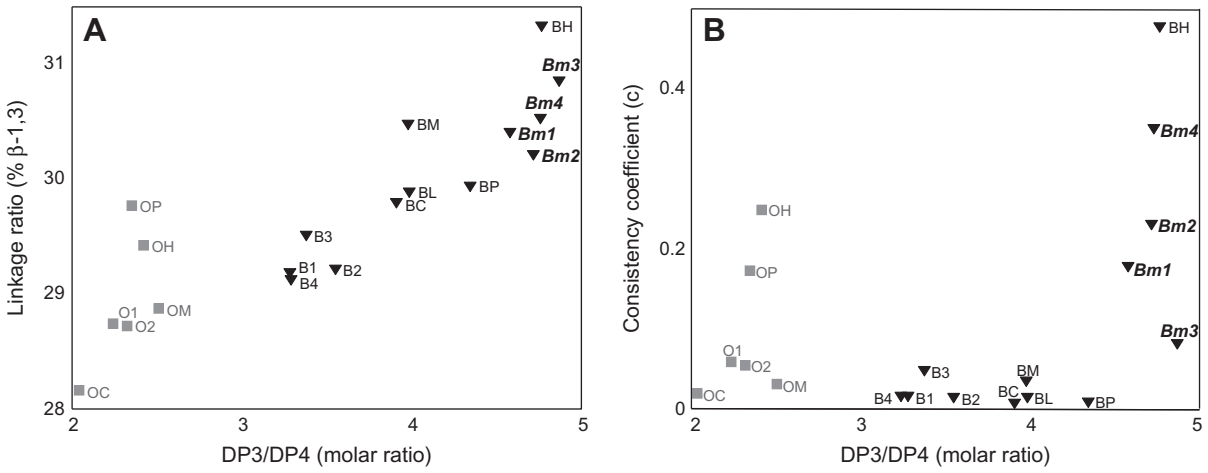

Fig. 4. Correlation plots of β-glucan structural (A: DP3/DP4 ratio versus % β-1,3 linkages) and functional (B: DP3/DP4 ratio versus consistency coefficient) features.

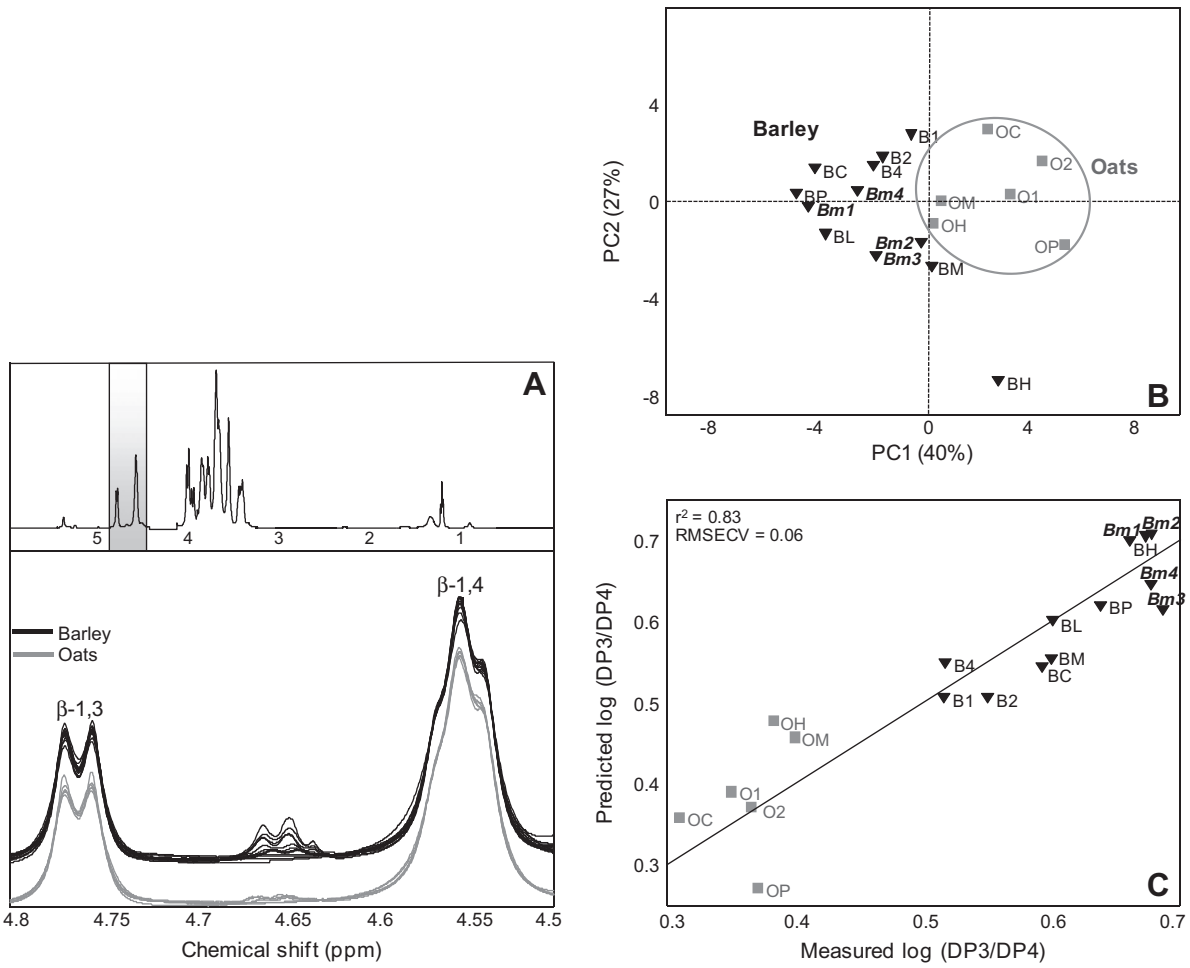

Fig. 5. (A) NMR spectra (6–0 ppm and 4.8–4.5 ppm regions) of β-glucan samples (sample B3 was excluded due to insufficient spectral quality). (B) PCA score plot of NMR spectral regions 4.8–4.7 and 4.6–4.5 ppm showing the first two principal components which explain 67% of the data variation. (C) PLSR result for spectral (4.8–4.7 and 4.6–4.5 ppm) prediction of log (DP3/DP4 ratio) using a 4 latent variable model.

The full Raman spectra of β-glucans (Fig. 6A, top) showed clear signals of compositional sample constituents, such as β- and α-glucans. PLSR results for Raman spectral (1800–800 cm^{-1}) prediction of β-glucan and starch content in samples using 3 latent variable models are listed in Table 2. The models showed good prediction capability when comparing the prediction errors (β-glucan; 4.4% and starch; 2.1%) to the measured ranges (β-glucan; 45–88% and starch; 1–21%) which demonstrate the potential for rapid Raman spectroscopy in purity screening of larger β-glucan sample sets. Comparing the Raman β-peak (Fig. 6A, bottom) of β-glucan

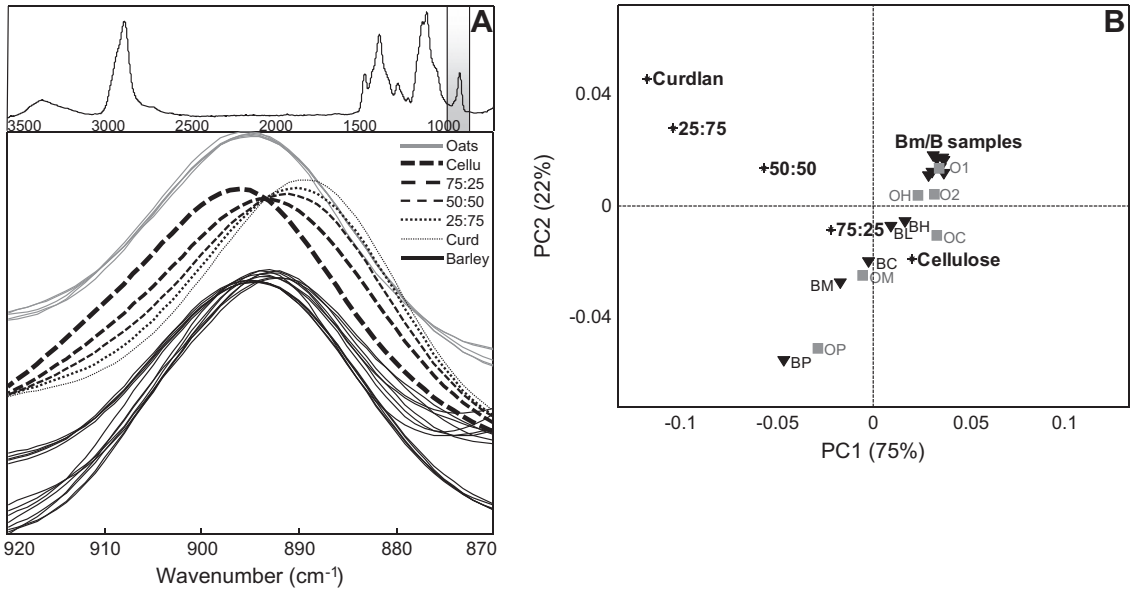

Fig. 6. (A) Raman spectra (full and β-peak region) of β-glucan and cellulose:curdlan blended samples. (B) PCA score plot of Raman spectral region 920–870 cm^{-1} showing the first two principal components which explain 97% of the data variation.

Table 2
Results of PLSR models based on MSC corrected Raman spectra of 18 β-glucan samples for prediction of β-glucan and starch contents.

	Range (%)	#PC	r^2	RMSECV
β-Glucan	45–88	3	0.91	4.4
Starch	1–21	3	0.90	2.1

samples and designed cellulose:curdlan blends confirmed a systematic, albeit very small, variation of the β-peak position related to the β-1,4 to β-1,3 linkage ratio. In the PCA score plot based on the Raman spectra the cellulose:curdlan blends spanned a straight line from curdlan (upper left corner) to cellulose (lower right corner), whereas the variation in the β-glucan samples were distributed orthogonal to this line in the middle of the range 75–100% cellulose (0–25% curdlan) in agreement with the β-1,4 to β-1,3 linkage ratio found by NMR spectroscopy. In contrast to liquid-state NMR spectroscopy, which is only sensitive to the mobile parts of the molecules, Raman spectroscopy will also measure longer "insoluble cellulose-like segments" in the β-glucan structures. This can sometimes lead to a discrepancy between the methods as observed for alginates (Salomonsen, Jensen, Larsen, Steuernagel, & Engelsen, 2009). In view of this, a Raman based approach for prediction of β-glucan linkage characteristics is promising.

4. Conclusion

In summary, we succeeded in extracting large batches of pure, comparable and reproducible barley and oat β-glucan isolates with similar molecular masses and specific genotypic structural characteristics. The combination of high performance anion exchange chromatography (HPAEC) with advanced spectroscopy such as FT-Raman and ^{1}H NMR and multivariate data analysis proved to be a strong tool in the study of complex relations contained in wide-ranging β-glucan data types. We found considerable variation in the β-glucan structures of barley and oat, but also among the two barley lines, suggesting that β-glucan functionality can be modulated by screening and selection of different barley lines. This type of molecular investigation will be of paramount importance in future studies linking positive dietary effects and specific structural and functional features of β-glucan fibres.

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Paper III

Simonsen HT, Nielsen MS, Christensen NJ, Christensen U, La Cour TV, Motawia MS, Jespersen BM, Engelsen SB, Møller BL

Molecular interactions between barley and oat β-glucans and phenolic derivatives

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Molecular Interactions between Barley and Oat β-Glucans and Phenolic Derivatives

HENRIK TOFT SIMONSEN,[†] METTE S. NIELSEN,[†,‡] NIELS J. CHRISTENSEN,[‡] ULLA CHRISTENSEN,[†] THOMAS V. LA COUR,[†] MOHAMMED SADDIK MOTAWIA,[†] BIRTHE P. M. JESPERSEN,[‡] SØREN B. ENGELSEN,[‡] AND BIRGER LINDBERG MØLLER*,[†]

Plant Biochemistry Laboratory and Laboratory for Molecular Plant Biology, VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark, and Quality and Technology, Department of Food Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark

Equilibrium dialysis, molecular modeling, and multivariate data analysis were used to investigate the nature of the molecular interactions between 21 vanillin-inspired phenolic derivatives, 4 bile salts, and 2 commercially available β-glucan preparations, Glucagel and PromOat, from barley and oats. The two β-glucan products showed very similar binding properties. It was demonstrated that the two β-glucan products are able to absorb most phenolic derivatives at a level corresponding to the absorption of bile salts. Glucosides of the phenolic compounds showed poor or no absorption. The four phenolic derivatives that showed strongest retention in the dialysis assay shared the presence of a hydroxyl group in *para*-position to a CHO group. However, other compounds with the same structural feature but possessing a different set of additional functional groups showed less retention. Principal component analysis (PCA) and partial least-squares regression (PLS) calculations using a multitude of diverse descriptors related to electronic, geometrical, constitutional, hybrid, and topological features of the phenolic compounds showed a marked distinction between aglycon, glucosides, and bile salt retention. These analyses did not offer additional information with respect to the mode of interaction of the individual phenolics with the β-glucans. When the barley β-glucan was subjected to enzyme degradation, the ability to bind some but not all of the phenolic derivatives was lost. It is concluded that the binding must be dependent on multiple characteristics that are not captured by a single molecular descriptor.

KEYWORDS: β-Glucan; barley; oat; bile salts; phenolic derivatives; β-glucosides

INTRODUCTION

The first publication on the relationship between dietary fibers and small molecules, namely, bile salts, was published by Cooksoon et al. in 1967 (*1*). Since then, the health-promoting effect of dietary fibers and the influence of dietary fibers in food mixtures has been investigated in a large number of studies. The health-promoting effects of dietary fibers are now well documented (*2*−*5*). Knowledge obtained in these studies has inspired this study and has served as a platform in the investigation of the interaction of a different set of small molecules with β-glucans. It has been observed in animal as well as human models that an increase in soluble, viscous nonstarch polysaccharides (e.g., β-glucan) in the diet is accompanied by an increase in fecal sterols, suggesting that these fibers interact with bile salts and cholesterol in the gastrointestinal (GI) tract (*6, 7*). Cellulose was shown not to possess any of these effects, but it was not possible to define the properties responsible for binding or retention (*8*). The adsorption capacities of different fiber types were shown to vary, and the drug colestyramine, a bile acid sequestrant, has been adopted as a standard for these measurements (*8*). Eastwood et al. (*9*) suggested a simple method to establish strong and reversible adsorption. A linear relationship between the percentage of bile acid adsorbed and the amount of fiber used regardless of the bile acid concentration was observed. However, differences between the adsorption of different bile acids made final conclusions difficult. Dietary fibers from different sources have been tested and shown to adsorb bile salts, but the adsorption was not correlated with the ability of the fiber to alter the cholesterol level in vivo. This suggests that several factors influence the properties that account for the adsorption of the bile acids and the lowering of blood cholesterol levels (*10, 11*).

* Corresponding author (telephone 353 33352; fax 353 33333; e-mail blm@life.ku.dk).
[†] Department of Plant Biology and Biotechnology.
[‡] Department of Food Science.

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Table 1. Percentage Dialysate Retention Based on the Asymptotic UV Absorbance (Δ*A*) and Dialysis Rate Constants (k_d) from Mixed Solutions of 2.5% (w/v) Glucagel and the 21 Selected Phenolic Derivatives (Means of Minimum Two Replicates)

	dialysate retention[a] (Δ*A*, %)			dialysis rate constant[b] (k_d)		
compd (name/no.)	day 1	day 2	Δ (2 − 1)	day 1	day 2	Δ (2 − 1)
4′-hydroxy-3′-methoxyacetophenone (**1**)	23	34	11	0.008	0.009	0.001
2-hydroxy-4-methoxybenzaldehyde (**2**)	10	8	−2	0.008	0.007	−0.001
2,6-di-*tert*-butyl-4-methoxyphenol (**3**)	6	3	−3	0.013	0.011	−0.002
2,6-di-*tert*-butyl-4-methylphenol (**4**)	12	18	6	0.012	0.011	−0.001
4-hydroxy-3-methoxybenzyl alcohol (**5**)	12	18	6	0.016	0.012	−0.004
3-hydroxy-4-methoxybenzyl alcohol (**6**)	13	17	4	0.015	0.011	−0.004
3-ethoxy-4-hydroxybenzaldehyde (**7**)	14	25	11	0.008	0.010	0.002
3,5-dimethoxyphenol (**8**)	14	21	7	0.011	0.012	0.001
2,3-dimethylphenol (**9**)	5	−2	−7	0.011	0.009	−0.002
2,5-dimethylphenol (**10**)	13	18	5	0.008	0.010	0.002
2′-hydroxy-4′-methoxyacetophenone (**11**)	19	13	−6	0.009	0.008	−0.001
ethyl 4-ethoxy-2-hydroxybenzoate (**12**)	5	10	5	0.013	0.011	−0.002
3,5-dimethoxy-4-hydroxybenzaldehyde (**13**)	35	38	3	0.009	0.010	0.001
3,5-dimethoxy-4-hydroxybenzoic acid (**14**)	40	34	−6	0.010	0.006	−0.004
2,3,5-trimethylphenol (**15**)	2	−4	−6	0.011	0.008	−0.003
2,3,6-trimethylphenol (**16**)	6	5	−1	0.012	0.010	−0.002
4-hydroxy-3-methoxybenzaldehyde (**17**)	12	4	−8	0.011	0.009	−0.002
4-hydroxy-3-methoxybenzoic acid (**18**)	2	15	13	0.010	0.011	0.001
methyl 4-hydroxy-3-methoxybenzoate (**19**)	5	0	−5	0.009	0.008	−0.001
ethyl 4-hydroxy-3-methoxybenzoate (**20**)	34	32	−2	0.010	0.008	−0.002
4-hydroxybenzyl alcohol (**21**)	17	20	3	0.012	0.009	−0.003

[a] Compounds **1**, **2**, **7**, **11**, **13**, **14**, **17**−**27** were measured at 280 nm and compounds **3**−**6**, **8**−**10**, **12**, **15**, and **16** were measured at 220 nm. [b] Values represent k_d values from mixed solutions of vanillin derivatives and Glucagel.

Fiber-induced changes in fecal bile salt concentrations or composition may not be the sole mechanism involved in the lowering of serum cholesterol. No direct correlation between the viscosity of the matrix and the adsorption has been observed, whereas adjustments of pH and salt strength have been observed to alter the adsorption properties of the fibers (*12*). Multiple adsorption mechanisms are possible, mediated by the same, partly overlapping, or different molecular parameters contributing to viscosity. One major mechanism could be the formation of micelles by the bile acid and the adsorption of these within the fiber (*13, 14*).

β-Glucans are known as hydrocolloid-forming glucose polymers and are used as texture-enhancing additives in the food industry. Several studies have shown that hydrocolloids influence the rate and intensity of flavor release in foods (*15*−*17*). It is recognized that viscosity affects overall flavor perception (*15, 18*). Thickened solutions of similar viscosity do not necessarily offer the same flavor perception. This demonstrates that viscosity as well as adsorption affects flavor release and perception (*19*). The molecular mechanisms that govern the functionality of β-glucans in human health and in food matrices thus remain elusive. Knowledge of the physicochemical interactions that occur between aroma compounds and food constituents is required to be able to describe the behavior of aroma and flavor compounds in food products.

To study interactions between β-glucans and small molecules such as aroma compounds, phenolic derivatives, and bile salts, several different methods have been used, including static headspace, NMR, dynamic exponential dilution, and size exclusion chromatography (*9, 19*−*22*). Thus, thermodynamic and other dynamic approaches have been used to study the behavior of aroma compounds in model complex media that possess different microstructure.

In equilibrium dialysis, a liquid is partitioned through a semipermeable membrane that separates a cell into two compartments, a sample and an assay chamber, of which one contains the dietary fiber. If the interactions that occur between two components (e.g., small molecules such as aroma compounds and macromolecules such as dietary fiber) are strong enough, only the nonretained small molecules will participate in the equilibrium. If the total concentration of a small compound in the two compartments at equilibrium is known, it is possible to calculate the quantity that is adsorbed or retained by the macromolecule matrix (*23*). This enables quantitative assessment of molecular interactions between small compounds and macromolecular food constituents (e.g., β-glucan). Molecular affinities and mechanisms by which β-glucans function may be elucidated by combined studies of small compound retention in β-glucan matrices, molecular modeling, and multivariate data analysis. This knowledge can be correlated to the interactions of other small molecules and β-glucans and provide ideas on how β-glucans affect the aroma of foods and function as health promoters in the intestine.

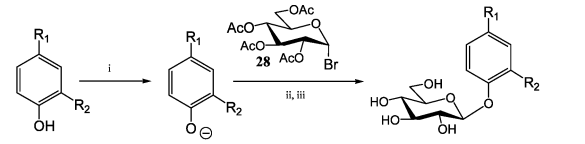
The objective of the present study was to determine the possible interaction between 21 different vanillin-inspired phenolic derivatives, 6 glucosides of these derivatives, and 4 bile salts and specific barley β-glucan (Glucagel) and oat β-glucan (PromOat) preparations using equilibrium dialysis. Differences in the β-glucan-induced retention of the small molecules were related to the specific physicochemical properties of these molecules using molecular modeling and multivariate data analysis.

MATERIALS AND METHODS

Phenolic Derivatives and Bile Acids. Vanillin and 20 different related phenolic structures (**1**−**21**), tryptophan, and the 4 bile acids deoxycholic acid, glycocholic acid, taurocholic acid, and cholic acid were obtained from Sigma-Aldrich (Copenhagen, Denmark) and selected for screening.

Glucosylation of Phenolic Compounds. The investigated phenolic glucosides **22**−**27** (**Table 2**) used in this study were chemically synthesized as shown in **Figure 1**.

Glucosylation of the aglycons **1**, **7**, **17**, and **19**−**21** with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**28**) was performed in aqueous organic basic media using homogeneous reaction conditions and aqueous NaOH with acetone as the organic cosolvent (*24*) to provide the related aryl O-protected-glucosides, which by Zémplen deacetylation afforded the phenolic compounds **22**−**27**. The purity and structural conformation was verified by NMR spectroscopy.



Comp. No.	R_1	R_2
22	CHO	OCH_3
23	$COOC_2H_3$	OCH_3
24	COCH3	OCH_3
25	$COOCH_3$	OCH_3
26	CH2OH	H
27	CHO	OCH3

Figure 1. Chemical synthesis of the phenolic glucosides **22**−**27**: (i) NaOH, H_2O, <10 °C, 15 min; (ii) acetone, room temperature, 24 h; (iii) MeOH, MeONa/MeOH, room temperature, 1−2 h.

Barley *β*-Glucan. Glucagel, a commercial soluble β-glucan extracted from hull-less barley, was obtained from GraceLinc Ltd. (Christchurch, New Zealand). Glucagel has a declared content of ≥75% β-glucan, <18% starch, <10% moisture, <5% protein, <2% ash, and <2% fat. The β-glucan is of moderate molecular weight, $(0.12-0.18) \times 10^6$.

Oat *β*-Glucan. PromOat, a commercial soluble β-glucan extracted from oat, was obtained from Biovelop (Kimstad, Sweden). PromOat has a declared content of 30−40% β-glucan, 6% pentosans, 49% carbohydrates (described as dextrins by the supplier), 4.5% moisture, <2.5% protein, 3.5% ash, and 0.5% fat. According to the supplier, molecular weights of 1.0×10^6 are routinely achieved, which characterizes PromOat as a high molecular weight β-glucan.

Equilibrium Dialysis Assays with Glucagel. The phenolic compounds and β-glucan were dialyzed in sterile 0.5% DMSO and 10 mM Tricine buffer (pH ~7) using 1 mL in-line equilibrium dialysis cells (Bel-Art Products, Pequannock, NJ) and dialysis membranes with size exclusion of 6−8 kDa for globular molecules (Spectrum Laboratories Inc., Breda, The Netherlands). According to the manufacturer, this cutoff is calculated for proteins, and for dextrans the cutoff is calculated to be 1−1.5 kDa. Glucagel 5% (w/v) was dispersed in distilled water in a 50 mL conical flask covered with aluminum foil and then heated for 30 min at 80−82 °C using a hot plate stirrer controlled by a thermostat. A sufficient quantity of phenolic derivatives or bile salts was dissolved in 1 mL of DMSO. Two 5 mL samples of 2.5% (w/v) β-glucan were prepared in the described buffer. To one sample were added phenolic derivatives or bile salts to achieve 2 and 10 mM final concentrations, respectively. Additional 5 mL samples of 2 mM phenolic derivative or 10 mM bile salt were prepared in buffer. The concentrations of the phenolic derivative relate to the maximum possible concentration to be kept in solution. The concentrations of the bile salts were chosen as those previously used in similar experiments (*14, 20*). All samples were stored at 4 °C prior to performance of the dialysis experiment. To minimize the differences in the gel-setting samples, the dialysis experiments were initiated 12 and 36 h (1 and 2 days) after sample preparation. All samples were then heated to 25 °C and thoroughly mixed using a vortex mixer, again to keep the gel setting to a minimum. One milliliter of each sample was applied into two individual sample chambers of the dialysis cells under aseptic conditions and subsequently dialyzed against 1 mL of sterile buffer in the assay chambers. The time of application of the first sample was denoted time zero, and a time gap of 30 s between applications of samples was maintained, thus keeping track of the exact dialysis time for each sample. After application, the dialysis cells were quickly transferred to a thermostat-regulated rotating water bath at 37 °C. Preliminary kinetic studies showed that dialysis equilibrium was reached in <5 h. Aliquots (10 μL) were withdrawn from the assay chamber after 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, and 300 min (5 h), transferred to a 96-well plate (Nunc A/S, Roskilde, Denmark), and diluted with distilled water in the ratio 1:9. Absorbance of the diluted dialysate sample and of a reference of distilled water was measured at 210, 220, and 280 nm on a Spectra Max 190 plate reader (Molecular Devices Corp., Sunnyvale, CA) depending on the wavelength providing maximum absorbance for the phenolic derivative used in the individual experiments. All compounds were tested four times; that is, two replicates were dialyzed 12 h after mixing and two replicates of the same sample were dialyzed 36 h after mixing. The 12 h (day 1) and 36 h (day 2) dialysis experiments were treated as two separate experiments.

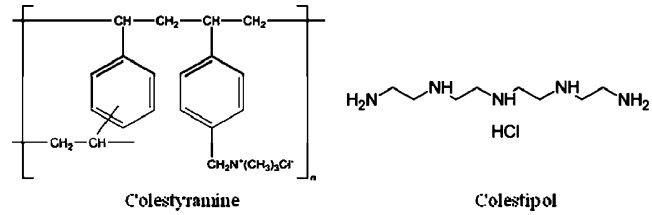


Figure 2. Chemical structures of colestyramine and colestipol.

Enzymatic Breakdown Studies. The studies were performed using Glucagel and the methodology reported above. After aliquot sampling at 240 min, 2 μL of 50 mU/mL Lichenase enzyme (Megazyme, Ireland) or 2 μL of a 1:100 dilution of the original product solution of Viscozyme L (batch KTN02140, Novozymes, Denmark) was added to the β-glucan-containing compartment of the dialysis instrument. Aliquots (10 μL) were withdrawn from the assay chamber after 255, 270, 285, 300, 315, 330, 360, 390, 420, and 450 min from the start of the experiment. The aliquots were analyzed as described above.

Comparison of Glucagel and the Bile Salt Sequestrant Drugs Colestyramine and Colestipol Hydrochloride. The studies were performed using the methodology reported above and a 2.5% (w/v) assay concentration of Glucagel. Using the commercial drug formulations, 1% (w/v) solutions of colestyramine (Questran, Bristol-Myers Squibb) and colestipol hydrochloride (Lestid, Pfizer) were prepared. Chemical structures are shown in **Figure 2**. The drugs were obtained directly from the manufacturer through the pharmacy at the University of Copenhagen, and the 1% (w/v) concentration used reflects the recommend dose of the drugs.

Comparative Equilibrium Dialysis Assays with Glucagel and PromOat. This series of dialysis experiments was carried out as above except that the concentrations of both β-glucan preparations were reduced to 1% (w/v) (the glucan concentration in the assay) to circumvent handling problems related to the high specific viscosity of PromOat.

Exponential Curve Fitting of Dialysis Data. Initial data handling was conducted in Excel (Microsoft Office 2003) where measured absorbencies were corrected for the background absorbance, mean values were calculated for the two replicates, and moving averages were calculated over five continuous measurements. Regression analysis procedures were employed to explore the relationship between dialysate absorbance and dialysis time. Analysis of moving averages reduces the impact of nonrelevant dialysis information, leads to simpler and more robust data sets for regression models, and improves interpretation of the dialysis data. Generally, linearity of the absorbance to concentration relationship over the concentration range of 0−2 mM for the phenolic compounds was observed when measured in the 210−280 nm range. Subsequent mathematical modeling was conducted in SigmaPlot (version 4.01). A curve derived from the exponential equation

$$\text{absorbance} = A(1 - e^{-kt}) \quad (1)$$

was fitted to data from each individual dialysis experiment, where A is the asymptotic or equilibrium UV absorbance value, t is the dialysis time, and k is the dialysis rate constant. All model fits were evaluated using correlation coefficients (r^2). Asymptotic values were compared between dialyses of each of the pure phenolic derivatives and for the phenolics mixed with β-glucan to quantitatively determine the level of dialysate retention (ΔA) by β-glucan. Asymptotic values derived from the pure β-glucan samples were subtracted from all of the β-glucan/aroma compound mixtures to account for the dialysate from β-glucan alone.

Multivariate Data Analysis. Dialysate retention data from day 1 and day 2 dialysis experiments were subjected to principal component analysis (PCA) and partial least-squares (PLS) regression.

In PCA, a data matrix is decomposed by consecutive orthogonal extraction of the largest variation (principal components, PCs) in data

Table 2. Percentage Dialysate Retention Based on the Asymptotic UV Absorbance (Δ*A*) from Mixed Solutions of 2.5% (w/v) Glucagel and Six Selected Glucosides of Phenolic Compounds (Means of Minimum Two Replicates, All Meseaured at 280 nm)

compd (name/no.)	aglycon no.	dialysate retention (Δ*A*, %)		
		day 1	day 2	Δ (2 − 1)
4-β-D-glucopyranosyloxy-3-methoxybenzaldehyde (**22**)	17	7	9	2
ethyl 4-β-D-glucopyranosyloxy-3-methoxybenzoate (**23**)	20	−8	−5	3
4′-β-D-glucopyranosyloxy-3′-methoxyacetophenone (**24**)	1	−3	2	5
methyl 4-β-D-glucopyranosyloxy-3-methoxybenzoate (**25**)	19	−12	−10	2
4-β-D-glucopyranosyloxybenzyl alcohol (**26**)	21	−10	−9	1
3-ethoxy-4-β-D-glucopyranosyloxybenzaldehyde (**27**)	7	−9	−7	2

until the variation left is unsystematic. The loading vectors can be considered as pure hidden profiles that are common to all measurements. Two-dimensional scatter plots of the score vectors show the covariance between samples, providing a characterization of data. The purpose of PLS regression is to build a linear model enabling prediction of a desired chemical/physical characteristic (*Y*) from measured data (*X*). During the regression, *X* is decomposed as in PCA, but the PCs are found as the underlying structures that covary best with the *Y* variable (*25*).

All molecular structures in this study were optimized with MM3* in MacroModel (*26*). A total of 234 molecular descriptors were calculated for the phenolic derivatives and glucosides with CDK (*27*) and QikProp (*28*). CDK descriptors are divided into five major classes: electronic (atomic polarizabilities, bond polarizabilities, charged partial surface areas, hydrogen bond acceptors, and hydrogen bond donors), geometrical (geometrical index, length over breadth, moments of inertia, and Petitjean shape indices), constitutional (*A*log*P*, bond, element, and atom type counts, largest chain, Lipinski's rule of five, rotable bonds count, *X*log*P*, molecular weight), hybrid (BCUT and WHIM), and topological (carbon types, Chi indices, eccentric connectivity index, fragment complexity, Kier and Hall molecular shape indices, topological polar surface area, Wiener numbers, Zagreb index, and Moreau-Broto autocorrelation descriptors). QikProp provides approximately 40 descriptors, of which several (e.g., predicted brain/blood partition coefficient, QPLogBB) are of pharmaceutical relevance, whereas others [e.g., PM3 calculated ionization potential, IP(eV)] are of a more general nature. Without any a priori knowledge of the mechanisms involved, we anticipate that this diverse set of molecular descriptors captures information relevant to the dialysis characteristics. To reduce the amount of noise in the descriptor matrix and improve the subsequent interpretation of PCA plots and PLS regressions, a simple two-step variable selection scheme was employed. First, descriptors were deleted unless they assumed distinct values for at least 12 samples. Second, the Pearson product moment correlation coefficient, *r*, between response (Δ*A* or k_d) and the descriptors was evaluated. In cases when *r* fell below a certain threshold (0.5 for Δ*A*, 0.2 for k_d), the corresponding descriptor was deleted. The lower threshold for k_d was required, because response−descriptor correlations were very low in this case. The number of descriptors produced by the variable selection was 62 for day 1 Δ*A*, 16 for day 2 Δ*A*, 82 for day 1 k_d, and 91 for day 2 k_d. The variables were autoscaled prior to data analysis, and full (leave-one-out) cross-validation was used. The PLS models were evaluated on the basis of the root-mean-square error of cross-validation (RMSECV), which is the estimation of the error of the predicted values. Multivariate data analysis was performed using Unscrambler (*29*), Matlab (*30*), and Latentix (*31*).

Molecular Mass Determination. The molecular mass of the β-glucans was estimated by size exclusion chromatography (SEC) using a Superdex 200 column (16 mm × 60 cm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) fitted with a refractive index detector. Superdex 200 is a cross-linked agarose and dextran material with a nominal bead size of 13 μm, a pore size of 100−120 Å, and an optimal separation range of 10,000−600,000 Da. The mobile phase consisted of 50 mM ammonium formate and 200 mM NaCl, and the column was eluted at room temperature at 1.6 mL/h. Dextrose standards of 5, 12, 25, 50, 80, and 150 kDa (Fluka, Buchs, Switzerland) were used for calibration.

RESULTS

Vanillin and 20 other phenolic derivatives (**1**−**21**), 6 phenolic glucosides (**22**−**27**), and 4 bile salts were tested with respect to their ability to bind to Glucagel using the dialysis setup. The phenolic derivatives can be divided into different chemical classes according to the functional groups present in the different compounds. All of them possess a central benzene ring as the core structure. The benzene ring is substituted with a minimum of two groups, of which one would be an oxy group. The aglycon structures may be grouped into 2 ketones (**1**, **11**), 4 aldehydes (**2**, **7**, **13**, **17**), of which 2 were hydroxylated on the benzene ring (**2**, **17**), 10 phenols (**3**−**6**, **8**−**10**, **15**, **16**, **21**), of which 2 had one additional hydroxyl group (**5**, **6**), 3 esters of either ethyl or methyl character (**12**, **19**, **20**), and 2 aromatic acids (**14**, **18**) (**Tables 1** and **2**). The molecules cover a broad range of log *P* values ranging from −0.5 for the glucosides to 5.1 for compound **4**. With respect to their physical dimensions, the phenolic derivatives and bile salts also span a significant range from the large bile salts (18−19 Å in diameter) to the smallest, being compound **21** (7 Å in diameter).

The ability of all of the compounds **1**−**27** to bind to Glucagel was analyzed in dialysis experiments. Exponential curve fitting of all dialysis data was performed to fit eq 1. Dialysis curves of **8** and **10** in the presence of 2.5% (w/v) Glucagel as well as the exponential curve fit are presented in **Figure 3** as typical examples. Dialysate retentions (Δ*A*) and dialysis rate constants (k_d) from the screening of all 21 phenolic derivatives are presented in **Table 1**. The values represent the mean of two replicates. Values obtained by dialysis of β-glucan in the absence of any added compound were subtracted from the values obtained with added compounds. All dialysis experiments exhibited patterns similar to those presented in **Figure 3**. The patterns are composed of an initial steep slope during the first ∼15−90 min and an asymptotic convergence toward a maximum after 90−300 min. Generally, there was good agreement between the dialysis curve and the exponential fit. Correlation coefficients from the curve fits were >0.95 in all dialysis experiments. At equilibrium/asymptotic level, the absorbance of **8** reaches a value of 0.96, whereas the asymptotic value of **8** + 2.5% (w/v) Glucagel is 0.76. This gives a relative difference of 22%, which corresponds to the retention of dialysate (**8**) by the β-glucan, as seen in **Table 1**. The different classes of chemical compounds within the 21 phenolic derivatives such as ketones, aldehydes, phenols, esters, and acids did not differentiate significantly from each other and within the groups with respect to being retained by the barley β-glucan. Only four compounds (**1**, **13**, **14**, **20**) gave rise to a Δ*A* > 30. All four compounds shared the presence of a hydroxyl group at position 4 and a CHO group at position 1. However, other compounds such as **18**, **5**, and **19** also possess these features and give rise to much lower Δ*A* values. The difference between day 1 and day 2 retentions (Δ 2 − 1) showed a weak tendency of increased

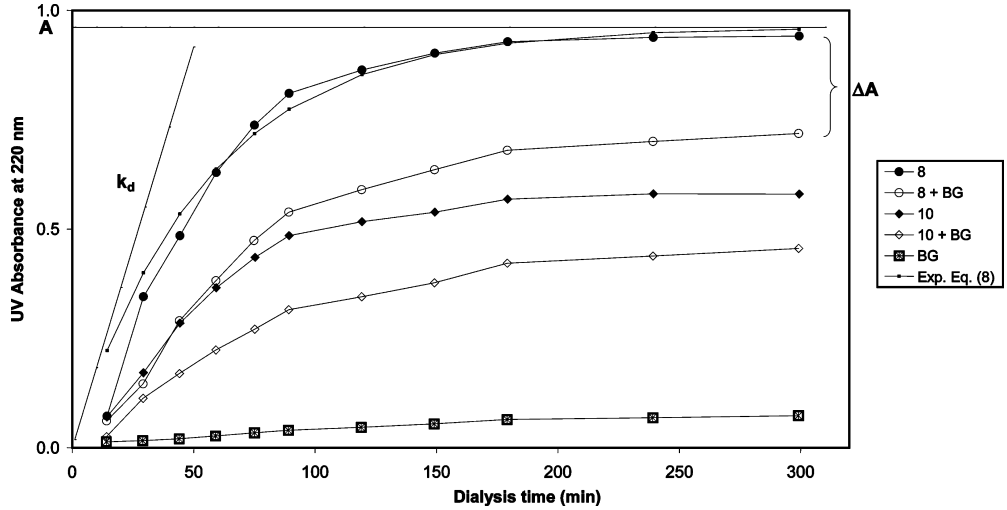


Figure 3. Dialysis curve of compounds **8** and **10** in the presence of 2.5% (w/v) Glucagel (BG). The line of k_d is shown, and the horizontal line illustrates the maximum absorbance to be obtained at equilibrium of the dialysis of the exponential data curve.

Table 3. Percentage Dialysate Retention Based on the Asymptotic UV Absorbance (ΔA) and Dialysis Rate Constants (k_d) from Mixed Solutions of 2.5% (w/v) Glucagel and Four Different Bile Salts and Tryptophan (Means of Minimum Two Replicates)

	dialysate retention (ΔA, %)			dialysis rate constant (k_d)		
compd	day 1	day 2	Δ (2 − 1)	day 1	day 2	Δ (2 − 1)
deoxycholic acid	69	76	7	0.008	0.009	0.001
glycocholic acid	78	86	8	0.008	0.007	−0.001
taurocholic acid	73	81	8	0.013	0.011	−0.002
cholic acid	38	42	4	0.012	0.011	−0.001
tryptophan	23	28	5	0.005	0.004	−0.001

dialysate retention on day 2, that is, positive Δ values. Comparison of the k_d values from days 1 and 2 (Δ 2 − 1) indicated lower dialysis rates at day 2. For the more hydrophilic phenolic derivative glucosides (**Table 2**) no or only very weak retention was observed. Compared with the values obtained for the aglycons, the glucosides are significantly less adsorbed by the fiber.

Four bile salts were tested in the dialysis equilibrium system (**Table 3**). All four bile salts showed good retention in the system with ΔA from 42 to 86. This confirmed the published in vivo evidence for the ability of dietary fibers to adsorb bile salts (*4, 5*). Tryptophan has previously been used as a reference molecule in dialysis tests (*14*) and was also tested in this study and showed a retention of 23 with is compliance with previous studies (*14*).

Comparison of Glucagel and PromOat. To evaluate the ability of a different commercial β-glucan to bind phenolic derivatives, the oat β-glucan product PromOat was investigated and compared to the barley β-glucan product Glucagel. The results (**Table 4**) show no significant differences between the two products with respect to retention of phenolic derivatives under the conditions used in our dialysis equilibrium system. The secondary structure, product composition, and viscosity of these two fiber preparations are not the same (*32*), which may be important for the adsorption. However, this is not captured by our dialysis assay.

Comparison with Commercial Bile Salt Sequestrant Drugs. To evaluate the efficacy of the fiber to adsorb bile salts and other small compounds, a comparative study with the commercial drugs colestyramine and colestipol hydrochloride was carried out. The results presented in **Table 5** show that for

Table 4. Comparison of Glucagel and PromOat (Means of Minimum Two Replicates)[a]

	dialysate retention	
compd (name/no.)	Glucagel	PromOat
3-ethoxy-4-hydroxybenzaldehyde (**7**)	23	26
ethyl 4-hydroxy-3-methoxybenzoate (**20**)	15	19
4-hydroxy-3-methoxybenzaldehyde (**17**)	14	17
4′-hydroxy-3′-methoxyacetophenone (**1**)	10	14

[a] Percentage dialysate retention based on the asymptotic UV absorbance (ΔA) from mixed solutions of 1% (w/v) β-glucan solutions and four different phenolic compounds.

Table 5. Percentage Dialysate Retention Based on the Asymptotic UV Absorbance (ΔA) of Four Phenolic Compounds and Four Bile Salts by Glucagel, Colestyramine, and Colestipol Hydrochloride

	dialysate retention		
compd (name/no.)	Glucagel	colestyramine	colestipol hydrochloride
3-ethoxy-4-hydroxybenzaldehyde (**7**)	23	2	4
ethyl 4-hydroxy-3-methoxybenzoate (**20**)	15	3	6
3,5-dimethoxy-4-hydroxybenzoic acid (**14**)	40	95	93
4-hydroxy-3-methoxybenzoic acid (**18**)	10	96	92
deoxycholic acid	69	89	85
glycocholic acid	78	95	97
taurocholic acid	73	94	93
cholic acid	38	96	95

nonacid compounds (**7**, **20**), the fibers have higher retention capability than the drugs. The drugs are characterized as having an anionic exchange nature. In agreement, the two drugs were able to retain the acidic compounds (**14**, **18**) up to 9 times better than the fiber. This demonstrates that ionic forces are not the main property responsible for the adsorption of small molecules to the fiber. This also confirms that the dialysis assay works with matrices other than the fibers.

Enzymatic Breakdown of the Fibers. To investigate the importance of fiber molecular mass for the adsorption ability, the rerelease of the small compounds from the fiber matrix was measured after partial enzymatic breakdown of the fibers (**Figure 4**). Some, but not all, of the compounds retained in the experiments with intact fibers were released as a result of enzyme breakdown of the fibers and moved freely between the two dialysis compartments. This shows that different parameters contribute to retention of the compounds. The fibers were broken

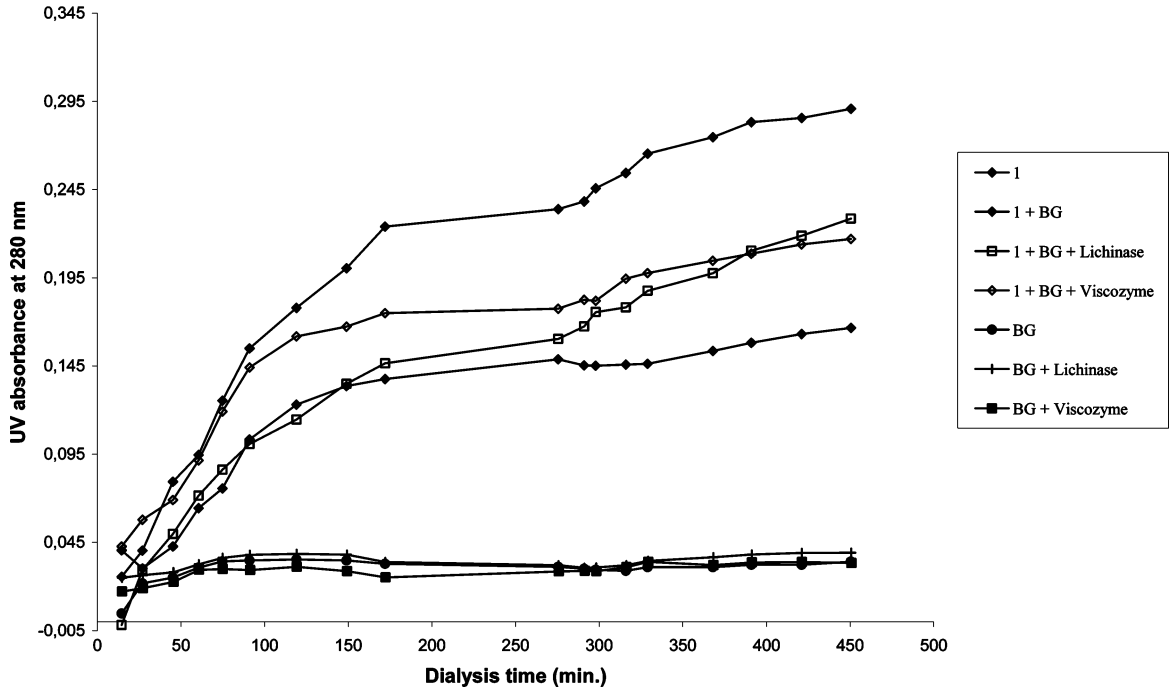


Figure 4. Effect of enzymatic breakdown of the barley β-glucan on its ability to bind phenolic derivatives as determined by dialysis assays. The experiment was carried out using 2.5% (w/v) barley β-glucan Glucagel (BG) as fiber matrix and 4′-hydroxy-3′-methoxyacetophenone (**1**) in the 2.5% (w/v) Lichenase and Viscozyme enzymatic breakdown of the fibers.

down to masses of <5000 Da, which supposedly disrupt the majority of the tertiary structures and some of the secondary structures of glucans. **Figure 4** also shows that even though the fibers were broken down, they were retained in the sample champers, indicating that they could not move freely as the small molecules can.

Multivariate Data Analysis. PCA was conducted using the dialysate retention data sets obtained at day 1 as well as day 2 using the dialysate retentions (ΔA) and rates (k_d). The correlation plot of the free aglycone, glycoside, and of the bile salt data (ΔA and k_d) (**Figure 5**) showed a marked distinction between these three groups of compounds according to ΔA. Additionally, the plot shows a tendency of decreasing k_d values between the data obtained at days 1 and 2 (arrows directed downward) and a weak trend of increasing ΔA values from day 1 to day 2 (arrows directed to the right). No obvious groupings within the 21 tested structures were observed. **Figure 6** shows the PCA score plot for the 21 phenolic derivatives and 6 glucosides represented by 62 descriptors selected using the value of day 1 ΔA as explained under Materials and Methods. The samples have been colored according to the value of ΔA. Although phenolic derivatives with high and low values of ΔA have scores on the extreme left and extreme right of PC1, respectively, the change in ΔA along PC1 is not systematic. Inspection of the remaining PCs did not reveal any improvements in describing the variation of ΔA. The PCA plots based on descriptors selected for the explanation of differences between day 1 and day 2 k_d values did not reveal any structure. The fact that a lowered threshold for correlation with the dependent variable had to be employed in the descriptor selection step supports the notion that a proper explanation of k_d cannot be achieved with the current set of descriptors. PLS regression for the prediction of the day 1 retention value seemed initially to hold some promise, but permutation testing of the model revealed that it was based on a chance correlation. The low quality of the model was also reflected in similar regression coefficients, indicating that no important descriptors could be singled out. The situation did not improve with respect to the remaining independent variables for days 1 and 2. In conclusion, the physical mechanisms involved in fiber retention of the compounds as monitored in the dialysis experiments cannot be explained with our current multivariate data analytical approach. Data analytical exploration of fiber-binding properties has to await the development of a more suitable set of descriptors. At this point the nature of such descriptors is unknown.

DISCUSSION

The metabolic health benefits and viscous properties of β-glucans have been reported by several investigators (*2*−*8*). However, their potential uses, mechanisms of action, and means of incorporation into foods and diets require further exploration. The aim of the present study was to investigate the molecular interactions between β-glucans and selected classes of small molecules. The approach of equilibrium dialysis was chosen because it has been proven useful in interaction studies on bile salts and barley β-glucan (*14*) and because the approach offers a relatively fast method for the analysis of large series of small samples. Dialysis conditions were set to mimic physiological conditions in the sense of continuous movement, a temperature of 37 °C, and pH of 7, which could provide evidence for β-glucan behavior in solutions in the GI tract independent of enzymatic degradation. However, this very simple in vitro study cannot provide an exact description of the physiological actions of β-glucans but rather affords knowledge on their molecular affinities toward small molecules. The effect of hydrocolloids on aroma release from food may be due to numerous mechanisms; one is the physical entrapment of aroma within the food matrix (*15*). Another mechanism involves chemical interaction between the aroma compound and the hydrocolloid components, for example, β-glucan (*16*). In the current study, both types of interactions were studied experimentally.

Along with the test of the 21 phenolic compounds, 6 β-glucosides of these compounds were tested. This would

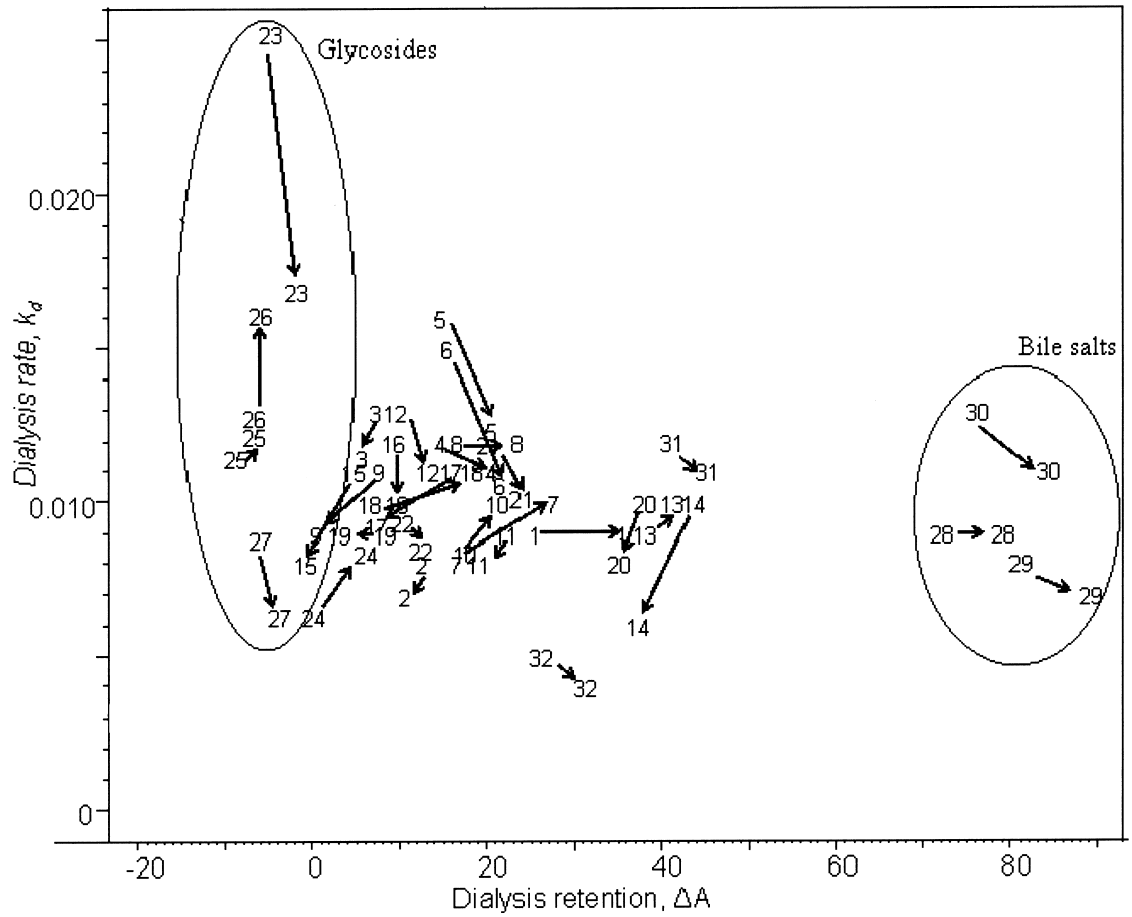


Figure 5. Correlation plot of day 1 dialysate retentions (ΔA) and dialysis rates (k_d). Numbers refer to the compounds listed in **Tables 1**−**3**, and arrows show the direction of movement in the plot between day 1 and day 2 data.

represent the native form in which the aroma compounds are present in the plants, and it was therefore of interest to test whether they are retained in the same fashion as the aglycons. As seen in **Table 2**, the glucosides are not retained or are retained at only a very low degree compared to the retention of the aglycons. This indicates that hydrophobic properties of the small molecules are of importance. Boland et al. (*15*) observed that of 11 flavor compounds tested, the hydrophobic compounds had the significantly lowest partition coefficients, that is, aroma release in a gelatin gel. In this study, an observed increased percentage of retention as a result of increased log *P* values for compounds **3** and **4** would suggest a similar contribution of hydrophobic properties to fiber retention. No such easily envisioned trends were observed in the present study using either simple curve fitting of retention versus log *P* or multivariate data analysis. Story and Kritchevsky (*8*) stated that the hydrophobic properties of the bile salts tested in their publication did not correlate directly with the binding to the tested fibers. This is in good agreement with the observations in our study both for the bile salts and for the phenolic compounds.

The differences observed in percentage of fiber retention of the different phenolic derivatives tested in the day 1 experiment (**Table 1**) do indeed indicate that the ability of the fibers to retain the different derivatives could be of a different nature depending on the physicochemical properties of each individual phenolic derivative. The corresponding glucosides have a significantly lower log *P* value than the aglycons, whereas at the same time containing a sugar moiety that might be expected to be able to interact with the β-glucan via hydrogen bond formation. As seen in **Table 2**, the glucosides are not retained by the fiber. High water solubility could therefore be one of the properties of the small molecule that would reduce possible interactions with the fiber. Among the tested phenolics the four compounds **1**, **13**, **14**, and **20** had significantly higher retentions than the others. The four molecules possess different functional groups at position 1, but all share a hydroxyl group at position 4. The log *P* values for the four molecules range from 0.83 to 1.68 and cannot be correlated to the significantly higher retention of these molecules compared to the remaining 17 compounds. The four molecules do not possess different physical dimensions, nor do they contain other functional groups compared to the rest of compounds tested. This indicates that multiple different binding properties are of importance for the retention of the small molecules to the β-glucan fiber.

The differences between the results obtained at day 1 and day 2 are most likely due to time-dependent changes in the β-glucan matrix, for example, network formation and increased rigidity in the β-glucan solution induced by the increased incubation time prior to dialysis of the day 2 samples (*32*). In a preliminary study on the viscous properties of a 2.5% (w/v) Glucagel solution, the viscosity (at 37 °C and 30 s^{-1}) of the sample increased approximately 6-fold from day 1 to day 2, which would agree with the slower dialysis rates observed for the day 2 samples. The matrix-dependent changes in dialysate retention indicate that some of the retention is due to the hygroscopic and tertiary structure of the β-glucan. The current findings are in agreement with those of Boland et al. (*15*), who found that flavor release was significantly affected by the texture of gelatin, starch, and pectin gels. The most rigid gel showed the lowest flavor release.

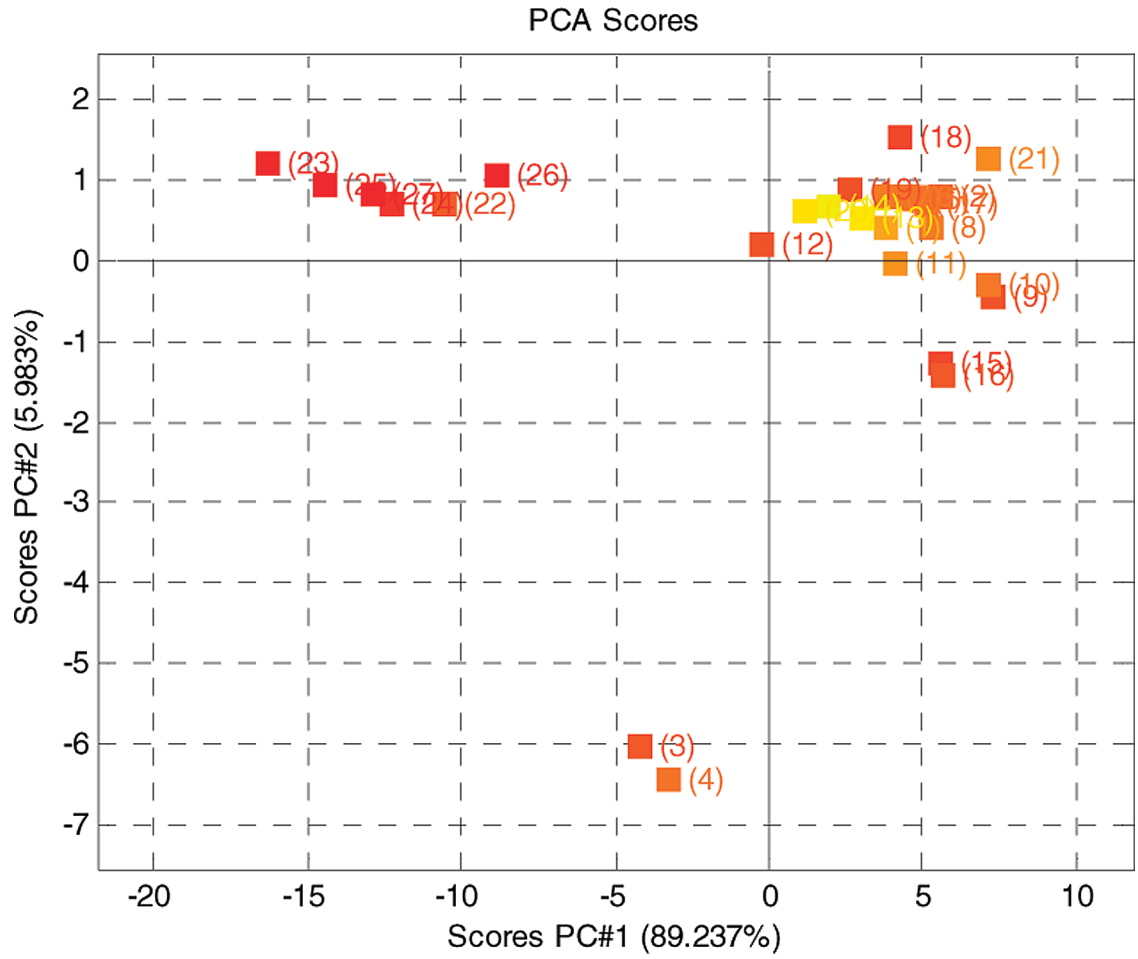


Figure 6. PCA score plot. The value of ΔA on day 1 has been used to color the samples (21 phenolic derivatives and 6 phenolic glucosides). The PCA is based on 62 calculated physicochemical descriptors, selected as described under Materials and Methods. Numbers refer to the compounds listed in **Tables 1** and **2**.

Because the term β-glucan is not a uniform definition, this study included a comparative study of the two commercial products Glucagel (barley) and Promoat (oat). The two matrices of these products are chemically quite different, for example, with respect to purity and molecular masses (*32*). Despite these differences, the two β-glucans show nearly identical properties with respect to their ability to retain phenolic derivatives in the dialysis equilibrium assays. This suggests that the retention of the different phenolics is due to several and interacting properties of the fiber matrix and not a few single parameters.

The efficacy of the β-glucan fibers to adsorb bile salts and other small compounds was also evaluated in comparison with the commercial drugs colestyramine and colestipolhydrochloride. This study indicated that ionic interactions are not the main property responsible for the adsorption of small molecules to the fiber, again confirming that the retention is composed of multiple properties. This also confirms that the dialysis assay works with matrices other than the fibers.

The importance of the fiber molecular mass and thereby the physical size of the molecules for the adsorption ability was also evaluated. A clear rerelease of some, but not all, of the retained compounds was observed upon enzymatic degradation of the barley β-glucan-based matrix. Partial rerelease of the small compounds again indicates that various factors contribute to retention of the different phenolic derivatives. The fibers were broken down to an extent that would disrupt the tertiary structure along with some parts of the secondary, which can explain why some phenolic derivatives were retained.

PCA and PLS regression analyses are powerful tools for extraction of important variances in a data matrix consisting of many variables. In the present study, comparison of β-glucan binding of different phenolic derivatives was conducted using molecular modeling and multivariable data analysis. No strong tendency of sample grouping was found by PCA either for day 1 or for day 2. This implicates that the employed set of descriptors is not well suited for explaining the variation in ΔA and k_d. Additionally, no reliable model fit was found using PLS regression to dialysis data. This could be due to the use of insufficient or ineffective descriptors, the need for more data (a larger number of phenolics screened in the dialysis assay) to strengthen the robustness of prediction, or measurement errors within the present data set from the dialysis assay. Even though no reliable prediction model was found, indications of a correlation between the binding data and some of the molecular descriptors were evident. This indicates that multiple parameters are involved in determining the binding of small molecules to fibers and that the multiplicity of parameters involved obscures the correlations of the observed binding to specific descriptors.

The dialysis data presented provide some information on the complex mechanisms controlling the ability of β-glucans to bind low mass compounds. To more accurately determine the nature of the interactions between β-glucans and low mass

compounds, a range of advanced spectroscopy and molecular modeling methodologies will need to be introduced.

In conclusion, the retention of aroma compounds by β-glucan is of great interest from a food composition point of view and from a health perspective. In recent years, the increased health consciousness among consumers has led to extended additional use of hydrocolloids as replacements for fats. The food industry would benefit greatly from an improved understanding of the mechanisms involved in the flavor retention and release from β-glucan matrices along with the nature of the interactions occurring in the GI tract. Knowledge of the action of model compounds in β-glucan matrices can provide general information on the β-glucan affinity toward small molecules, which would be applicable in the studies of, for example, the bioavailability of natural compounds found in association with β-glucans and for health claims on β-glucan.

It was confirmed that β-glucans from barley and oat are able to adsorb bile salts, and for the first time β-glucans' ability to absorb vanillin and other phenolic compounds was demonstrated. The retention in our newly developed dialysis assay depends on numerous and interacting physicochemical properties of the small molecules. The interaction could not be explained by simple correlation to any of the descriptors included in the multivariable data analysis, and the results could not confirm or disprove the previously described hydrophobic binding or micelle capture of the small molecules to the β-glucan.

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Paper IV

Mikkelsen MS, Jespersen BM, Mehlsen A, Engelsen SB, Frøkjær H

Cereal β-glucan immune modulating activity depends on polymer fine structure and in turn the polymer solubility and aggregation in solution

Molecular Nutrition & Food Research, 2012, Submitted

Cereal β-glucan immune modulating activity depends on polymer fine structure and in turn the polymer solubility and aggregation in solution

Mette Skau Mikkelsen[1*], Birthe Møller Jespersen[1], Anni Mehlsen[2], Søren Balling Engelsen[1], Hanne Frøkiær[2]

[1]*Quality & Technology, Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark;* [2]*Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Ridebanvej 9, 1871 Frederiksberg C, Denmark*

*Corresponding author: Mette Skau Mikkelsen; Tel: +45 35 33 35 07; Fax: +45 35 33 32 45; E-mail: skau@life.ku.dk

Abstract

The relationship between the immune modulatory properties of different mixed linkage β-glucans from microbial and plant origin and their structure and physico-chemical traits is elusive. This study addresses the importance of the physico-chemical properties of barley and oat β-glucans for their capacity to modulate immune responses. A total of 23 different β-glucan samples characterised with respect to compositional, molecular, structural and rheological properties was investigated for their immune modulatory properties. It was found that the β-glucans modulate LPS induced cytokine production in dendritic cells as IL-12 levels decreased and IL-10 levels increased. In dendritic cells stimulated with the probiotic *Lactobacillus acidophilus* the β-glucans up-regulated IL-10 production but showed no effect on the IL-12 levels. The extent to which β-glucan modulated the cytokine responses depended on the β-glucan block structure as expressed by the ratio of tri- to tetraosyl units, solubility and especially the polysaccharide aggregation structures in solution. Speculations on β-glucan fringed micelle interaction with dendritic cell receptors are presented. This comparative study reveals that the β-glucan fine structure and in turn their intermolecular organisation are key elements for understanding their immune modulatory capacity.

Keywords

β-Glucan, barley, oat, dendritic cell, immune modulation, cytokine

Introduction

β-Glucans comprise non-starch polysaccharides build of glucose as the structural component and interlinked by different and alternating β-glucosidic bonds (β-1,3; β-1,4 and β-1,6), resulting in a great variability in structural composition. It is well-established that many dietary β-glucans hold health promoting properties as they may reduce serum cholesterol levels and serve as prebiotics [1-3]. Recently, it has been proposed that β-glucans of both microbial and plant origin may hold immune modulatory properties [4;5]. The structure of the β-glucan largely depends on the origin of the β-glucan, e.g. branched β-(1,3)(1,6)-glucans are found in fungi and yeasts [6;7] while mixed linked β-(1,3)(1,4)-glucans are present in cereals such as barley and oat [8], or β-glucan may be straight β-(1,4)-glucans as cellulose or β-(1,3)-glucans as found in curdlan [9]. Thus, structural differences in β-glucans from microbial and plant origin exist and this may influence the immune modulatory properties.

Recognition of molecular motifs by the cells of the immune system is a key element in the mammalian defense against pathogens. The immune cells express different types of pattern recognition receptors (PRRs) on their surface including Toll like receptors (TLRs) recognising various microbial structures such as lipopolysaccaride (LPS) and peptidoglycan (PNG) and C-type lectins (CTLs) which recognise specific carbohydrates [10;11]. Upon ligation of the PRR, the cell responds by initiating the transcription of genes encoding cytokines and surface receptors of importance for the interaction with and signaling to other cells. The resulting response in the immune cell depends on the combination of PRRs that are being ligated as well as the cell type. β-Glucans from yeast and fungi have been demonstrated to bind to the CTL receptor dectin-1 as well as to Complement receptor CR3. In particular, β-1,3-glucan structures bind to dectin-1 [12;13], while the CR3 is more promiscuous, binding carbohydrates containing mannose, N-acetyl-D-glucosamine and glucose as well as non-carbohydrate molecules [14].

Antigen presenting cells of the innate immunity, are key cells in the recognition of microorganisms and foreign molecules and express a rich repertoire of PRRs, including the TLRs and CTLs. Of these cells, the dendritic cells (DCs) play a key role in initiation and orchestration of the innate as well as the adaptive immunity [15]. Through the ligation to PRRs, most bacteria and bacterial constituents are potent stimulators of cytokine production in the DCs. LPS is often used to assess the immune modulatory effect of microbes and bioactive molecules, but the use of other stimuli, e.g. other TLR ligands or whole bacteria

may also be used to stimulate the cells and may in fact induce different effects through different cellular mechanisms [16;17]. While many bacteria and microbial components induce maturation and cytokine production, some molecules of microbial as well as plant origin may act as antagonists when binding to the PRR. In particular ligation of CTLs has been suggested to down-regulate TLR induced cytokine production [13;18].

Although the plant derived β-glucan structures differ from those of microbial origin, it has been speculated whether the plant β-glucans are able to bind to the same receptors as the microbe derived β-glucans or to other receptors on immune cells. In fact, Brown and Gordon [12] demonstrated that β-(1,4) structures bound to dectin-1 tranfected HEK cells although the binding affinity was several fold lower than the binding of β-(1,3) and β-(1,6) structures. We have also recently shown that β-glucans purified from barley and oat modulated the cytokine production in LPS stimulated DCs albeit not as strongly as β-glucans of microbial origin [19]. The results indicated that linkage type, molecular mass and solubility impact the immune modulatory capacity of the β-glucans but the contribution of the different properties was not examined in details. Adams et al. [20] suggested that binding affinities of dectin-1 to various β-glucans vary according to the side-branching frequency and chain length of the polymer. In an immuno test system favouring complement activation via the alternative pathway aggregated barley β-glucan showed significant activity in contrast to a high molecular mass soluble β-glucan [21] whereas increased β-glucan immunological activity depending on higher β-(1,4)/β-(1,3) linkage ratios was indicated by Samuelsen et al. [22].

The present study specificly adress the physico–chemical properties for the immuno modulatory capacity of β-glucans from barley and oat. β-Glucans purified from different barley and oat varieties and characterised with respect to compositional, molecular, structural and rheological features [23] were tested for their ability to affect immune responses in DCs stimulated with either LPS or the probiotic bacterium *Lactobacillus acidophilus* NCFM.

Material and methods

Beta-glucan materials and sample preparations

A total of 19 concentrated barley and oat mixed linkage β-glucan samples along with curdlan (β-1,3), cellulose (β-1,4), lichenan (β-1,3; β-1,4) and yeast (β-1,3; β-1,6) reference β-glucans were tested. Sample compositional, structural and rheological properties were analysed by Mikkelsen et al. [23] and data are shown in Appendix A. Considerable variation in the β-

glucan structure of barley was found as compared to oat and low solubility of the barley β-glucan samples was ascribed the relative higher proportion of cellotriosyl units in barley β-glucans, which might form substructures consisting of longer repetitive cellotriosyl sequences that are prone to aggregate. Especially, the large-scale extracted barley (Bm1-4 and B1-4) β-glucans showed low solubility as compared to oat (O1-2) when using the AACC Total dietary fibre Method 32-07 where soluble (SDF) and insoluble dietary fibre (IDF) fractionation is based on filtration after β-glucan pre-solubilisation (35 min at 100°C). IDF is retained in the filtrate whereas SDF is comprised in the supernatant.

A schematic overview of the various β-glucan preparations tested in the immune assay is presented in Fig. 1. Dispensed β-glucan samples (1% v/w) were prepared from 20 mg sample in 2 mL distilled water and stirred for 30 min at 25°C. Additionally, a pre-solubilisation procedure was introduced to achieve standardised and fully dissolved β-glucans [23]. Solutions (1% w/v) were prepared from 20 mg sample in 2 mL distilled water with continuous stirring for 30 min at 80°C. The above corresponds to preparations used for experiment I. Furthermore, a subset of samples (Bm2, O1 and OM) was fractionated into SDF and IDF fractions using the AACC Method 32-07 until the filtration step. SDF and IDF preparations were tested in experiment II as dissolved or dispensed samples. Finally, the dissolved SDF fractions were either sedimented for 2 hours or centrifuged at 10,000 g to remove soluble aggregates and from this either the top fraction or supernatant was tested in stimulated DCs, which correspond to experiment III. All β-glucan samples were diluted × 30 prior to cell culturing.

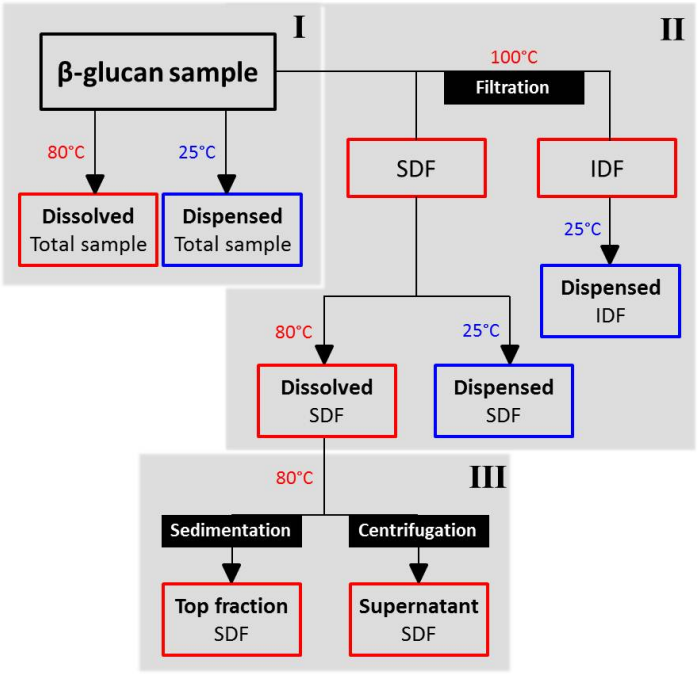


Fig. 1 β-Glucan sample preparation scheme with experimental work packages I, II and III. Dispensed and dissolved samples were prepared from stirring β-glucan into water for 30 min at 25°C or 80°C, respectively. Soluble and insoluble dietary fibres (SDF and IDF) were fractionated by filtration and SDF was either sedimented or centrifuged to remove aggregated β-glucan from the top fraction or supernatant, respectively. Arrows indicate tested fractions in the immune assay.

Stimulation of dendritic cells and cytokine quantification

Bone marrow cells were isolated from C57BL/6 mice (Taconic Europe, Denmark) as described previously [24]. To cultivate DCs, 10 mL cell suspension containing 2 $\times 10^5$ stem cells was seeded in 100-mm bacteriological petri dishes at day 0 (Greiner bio-one, Kremsmünster, Austria) and incubated for 8 days at 37°C and 5% CO_2. On day 3, additional 10 mL cell culture medium was added. At day 6, 8 mL cell culture medium was replaced by 9 mL fresh medium. On day 8, the non-adherent cells were gently pipetted from the petri dishes and centrifuged for 5 min at 280 g. The cells were resuspended in fresh cell culture medium without granulocyte/macrophage colonystimulating factor, and seeded in 48-well culture plates (Corning, Corning, NY) at 1×10^6 cells/500 µL well. DCs were incubated with the various β-glucans for 30 min prior to stimulating with LPS 1 µg/mL (*Escherichia coli* O26:B6; Sigma-Aldrich) or *L.acidophilus* NCFM 10 µg/mL. Cells added medium alone were used as untreated DCs. After stimulation for 18 hours, culture supernatants were collected and stored at -20°C until cytokine analysis. Cytokines IL-10 and IL-12 in culture supernatants were quantified using commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Data analysis

The variance structure of β-glucan physico-chemical data (Appendix A) and immunological responses was investigated by multivariate data analysis using principal component analysis (PCA) [25]. Interval Partial Least Squares Regression (iPLSR) [26] was performed in order to reveal the most important physico-chemical features correlated to the immunological responses. Prior to analysis, the data was auto-scaled and the reported model was validated using leave one out cross validation. The PCA and iPLSR was carried out using the PLS toolbox v.6.1. (Eigenvector Research Inc., Manson, WA, USA) as a routine for MatLab v.7.14.0.739 R2012a (Mathworks Inc., Natick, USA).

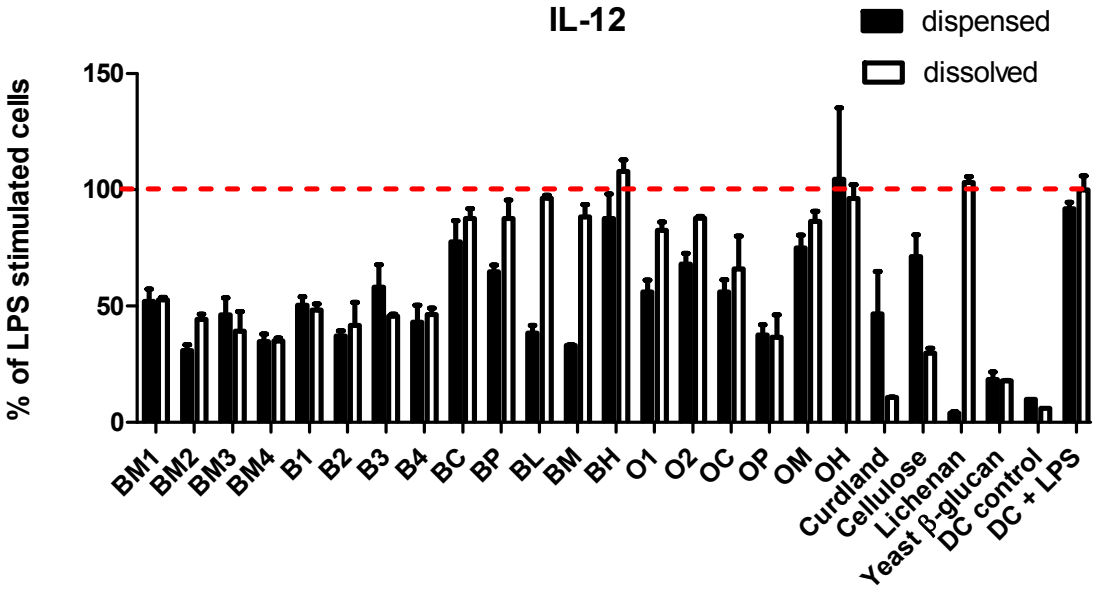
Results

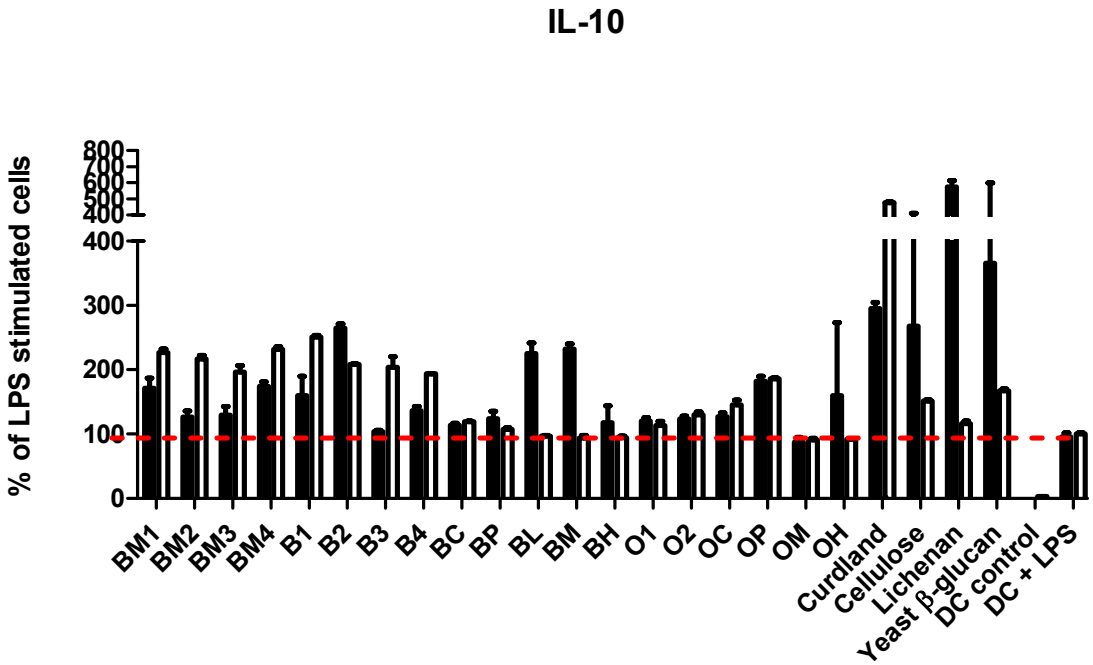
We have previously shown that β-glucan from barley and oat exhibit some immune modulatory effects on LPS stimulated DCs when dispensed in water resulting in an inhibition of the IL-12 production and a concomitant increase in IL-10 production [19]. In order to investigate in more details the role of physico-chemical parameters for this immune modulatory effect, a panel consisting of 23 β-glucan samples isolated from barley, oat, algae and various microorganism and characterised for structural-functional features (Appendix A) was investigated for their immune modulatory properties. For all samples it was examined if the immune modulatory property was affected by the microbial stimulus used to activate the cytokine production in the DCs. In addition to LPS stimulus which was previously used to assess the immune modulatory effects of β-glucan samples, we also investigated the modulatory effects of the β-glucans when the DCs were stimulated with whole gram positive bacteria, *L.acidophilus* NCFM (Fig. 2).

The following legend refers to the figure appearing at pages 6-8.

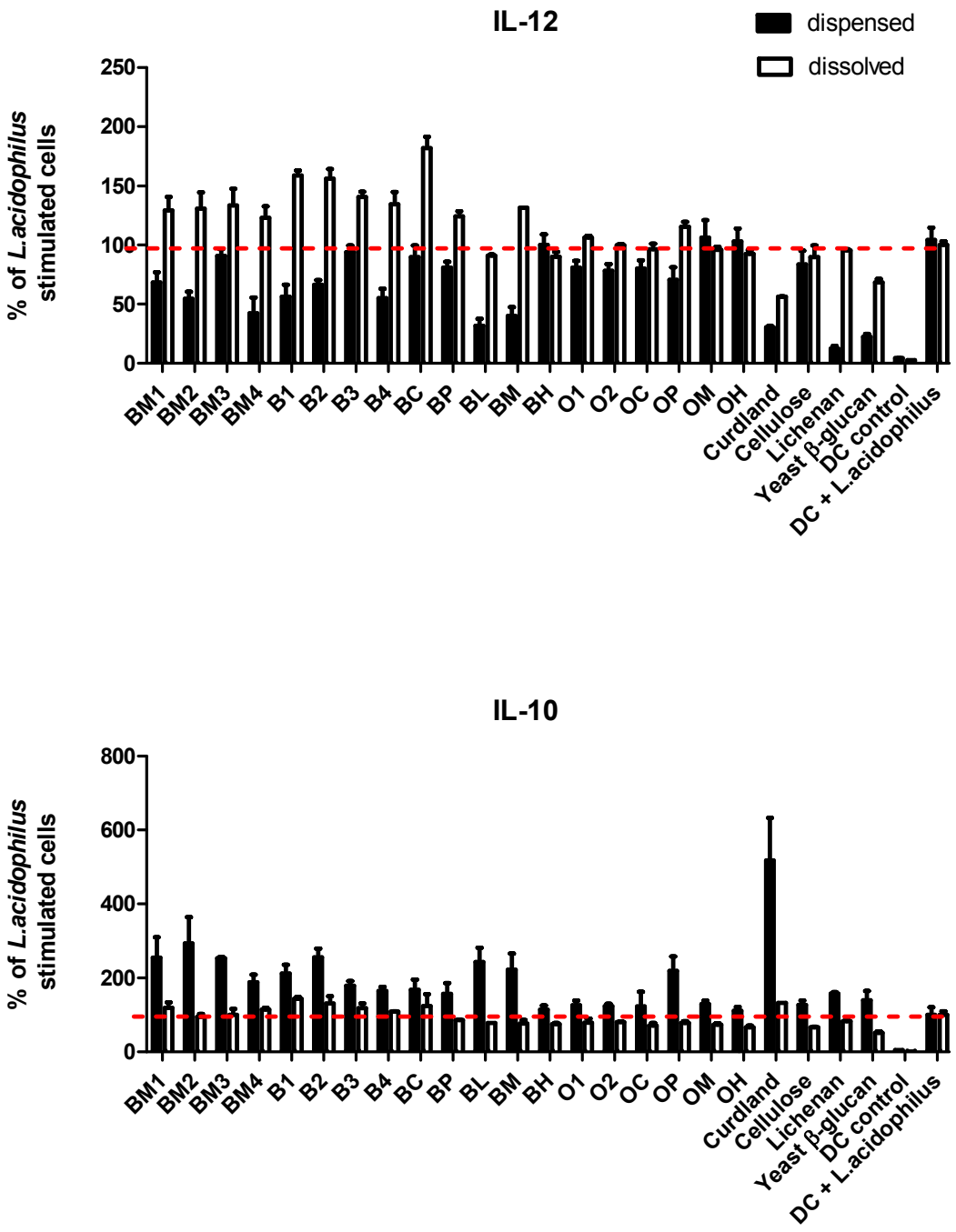
Fig. 2 Experiment I. Immune modulatory activity of β-glucan preparations from barley, oat or microbial sources. Dendritic cells were added the β-glucan samples prior to stimulation with LPS (A) or with live *L.acidophilus* NCFM (B). The production of the cytokines IL-12 (upper panels) and IL-10 (lower panels) was measured and is expressed as the percentages of the cytokine production of dendritic cells stimulated with *L.acidophilus* NCFM or LPS alone. Black bars depict the immune modulatory capacity of β-glucans samples dispensed in water and white bars when the samples were dissolved. The capacity of the β-glucan samples to stimulate cytokine production in dendritic cells *per se* is shown in (C).

A


IL-12
dispensed
dissolved
% of LPS stimulated cells
150
100
50
0
BM1
BM2
BM3
BM4
B1
B2
B3
B4
BC
BP
BL
BM
BH
O1
O2
OC
OP
OM
OH
Curdland
Cellulose
Lichenan
Yeast β-glucan
DC control
DC + LPS


IL-10
% of LPS stimulated cells
800
700
600
500
400
400
300
200
100
0
BM1
BM2
BM3
BM4
B1
B2
B3
B4
BC
BP
BL
BM
BH
O1
O2
OC
OP
OM
OH
Curdland
Cellulose
Lichenan
Yeast β-glucan
DC control
DC + LPS

B


IL-12
dispensed
dissolved
% of L.acidophilus stimulated cells
250
200
150
100
50
0
BM1
BM2
BM3
BM4
B1
B2
B3
B4
BC
BP
BL
BM
BH
O1
O2
OC
OP
OM
OH
Curdland
Cellulose
Lichenan
Yeast β-glucan
DC control
DC + L.acidophilus
IL-10
% of L.acidophilus stimulated cells
800
600
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BM1
BM2
BM3
BM4
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BL
BM
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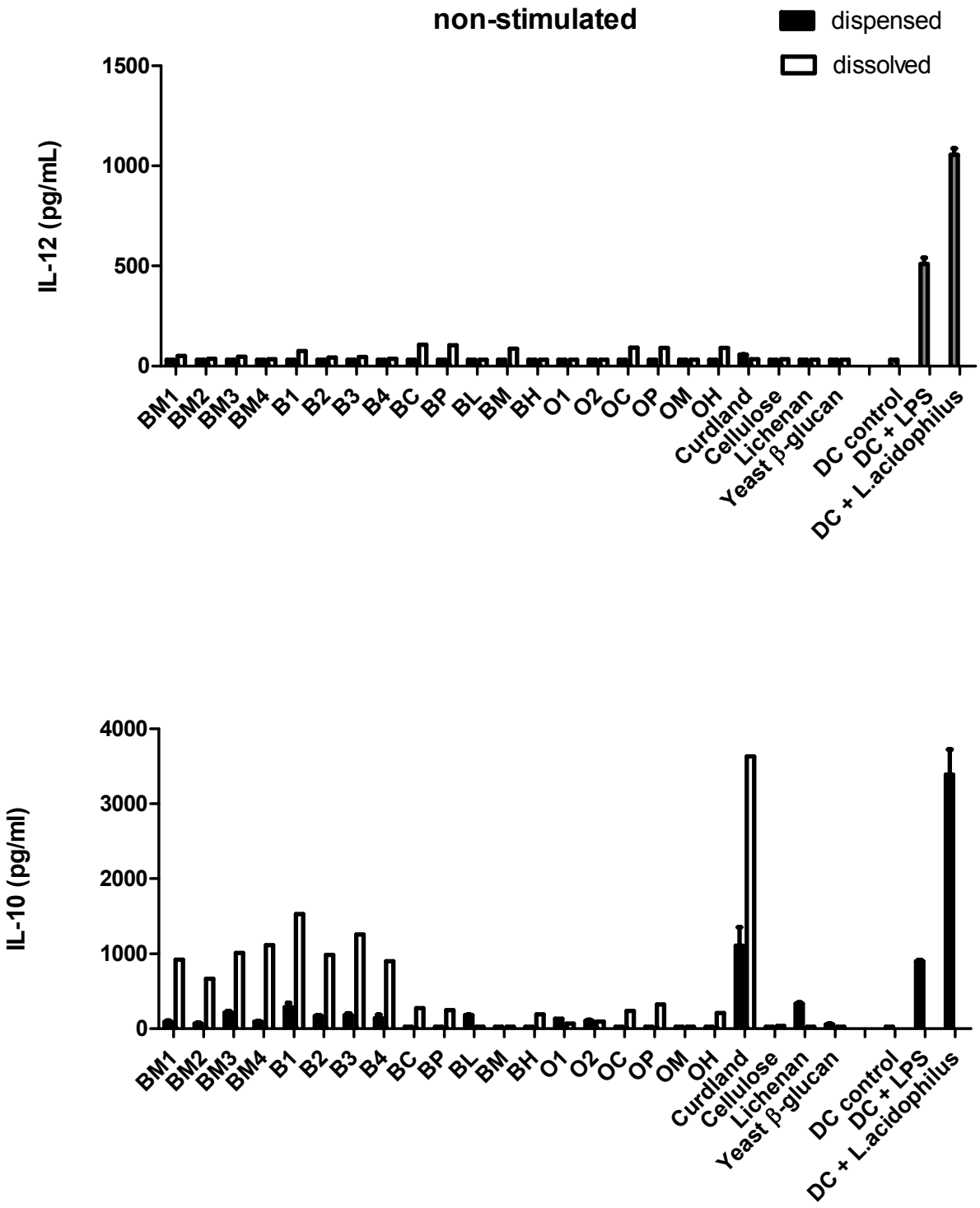
OH
Curdland
Cellulose
Lichenan
Yeast β-glucan
DC control
DC + L.acidophilus

C


non-stimulated
dispensed
dissolved
IL-12 (pg/mL)
1500
1000
500
0
BM1
BM2
BM3
BM4
B1
B2
B3
B4
BC
BP
BL
BM
BH
O1
O2
OC
OP
OM
OH
Curdland
Cellulose
Lichenan
Yeast β-glucan
DC control
DC + LPS
DC + L.acidophilus
IL-10 (pg/ml)
4000
3000
2000
1000
0

Effect of β-glucan preparation on immuno modulation

The procedure for preparation of the β-glucans for testing in the cellular system influenced the modulatory capacity of the β-glucans in microbially stimulated DCs (Experiment I). All β-glucan samples exhibited inhibitory activity on the LPS induced IL-12 production when added in a water dispensed form. When added after being solubilised (80ºC for 30 min), however, the IL-12 inhibitory capacity of some of the samples changed dramatically (Fig. 2A, upper panel). Also, the solubilisation procedure had highly diverse effects on the different β-glucans capacity to affect the LPS induced IL-10 production (Fig. 2A, lower panel). Pre-solubilisation of β-glucans significantly increased the IL-10 production in cells treated with Bm1-4 and B1-4 samples of low solubility suggesting an incomplete hydration of these fibres in the dispensed samples. Highly soluble BL and BM samples along with cellulose, lichenan and yeast β-glucans did not or only modestly affect the IL-10 production after the standardised solubilisation as compared to their dispensed counterparts which might indicate the presence of lumps or sediments as a source of variation in the dispensed samples. The solubilisation procedure especially affected the modulatory capability of Bm1-4 and B1-4 barley samples in the *L.acidophilus* NCMF stimulated DCs (Fig. 2B). The IL-12 production in cells treated with Bm1-4 and B1-4 β-glucans was inhibited by dispensed, however, stimulated by dissolved samples and the significantly increased IL-10 production as an effect of dispensed samples was almost eliminated in dissolved samples. In the PCA score plot on the barley and oat β-glucan physico-chemical properties and immunological responses (Fig. 3A) a mismatch is observed between the modulatory capacities of the dispensed (blue) and dissolved (red) samples as manifested by a non-systematic varying distance between replicate samples. This indicates the importance of the standardised pre-solubilisation procedure introduced to the dissolved samples for correct interpretation of β-glucan immune modulatory functionality and further interpretation of the results will be founded on the dissolved samples.

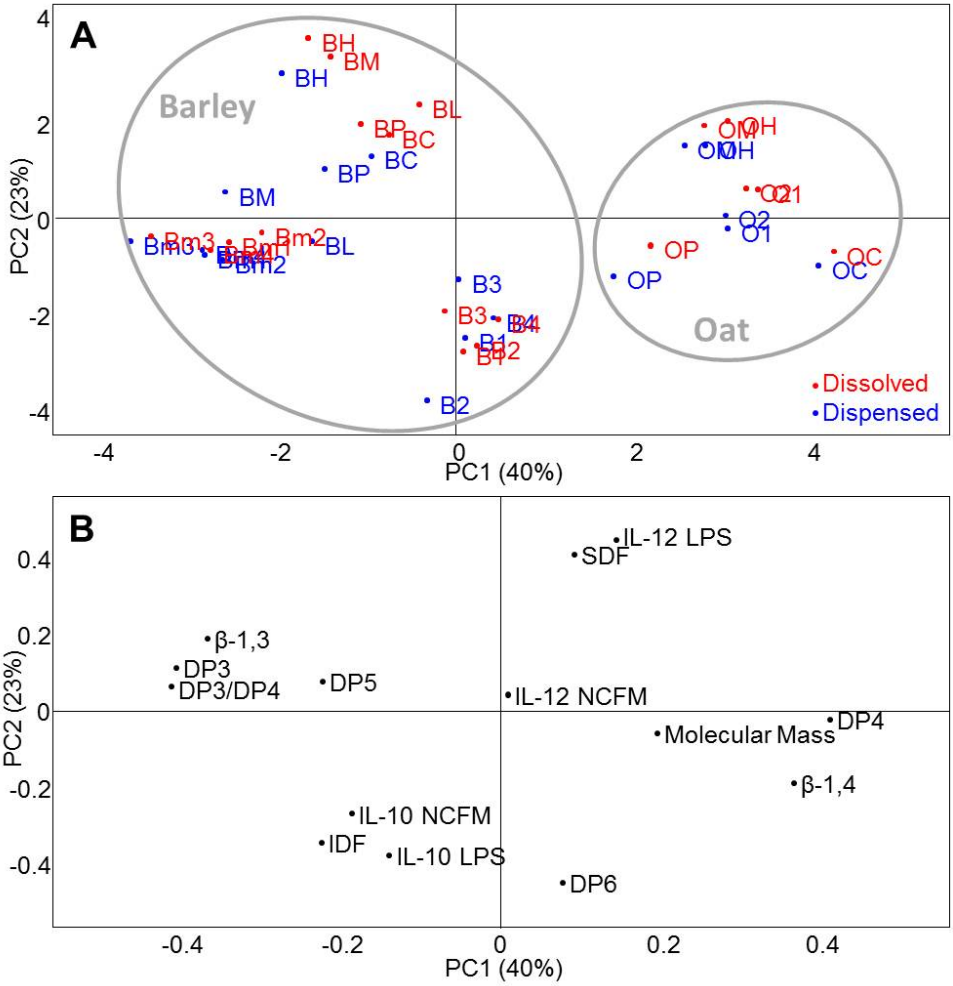


Fig. 3 PCA score (A) and loading (B) plot based on the auto-scaled physico-chemical properties of the barley and oat β-glucan samples (Appendix A) and immunological responses of dissolved and dispensed β-glucans. The first two components explain 63% of the data variation.

Influence of stimulant on immune modulatory properties

As demonstrated above, the cereal β-glucan preparations were found to modulate the LPS-induced cytokine production in DCs (Experiment I). Generally, the IL-12 levels decreased whereas the IL-10 levels increased (Fig. 2A, white bars), which is in agreement with previous findings [19;27]. In order to study if the stimulatory agent influences the immune modulatory effect of the β-glucan, DCs were alternatively stimulated with *L.acidophilus* NCFM. We have previously studied the immune stimulatory properties of this probiotic bacterium thoroughly and shown that it is a very strong inducer of IL-12 and employs cellular mechanisms distinct from LPS to induce IL-12 [16;28]. As in LPS stimulated cells, cereal β-glucans proved to modulate the *L.acidophilus* NCMF cytokine production in DCs where IL-12 cytokines were generally up-regulated and IL-10 levels showed insignificant changes as compared to the control (Fig. 2B, white bars). Generally, the *L.acidophilus* NCMF induced cytokine

production seemed to be more sensitive to the potential solution conformational differences of dispensed and dissolved samples as exemplified by Bm and B samples. Figure 2C depicts the immune stimulatory effects of the samples *per se*. Even though this revealed some dependency as to whether the samples were dispensed or dissolved, overall the samples except for curdlan showed no or only modest cytokine stimulatory capacities. Whereas none of the dissolved samples were capable of inducing a IL-12 production above 100 pg/mL (corresponding to less than 3 times the detection limit), Bm and B β-glucan samples induced an IL-10 production corresponding to that induced by LPS (900 pg/mL) and curdlan induced an IL-10 production slightly higher than that induced by *L.acidophilus* NCFM (3,300 pg/mL).

Effect of molecular structure, solubility and aggregation of β-glucans on modulatory capacity

The cereal β-glucan preparations generally exhibited great variability in their capacity to modulate the LPS and *L.acidophilus* NCFM induced cytokine production in DCs (Experiment I). This shows in the PCA score plot in Fig. 3 where barley and oat samples divide according to crop type (Fig. 3.A) and inspection of the loading plot (Fig. 3.B) confirms that the main variance among samples (PC1, 40%) can be ascribed to β-glucan block structural differences. The immunological variables and solubility features of the different β-glucans are spanned along PC2, explaining 23% of the systematic variance. The apparent correlation between responses to different stimuli and β-glucan solubility was further investigated in a PCA performed solely on data from the dissolved β-glucans. The combined score and loading plot in Fig. 4 shows that the β-glucan samples are systematically distributed between the stimulant and cytokine variables according to their content of insoluble fibres (PC1, 73%). Samples Bm1-4 and B1-4, high in IDF, are located near IL-10 LPS, IL-10 NCFM and IL-12 NCFM loadings corresponding to the up-regulating immune modulatory capacity of these samples. The IL-12 LPS loading is located opposite to the other cytokine responses due to the inhibitory activity of β-glucans on this cytokine.

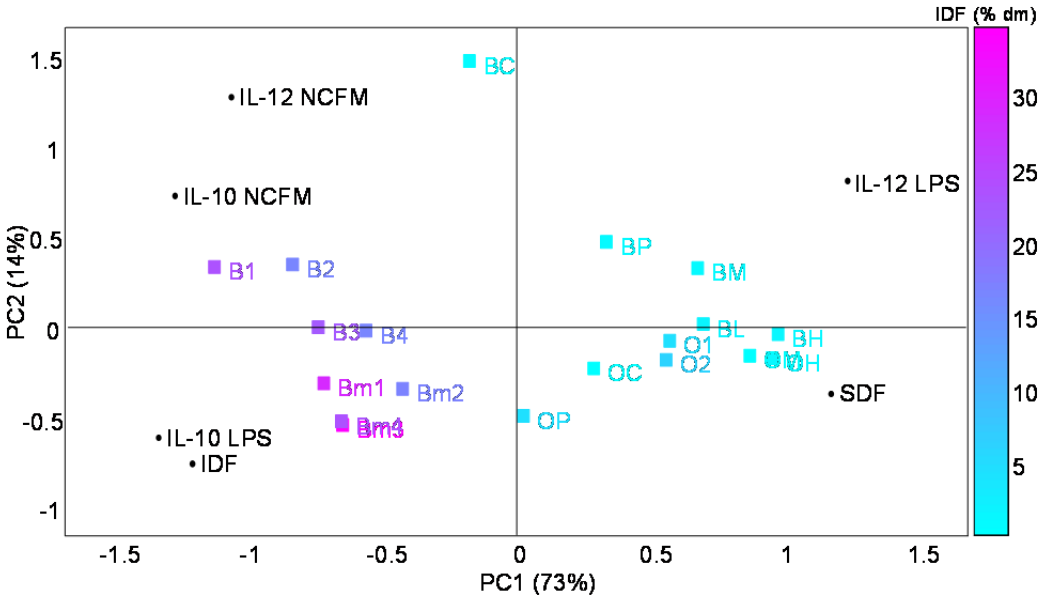


Fig. 4 PCA bi-plot based on the auto-scaled immunological responses and solubility properties of the dissolved barley and oat β-glucan samples. Samples are coloured according to content of insoluble dietary fibre (IDF, % dm). The first two components explain 87% of the data variation.

As β-glucan immune regulatory functionality has been speculated to relate to the structural basis of the polysaccharides, iPLSR variable selection was performed in order to reveal the most important β-glucan physico-chemical features correlated to the immunological responses. The result of the iPLSR of the barley and oat β-glucan samples is presented in Fig. 5. The calibration errors (RMSECV) for each of the 16 physico-chemical variables (Appendix A) are presented as bars and the calibration errors of two global models (1 and 2 latent variables) are seen as horizontal dotted lines (Fig. 5.A). All models were calculated using one PLSR component and IDF could be identified as the variable with the lowest RMSECV and hence as the most influential physico-chemical descriptor for prediction of the β-glucan immunological responses. This was further validated in Fig. 5.B., which shows that the β-glucan IDF content and the immune modulatory capacity on IL-10 LPS are highly correlated features. Inspection of correlations between β-glucan structural features (DP3/DP4 ratio, β-linkage, molecular mass) and immune modulatory effects did not reveal any relation (data not shown).

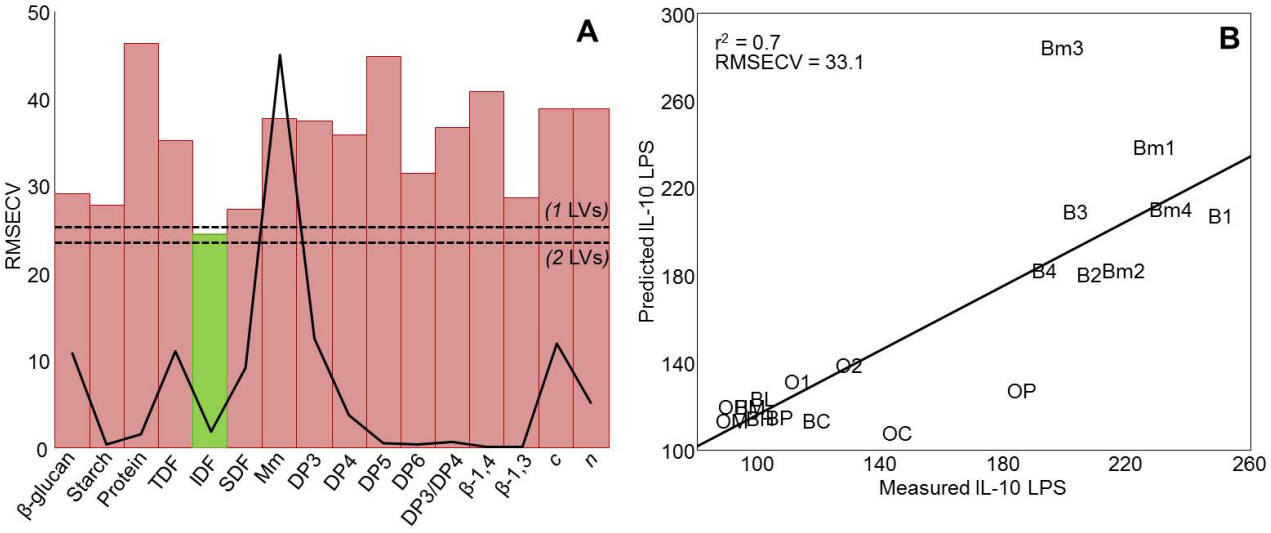


Fig. 5 Interval PLSR result (A) and measured versus predicted plot (B) of β-glucan IDF content prediction of IL-10 LPS immunological response. Horizontal lines in (A) show the performance of the full PLS model with one respective two components. The green variable IDF show the lowest PLSR prediction error to IL-10 LPS. This model is shown in (B), which have a Pearson correlation coefficient of 0.7.

Hromadkova and co-workers [29] found that β-glucan immune modulatory activities are highly dependent on the microstructure, which the polysaccharide achieves during the various isolation and drying processes. To further evaluate the importance of β-glucan fine structure on immune modulatory properties, results from equally processed β-glucans; Bm1-4, B1-4 and O1-2 were compared. As seen in Fig. 6, a general correlation between β-glucan block structural characteristics (DP3/DP4 ratio) and IL-10 LPS response is indicated. This is in good agreement with the suggested relation between β-glucan block structure and solubility properties and in turn the close relation between Bm samples, IDF content and IL-10 LPS response seen in Fig. 4. There was no correlation found between β-glucan block structure and other cytokine responses.

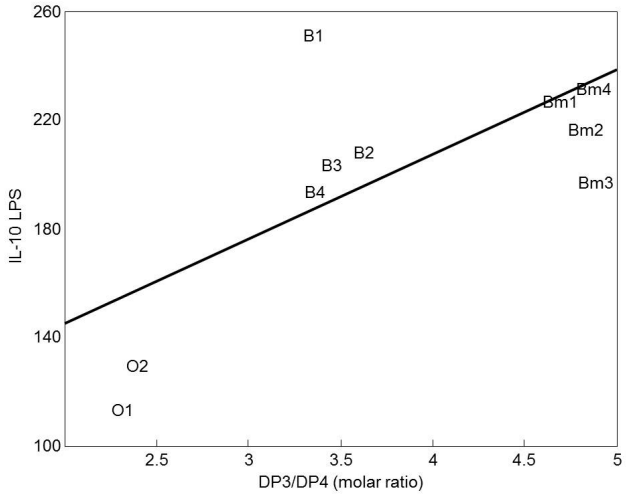


Fig. 6 Correlation between β-glucan block structure (DP3/DP4 ratio) and IL-10 LPS response.

High DP3/DP4 ratios are generally associated with β-glucan insolubility and aggregation tendency [30] and aggregated species were related to increased immune stimulatory activities as compared to soluble β-glucan [21]. The first experiment (I), did not address if the modulatory activity was related to the soluble or the insoluble fraction. Moreover, as there was a fine correlation between the solubility of the sample and its immune modulatory activity, it was hypothesised that samples with a high proportion of insoluble matter also contain a high amount of aggregates of β-glucans in solution. To investigate if the higher effect from Bm samples, high in DP3/DP4 ratio, depend on aggregated species, three approximately equally sized β-glucans (Bm2, O1 and OM) with different solubility were fractionated into SDF and IDF and tested as dispensed or dissolved polysaccharides (Experiment II). Results from SDF and IDF Bm2 samples are shown in Fig. 7A. A significant modulatory effect of Bm2 is seen for two SDF samples as compared to IDF fraction with the dissolved SDF showing the strongest effect. In accordance with previous data on solubilisation, OM and O1 did not result in appreciable IDF fractions by filtration. Moreover, OM and O1 SDF samples showed a weaker immune modulatory activity as compared to Bm2 and they showed only minor differences between the dissolved and dispensed SDF samples of experiment II (data not shown).

To remove potential aggregates from the dissolved SDF sample, it was allowed to sediment or centrifuged and the top fraction or supernatant, respectively, were assessed for immune modulatory effects on LPS stimulated DCs (Experiment III). By the centrifugation, it was expected that aggregates of a size that allows them to solubilise are removed, while by the

sedimentation procedure such aggregates will stay in solution. As observed in Fig. 7B, centrifugation of the dissolved SDF samples strongly reduced the immune modulatory activity of the samples while this was retained in the top fraction after sedimentation of all three samples.

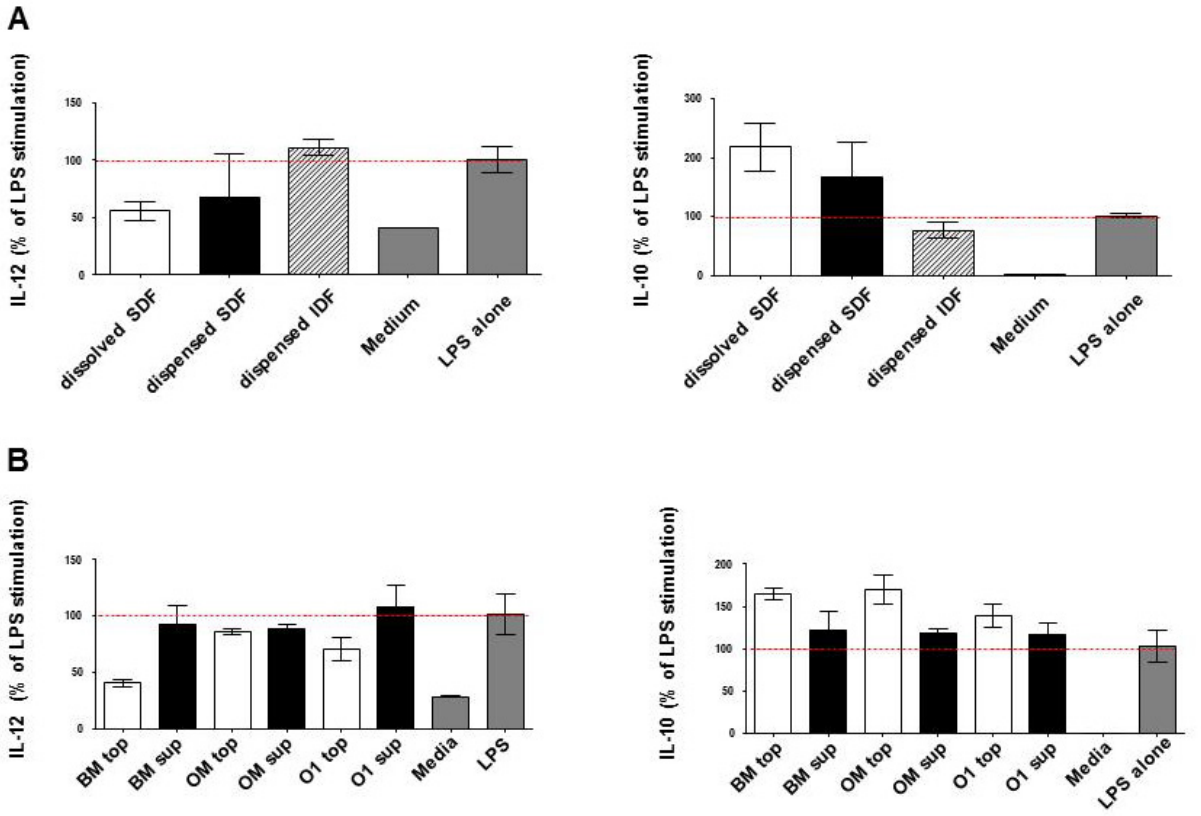


Fig. 7 (A) Experiment II. Immuno modulatory effect of Bm2 insoluble (IDF) dispensed and soluble (SDF) fractions that are dissolved or dispensed. (B) Experiment III. Immuno modulatory effects of Bm2, OM and O1 SDF samples. Samples were dissolved at 80ºC and subsequently allowed to sediment for 2 hours (top) or centrifuged at 10,000 g (sup). For all experiments, the β-glucan samples were added to DCs 30 min prior to stimulation with LPS or *L.acidophilus* NCFM and IL-10 and IL-12 were analysed in the supernatants harvested 18 hours post stimulation.

Discussion

The aim of the this study was to address the importance of structure-functional relationship for immuno regulatory capacity in DC amongst mixed linkage barley and oat β-glucans that are part of our common foods and claimed to lower blood cholesterol levels and reduce the risk of heart diseases [31;32]. A total of 19 fully characterised barley and oat β-glucans was compared in order to identify potential physico-chemical properties that may affect the immune modulatory efficiency.

In fungal β-glucans, molecular mass, fine structure, solubility and polymer conformation in solution influence immunological responses [33]. These are parameters that are highly related to each other and vary even within the same β-glucan sample thus making the establishment of structure-functional relationships particularly difficult. The molecular mass and fine structure of cereal β-glucans influence their solubility and conformation in solution [30;34] and solubilised β-glucan has been shown to consist of a rather heterogeneous group of polysaccharide conformations: low molar mass species with elongated single chain conformation, intermediate size species with random coil conformation and high molar mass species consisting of fringed micelle aggregates [35]. Regular mixed linkage β-glucans form helical structures introducing more kinks when increasing the ratio of β-(1,3) linkages, which initially will prevent the polymer from aggregating and provide a more water soluble polymer whereas pure β-(1,3) polymers will aggregate and precipitate. In case of regular cellotriosyl and cellotetrasyl block structures the mixed linkage β-glucan will adopt open helical forms, which can promote interchain interactions and introduce gel-like functionality [36]. More irregular β-glucans in solution are generally expected to behave like radom coil polysaccharides [37], but Hromadkova et al. [29] found that supramolecular aggregated species remain present even in very dilute β-glucan solutions.

In this study it was found that DC modulatory potency is highly influenced by the β-glucan preparation. The dissolved samples gave rise to a slighly higher IL-10 production on the LPS stimulated DCs, which may be due to an increased β-glucan concentration from the pre-solubilisation procedure. Interestingly, the effect from the pre-solubilisation had the opposite consequence for IL-10 NCFM where responses decreased with more β-glucan available in solution. Dissolved samples, especially Bm and B of low solubility, up-regulated while the dispensed samples down-regulated the *L.acidophilus* NCFM induced IL-12 response. These disparate effects of the two differently prepared samples indicate that solubility is a key determinant of the immune modulatory properties of the β-glucans and moreover, that especially stimulation with *L.acidophilus* NCFM seems to be influenced by the presence of insoluble matter. This underlines the important role of the immune stimulatory agents, which may employ distinct mechanism to induce the cytokines as previously reported [16].

Both high and low molecular mass β-glucans have shown immuno activating properties *in vitro* and *in vivo*. Some authors suggest that β-glucan *in vivo* activity dependent on intestinal uptake might be favouring high molecular mass β-glucans [5;38], while others suggest that *in*

vitro functionality related to receptor interaction might favour lower molecular mass fractions [39;40]. In the present study, the β-glucans molecular masses did not differ significantly (130-410 kDa) and no correlation was found between immune reposes and polymer size.

The extent to which β-glucan modulated the DC immune responses was found to depend on the β-glucan solubility. β-Glucans of low solubility exhibited the highest activities. This is in accordance with a pre-study where DCs were treated with SDF only and reduced or no modulatory effect was found. This result suggests that soluble β-glucan is not a prerequisite for bioactivity in immune cells [19] as indicated in various *in vivo* human studies investigating the efficiency of cereal β-glucans to modulate the risk of cardiovascular disease [41]. Higher DP3/DP4 ratios of equivalently processed and equally sized barley and oat β-glucans (Bm>B>O) showed to correlate with increased IL-10 LPS cytokine production. Likewise, a greater amount of β-(1,3) linkages (Bm>B>O) possessed higher immune modulating capacity (data not shown). This is in good agreement with the documented higher binding affinity of β-(1,3) structures to the dectin-1 receptor as compared to β-(1,4) [12] and supported by the fact that curdlan (100% β-(1,3)) and lichenan (70% β-(1,3)) were found among the most potent β-glucans tested in the present study. In contrast, cellulose (pure β-(1,4) linkages) did only modestly affect the production of IL- 10 and IL-12.

Elevated immunological activity has been associated with β-glucan high frequency of side-branching [20] and aggregation [21], which are molecular features contributing to an extended polysaccharide conformation in solution. Thus, the high immune responses of the Bm and B β-glucans of low solubility is speculated to arise from fringed micelle structures of the polymer aggreagtes favoured by the DC receptors as compared to receptor interaction with single chain polysaccharides. Comparison between the effects of dissolved SDF samples that were either allowed to sediment or were centrifuged established that the immune modulatory components in the solutions, although forming stable soluble aggregates, are so large that they precipitate at 10,000 g (Experiment III), which supports our hypothesis. It has previously been demonstrated that interaction between yeast β-glucan and dectin-1 receptors results in aggregation of multiple dectin-1 receptors in the cell membrane, in turn giving rise to changes in the β-actin skeleton and intracellular signalling [42]. A hypothetical presentation of mixed linkage β-glucan interaction with DC receptors is presented in Fig. 8.

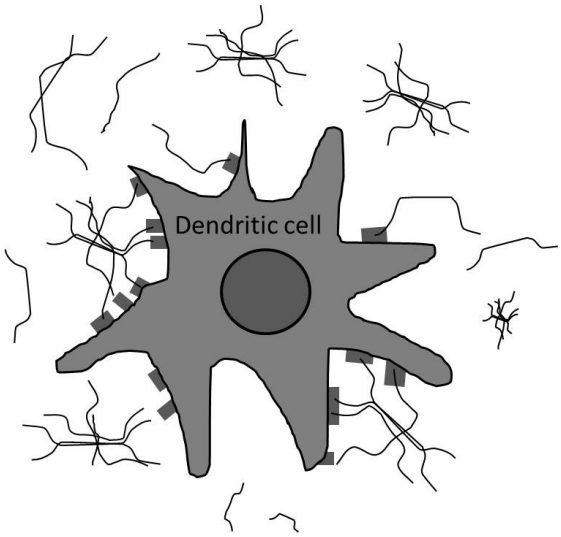


Fig. 8 Hypothetical illustration of mixed linkage cereal β-glucan interactions with dendritic cell receptors. Various β-glucan polymer conformations are possible: elongated single chains, radom coils and aggregates in a fringed micelle structure.

The study also shows that the IDF of Bm2 alone did not possess any immune modulatory activity in LPS stimulated DCs while both dispensed and dissolved SDF did. In addition, a strong inverse correlation between the solubility of a sample and its immune modulatory potential was found. Together, the results indicate that the low solubility not only gives rise in a high proportion of IDF but also that more aggregates are present in the dissolved fraction.
Bm2, O1 and OM samples varying in solubility (OM>O1>Bm) was fractionated into SDF and IDF, but only Bm2 gave rise to appreciable amounts of IDF reflecting the lower solubility of this sample compared to the oat samples. Moreover, the immunological activity of dissolved SDF from Bm was higher as compared to the dissolved SDF from the oat samples. In general no differences in cytokine modulating effects were seen between high SDF barley and oat β-glucans as suggested elsewhere [19], which indicate that small structural differences in the β-(1,3) to β-(1,4) linkage ratios and hence specific dectin-1 receptor interaction is of minor importance compared to the β-glucan aggregate structure in solution for DC interaction with β-glucans. Together, this suggests that the dissolved oat samples contain less aggregated species than the Bm2 sample and accordingly, despite that monomeric or oligomeric β-glucans may be able to bind to the cell receptor, this may not cause significant immune modulation (Fig. 8).

In conclusion, our study demonstrated, that the extent to which mixed linkage cereal β-glucans modulate the immune response is found to vary with the sample preparation, β-glucan solubility and fine structure and in turn the polysaccharide aggregation in solution.

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Appendix A. Compositional, molecular, structural and rheological features of β-glucan samples. From Mikkelsen et al. [23]

	Sample name	Specification	β-glucan (% dm)	Starch (% dm)	Protein (% dm)	TDF (% dm)	IDF (% dm)	SDF (% dm)	Molecular mass (kDa)	DP3 (molar ratio)	DP4 (molar ratio)	DP5 (molar ratio)	DP6 (molar ratio)	DP3/DP4 (molar ratio)	Linkage ratio (% β-1,4)	Linkage ratio (% β-1,3)	Consistency coefficient, *c* (Pa s)	Flow behavior index, *n*
Barley	Bm1	Mutant line	59.8 ± 0.3	4.8 ± 0.0	3.8 ± 0.0	69.5 ± 3.3	29.3 ± 0.9	40.2 ±2.9	270	78.8	17.2	2.8	1.2	4.6	69.6	30.4	0.18	0.43
	Bm2		56.0 ± 0.3	5.9 ± 0.1	3.7 ± 0.0	63.4 ± 7.7	17.3 ± 2.8	46.1 ± 7.1	280	78.6	16.7	3.0	1.7	4.7	69.8	30.2	0.23	0.47
	Bm3		61.8 ± 1.7	6.2 ± 0.3	4.0 ± 0.0	79.4 ± 8.1	34.7 ± 4.8	44.7 ± 7.2	170	79.4	16.3	2.8	1.5	4.9	69.1	30.9	0.08	0.61
	Bm4		54.0 ± 3.9	5.1 ± 0.0	3.8 ± 0.0	72.1 ± 11.8	23.8 ± 2.6	48.3 ± 9.9	290	78.9	16.6	3.0	1.6	4.8	69.5	30.5	0.35	0.31
	B1	Mother line	45.3 ± 2.0	18.3 ± 1.5	3.9 ± 0.0	39.9 ± 4.2	23.8 ± 1.0	18.5 ± 1.1	260	71.8	21.9	2.7	3.5	3.3	70.8	29.2	0.02	0.87
	B2		44.8 ± 0.6	21.1 ± 0.5	3.5 ± 0.0	44.3 ± 7.9	16.8 ± 1.0	27.6 ± 8.1	260	72.4	20.4	2.5	4.8	3.6	70.8	29.2	0.01	0.80
	B3		47.2 ± 1.5	16.6 ± 0.5	3.9 ± 0.1	48.8 ± 8.4	22.8 ± 0.4	26.0 ± 8.0	270	72.6	21.5	2.7	3.1	3.4	70.5	29.5	0.05	0.67
	B4		47.1 ± 0.6	18.2 ±0.0	3.4 ± 0.0	41.4 ± 2.4	16.9 ± 1.3	24.5 ± 3.4	250	71.5	21.8	2.9	3.9	3.3	70.9	29.1	0.02	0.82
	BC	Crude	64.3 ± 0.5	10.0 ± 0.0	4.8 ± 0.1	41.6 ± 15.5	0.8 ± 0.9	40.9 ± 15.4	130	76.4	19.5	3.2	0.9	3.9	70.2	29.8	0.01	0.97
	BP	Pure	81.1 ±8.9	1.8 ± 0.0	1.8 ± 0.0	38.5 ± 4.3	1.1 ± 1.4	37.7 ±4.6	130	78.3	18.0	2.8	0.9	4.4	70.1	29.9	0.01	0.99
	BL	Low viscosity	81.9 ± 0.2	0.9 ± 0.0	0.2 ± 0.0	57.8 ± 27.2	1.7 ± 1.5	56.1 ± 28.7	140	77.3	19.3	2.6	0.8	4.0	70.1	29.9	0.01	0.98
	BM	Med. viscosity	88.2 ± 3.7	0.9 ± 0.0	0.3 ± 0.1	94.5 ± 3.7	1.2 ± 0.7	93.3 ± 3.0	200	75.7	19.0	4.5	0.9	4.0	69.5	30.5	0.03	0.98
	BH	High viscosity	84.8 ± 1.5	0.9 ± 0.0	0.2 ± 0.0	91.5 ± 14.7	1.4 ± 0.1	90.1 ± 14.8	390	79.8	16.8	2.5	0.9	4.8	68.7	31.3	0.48	0.86
Oats	O1		72.5 ± 0.8	5.7 ± 0.2	1.4 ± 0.0	81.5 ± 5.0	4.6 ± 3.4	76.9 ± 2.0	270	65.8	29.4	2.5	2.3	2.2	71.3	28.7	0.06	0.95
	O2		70.5 ± 1.2	7.9 ± 0.1	1.4 ± 0.0	85.2 ± 2.9	6.5 ± 2.1	78.7 ± 2.3	340	66.5	28.7	2.7	2.1	2.3	71.3	28.7	0.05	0.95
	OC	Crude	31.9 ± 1.7	43.1 ± 0.8	5.2 ± 0.1	25.4 ± 5.2	0.3 ± 0.3	25.3 ± 4.9	410	64.5	31.6	2.5	1.4	2.0	71.8	28.2	0.02	0.89
	OP	Pure	67.0 ± 1.0	7.3 ± 0.2	2.5 ± 0.0	66.2 ± 15.2	4.8 ± 3.1	63.0 ± 15.2	410	67.2	28.6	2.1	2.1	2.4	70.2	29.8	0.17	0.90
	OM	Med. viscosity	81.4 ± 1.1	0.8 ± 0.0	0.1 ± 0.0	96.5 ± 2.1	0.3 ± 0.5	96.5 ± 3.0	190	69.0	27.5	2.4	1.2	2.5	71.1	28.9	0.03	0.97
	OH	High viscosity	81.2 ± 2.8	0.8 ± 0.0	0.2 ± 0.0	91.6 ± 7.9	1.5 ± 2.2	90.4 ± 5.1	380	68.5	28.3	2.0	1.2	2.4	70.6	29.4	0.25	0.91
	Crd	$Curdlan_{1,3\text{-}BG}$	0.3 ± 0.3	0.8 ± 0.0	1.3 ± 0.0	94.7 ± 0.3	94.2 ± 2.3	1.2 ± 1.7	-	-	-	-	-	-	-	-	-	-
	Cell	$Cellulose_{1,4\text{-}BG}$	0.1 ± 0.0	0.8 ± 0.0	0.1 ± 0.0	74.2 ± 10.0	75.7 ± 8.1	0.0 ± 0.0	-	-	-	-	-	-	-	-	-	-
	Lich	$Lichenan_{1,3/1,4\text{-}BG}$	69.9 ±1.7	0.9 ± 0.0	0.9 ± 0.1	43.0 ± 14.7	0.0 ± 0.0	44.7 ± 15.6	-	87.2	3.9	7.1	1.8	22.4	70.8	29.2	0.00	1.04
	Y	$Yeast_{1,3/1,6\text{-}BG}$	0.8 ± 0.1	0.8 ± 0.0	1.3 ± 0.2	87.8 ± 11.1	7.7 ± 0.5	80.1 ± 10.6	-	-	-	-	-	-	-	-	-	-

Total Dietary Fiber (TDF), Insoluble Dietary Fiber (IDF), Soluble Dietary Fiber (SDF), Degree of Polymerisation (DP).

Paper V

Ibrügger S, Kristensen M, Poulsen MW, Mikkelsen MS, Ejsing J, Knudsen KEB, Jespersen BM, Dragsted LO, Engelsen SE, Bügel S

Effect of oat and barley β-glucans on cholesterol metabolism in young healthy adults: a randomized controlled trial

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Effect of oat and barley β-glucans on cholesterol metabolism in young healthy adults: a randomized controlled trial

Sabine Ibrügger[1], Mette Kristensen[1*], Malene Wibe Poulsen[1], Mette Skau Mikkelsen[2], Johnny Ejsing[1], Knud Erik Bach Knudsen[3], Birthe Møller Jespersen[2], Lars Ove Dragsted[1], Søren Balling Engelsen[2], Susanne Bügel[1]

[1]Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, Denmark; [2]Quality & Technology, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Denmark; [3]Department of Animal Science, Aarhus University, Denmark.

*Corresponding author: Mette Kristensen, PhD; Tel: +45 3533 3536; Fax: +45 3533 2483; E-mail: mekr@life.ku.dk

ABSTRACT

Background β-glucans are known to exhibit hypocholesterolemic effects. Increased intestinal viscosity, as a function of β-glucan concentration and molecular mass, may be crucial for cholesterol-lowering effect. Structural differences, including the ratio of (1→3) to (1→4) glucan bonds (DP3:DP4 ratio) between β-glucans, may also be of importance for β-glucan functionality. **Objective** This work investigates the effects of three different β-glucan sources on blood lipids and fecal endpoints. **Design** Fourteen participants (8F/6M) completed this randomized, crossover, single-blinded study with four 3-wk periods with 3.3g/d of fibers: control and oat, barley, and barley mutant β-glucan of similar molecular mass. Before and after each period fasting and postprandial blood samples were drawn and 3-d fecal collections were made. **Results** No effect of treatment was observed for changes in total, LDL, and HDL cholesterol compared to control; however, consumption of 3.3g oat β-glucans for three weeks resulted in lower total (-0.29±0.09mmol/L; $P<0.01$), LDL (-0.23±0.07mmol/L, $P<0.01$), and HDL cholesterol (-0.05±0.0.3mmol/L, $P<0.05$) compared to baseline. No relationship between the β-glucan structure (DP3:DP4) and changes in LDL cholesterol could be established. Overall, treatment affected changes in fasting triacylglycerol ($P=0.05$) as well as total SCFA and acetate ($P<0.01$). Fecal dry and wet weight, stool frequency, fecal pH and energy excretion was not affected. **Conclusions** The results do not fully support the hypocholesterolemic effects by differently structured oat and barley β-glucans. However, a greater potential was observed for oat β-glucan, presumably due to higher solubility and viscosity. This underlines the importance of elusive structural β-glucan features for beneficial physiological effects.

This trial was registered at www.clinicaltrial.gov as NCT 01317264.
The study was carried out as a part of the research program of the UNIK: Food, Fitness & Pharma for Health and Disease. The UNIK project is supported by the Danish Ministry of Science, Technology and Innovation. The study was also supported by the strategic research program "BEST" at Faculty of Science, University of Copenhagen. Denmark. All authors declared no conflict of interest.

INTRODUCTION

Dietary fibers have been recognized for their positive effects on health for a long time (1). Observational studies have shown an association between dietary fiber intake and a reduced risk for the development of cardiovascular diseases (CVD) (2). This is partly due to their hypocholesterolemic properties, observed especially for soluble dietary fibers(3), as high serum cholesterol concentration represents a major cardiovascular risk factor and cholesterol reduction is a target in the management of CVD risk (4).

In a meta-analysis by Brown *et al*. it was shown that oat products lower LDL cholesterol by 0.037 mmol/ L per gram of soluble fiber; however, the included studies constitute a heterogeneous group of studies in terms of both study design and intervention product characteristics (3). Molecular mass (MM) and molecular structure of the β-glucan are assumed to be the most important determinants of physico-chemical properties, which in turn influence their physiological impact following consumption. Thus, differences in MM and structural characteristics constitute the most likely reason for the successful cholesterol lowering seen in some human intervention studies and the lack of effect in other studies on oat and barley β-glucans (5;6).

Both oat and barley are rich sources of soluble (1→3), (1→4)-β-D-glucan (β-glucan), a linear, non-starch polysaccharide consisting of about 70% of 1-4-O-linked and 30% 1-3-O-linked β-D-glucopyranosyl units (7). The polymer chain is composed of 85 - 90% of cellotriosyl and cellotetraosyl units connected by β-D-(1→3) linkages (7). The relative proportion of cellotriosyl and cellotetrasyl units is also referred to as the DP3:DP4 ratio (8), which differs between cereal genera as well as genotypes of the same genera and is considered a ‘fingerprint’ of the individual β-glucans (8). Generally, β-glucans derived from oat have been reported to exhibit smaller DP3:DP4 ratios (1.5 – 2.3) than those derived from barley (1.8 – 3.5) (8).

The primary aim of the present study was to relate structural characteristics of three β-glucans from oat and barley, but of similar MM (9), with cholesterol-lowering properties. Secondly, we report differences between the three β-glucans on blood pressure, body weight, and concentrations of triacylglycerol and plasma short chain fatty acid (SCFA) as well as fecal output, dry matter, fecal pH and energy excretion. Results on appetite regulation, tolerability,

insulin and glucose metabolism, product palatability, and plasma and urine metabolomics will be reported elsewhere.

SUBJECTS AND METHODS

Study subjects

Sixteen young adults were recruited through advertising at university campuses in the Copenhagen area. The exclusion criteria were as follows: known chronic illnesses (such as diabetes, hypertension, hyperlipidemia), smoking, excessive physical activity (>10h/wk), regular use of medication (oral contraceptives were allowed), use of dietary supplements, and food intolerances relevant to the protocol. All study subjects gave written consent after having received verbal and written information about the study. The study was carried out at the Department of Human Nutrition, Faculty of Science, University of Copenhagen, Denmark, and was approved by the Municipal Ethical Committee of The Capital Region of Denmark in accordance with the Helsinki declaration (H-4-2009-111) and registered in the database at http://www.clinicaltrials.gov (NCT 01317264).

Randomization and concealment

Subjects were randomly assigned to the order of the four treatments by simple randomization generated by a computer. Treatment assignment was done by a person (MK) not involved with the study subjects and assigned in the order of the day of enrolment. The subjects were instructed not to discuss the product appearance with the study staff to prevent un-blinding of the outcome assessors.

Study design

The study was designed as a single-blind randomized crossover trial examining four different intervention products during four 3-wk dietary intervention periods, which were separated by ≥2 weeks washout. The study was performed at the Department of Human Nutrition at the University of Copenhagen. During each of the four intervention periods, the subjects consumed both a beverage and a serving of yoghurt with/without added β-glucan derived from the three different sources. Participants did not know which study product they received. They were instructed to consume each serving of β-glucan with a meal. The study subjects received freshly prepared products twice weekly. Aside from the two daily doses of intervention products, they consumed their habitual diet and were asked to maintain their habitual activity level throughout the study. Further, they were instructed to abstain from oat

and barley products containing intact β-glucans. Any deviations from these instructions as well as cases of illness were reported in a diary.

Before and after each intervention period (day 1 and 22), the participants came to the Department for a meal test day after an overnight fast (>10 hours) and abstention from alcohol and physical exercise for 24 hours. Body weight was measured in light clothing to the nearest 0.05 kg (Tanita BWB-600, Japan) and height was assessed (only at the first day) to the nearest 0.5 cm using a wall-mounted stadiometer (Seca, Hultafors, Sweden). Blood pressure measurements were performed on the right arm in the supine position after 10 min of rest with an automatically inflated cuff (UA-787; A & D Co Ltd, Saitama, Japan). Two measurements were performed and the mean value was calculated. Thereafter, subjective appetite sensation was assessed using visual analogue scales (VAS) and a fasting blood sample was drawn. After all fasting measurements were performed, a breakfast meal including a daily dose of β-glucans was served together with 300 mL of water (~2000kJ; 14% of energy from protein, 22% of energy from fat and 64% of energy from carbohydrates), and the palatability of the meal was assessed using VAS. During the following 240 min, appetite sensation was assessed every 30 min and blood samples were drawn after 120 and 240 min. At the end of the meal test day, an *ad libitum* pasta Bolognese lunch meal was served.

The subjects were instructed to keep a weighed food record during the last four days before each meal test day. Further, all feces samples were collected in pre-weighed plastic containers during the three days before and the three last days of each treatment period. Also, 24-h urine was collected at the day before and at the last day of each period. At the end of each period, subjects evaluated gastrointestinal tract (GIT) comfort using VAS. Furthermore, they were asked to guess which of the four intervention products they had been consuming to assess the successfulness of blinding.

Intervention products

Formulation of intervention products

Three β-glucans, derived from oat (O), barley (B) and barley mutant (Bm) were extracted and characterized by Mikkelsen et al (2012) (9). Physico-chemical characteristics of the extracts are summarized in **Table 1**.

Table 1 Compositional and structural features of the β-glucan extracts and composition of the daily administered intervention product[1]

	C	O	B	Bm
β-glucan extract characteristics				
β-glucan (% dm)	-	72	46	58
TDF (% dm)	-	83	44	71
IDF (% dm)	-	6	20	26
SDF (% dm)	-	78	24	45
Molecular mass (kDa)	-	305	260	280
DP3/DP4 (molar ratio)	-	2.3	3.5	4.7
Daily dose of intervention product				
β-glucan (g/d)	-	3.3	3.3	3.3
TDF (g/d)	-	3.8	3.2	4.0
IDF (g/d)	-	0.3	1.4	1.5
SDF(g(d)	-	3.6	1.7	2.6

[1] DP3/DP4 = ratio between cellotriosyl and cellotetraosyl oligomer units in the β-glucan chain, IDF = insoluble dietary fiber, SDF = soluble dietary fiber, TDF = total dietary fiber.

Fresh 2% (w/v) β-glucan solutions were prepared from the dry β-glucan extracts by stirring for 30 min at 80°C to secure proper hydration of the fibers. The fresh solutions were then mixed into a beverage with blackcurrant syrup (Minimum®) and low-fat vanilla yoghurt (Arla Cheasy®) with a total content of 1.65g β-glucan in each preparation, corresponding to a daily dose of 3.3g of β-glucan. All four beverage preparations had an equal total volume of 250 mL whereas the volume of the vanilla yoghurt preparation ranged from 100-174 mL, as different amounts of β-glucan extract was added to 100 mL of yoghurt (Table 1).

Viscosity of intervention products

Viscosity measurements of the pure β-glucan solutions and the prepared intervention products were performed with a StressTech rheometer (Reologica Instruments AB, Sweden) using a cup (26.0 mm) and bob (25.0 mm) geometry over a shear rate range of 1-100 s^{-1} and temperatures of 10 and 37°C. The viscosity behavior was approximated with a consistency coefficient *c* (related to viscosity, Pas) and flow behavior index *n* from the Power Law model as $SS = c\ SR^n$, where SS (Pa) is shear stress and SR (s^{-1}) is shear rate. True replicate measurements of β-glucan drink and yoghurt intervention products, control samples, and pure β-glucan solutions were performed in separate weeks throughout the intervention period. Intervention product stability over time, i.e. viscosity and flow behavior, was monitored over a 4-d period corresponding to the time that test persons stored the products in their refrigerators. Prior to the measurements the intervention products were shaken according to the procedure the subjects were instructed to apply before consumption of the products.

Dietary intake assessment

All recorded foods and energy-containing beverages were entered into the Dankost 3000 dietary assessment software (Dankost 3000, version 2.5, Danish Catering Center, Herlev, Denmark) and mean total intake of energy, fat, carbohydrates, protein, alcohol and total dietary fiber was calculated for each 4-d registration period.

Analytical procedures

Blood samples

Fasting concentrations of serum total, and LDL cholesterol and both fasting and postprandial plasma triacylglycerol were assessed using colorimetric test kits (Roche TG, Roche Diagnostics GmbH, Mannheim, Germany); intra-assay variations were 0.6% and 0.9%, respectively. Serum HDL-cholesterol was measured using a homogeneous enzymatic colorimetric test kits (Roche HDL-C plus 2^{nd} generation, Roche Diagnostics GmbH, Mannheim, Germany); intra-assay variations were 1.8%. All analyses were performed on a COBAS MIRA Plus (Roche Diagnostic Systems Inc., Mannheim, Germany). Plasma was analyzed for SCFA (total SCFA, acetate, propionate, and butyrate) essentially as described by Brighenti_*et al.* (1998) using 2-ethyl butyrate (Fluka No. 03190; Sigma Aldrich, St. Louis, MO), rather than isovaleric acid, as an internal standard (10).

Fecal endpoints

All fecal samples were weighed and the mean wet weight of the 3-day collection period was calculated. One fecal sample from each subject in each 3-d collection period was mixed with demineralized water (1:1) and homogenized for measurement of pH (PH-208, Lutron Electronic Enterprise CO., Taipei, Taiwan). All fecal samples were freeze-dried, weighed, and homogenized. For each subject, all samples of each 3-day collection period were pooled. Fecal energy was measured by bomb calorimetry (Ika-calorimeter system C4000; Heitersheim, Germany).

Sample size

The number of study participants was based on a power calculation of two previous intervention studies on the effect of dietary fibers on fasting total cholesterol concentrations conducted in our laboratory (11;12). Here, the change in total cholesterol differed from control by -0.3 and -0.5 mmol/L, respectively, with corresponding standardized differences of 0.39 and 0.48 mmol/L. A total of 14 study subjects needed to complete the study in order to

have sufficient power (α=0.05; β=0.20) to detect a 0.4 mmol/L difference with a standardized difference of 0.5 mmol/L. In total, 16 subjects were recruited for the study to allow for a 10 % dropout.

Calculations and statistical analysis

The area under the curve (AUC) for triacylglycerol was calculated using the trapezoidal method. Energy digestibility was calculated as mean daily energy excreted based on the 3-d fecal collection divided by mean energy intake based on the 4-d weighed food record multiplied by 100.

All statistical analyses and calculations were performed using Data Analysis and Statistical Software, release 11 (StataCorp LP, College Station, TX, USA). All dependent variables were controlled for homogeneity of variance and normal distribution by investigation of residual plots and normal probability plots. When not normally distributed, log-transformation was applied. For all variables, an analysis of co-variance (ANCOVA) was performed using a random effect model (xtmixed) to investigate if there was an overall effect of treatment on changes from baseline to week three. The overall *P*-value for treatment was obtained by performing a likelihood ratio test with and without the treatment variable. If $P < 0.01$, pairwise comparisons of the three β-glucan treatments to control were made. Subject was modeled as a random variable, sex and period were included as fixed variables and dietary fiber intake at period baseline were included as co-variables. A likelihood ratio test was performed to test whether an interaction of treatment × sex or treatment × period should be included in the model. Moreover, an ANCOVA was performed to test for differences between period baseline and three weeks of treatment within each of the four treatments. Results are presented as means ± SEMs or as medians (95% CI), when data were log-transformed. Statistical significance level was defined as $p<0.05$.

RESULTS

Of the 16 enrolled subjects (10F/6M), 13 persons completed all four dietary periods (7F/6M). One person dropped out due to dislike of the intervention products and one for personal reasons not related to the study. Finally, one female subject dropped out before the fourth period due to pregnancy. Data from the three periods completed by this female was included in the statistical analysis. Subjects were enrolled in January and February 2010, and the last participant finished in October 2010. All 14 subjects were young healthy adults with BMI,

blood cholesterol concentration and blood pressure within the normal range (**Table 2**). During one period, one participant was provided a dry β-glucan powder for 10 days due to travel activities. No other deviations from the protocol were reported, thus compliance to the intervention was high. Based on the subjects' guesses as to which intervention product they were allocated in each period, the blinding was not entirely successful. When given the control products, 100% answered correctly, whereas they were not able to distinguish between the three β-glucan sources (data not shown).

Table 2 Baseline characteristics of study participants[1]

	All (*n* = 14)	**Women (*n* = 8)**	**Men (*n* = 6)**
Age (y)	22.9 ± 2.1	23.6 ± 1.3	22 ± 2.6
Body weight (kg)	70.3 ± 12.7	61.7 ± 5.3	84.2 ± 10.1
Height (m)	1.75 ± 0.12	1.67 ± 0.07	1.86 ± 0.09
BMI (kg/m^2)	22.8 ± 2.3	22.2 ± 2.4	23.6 ± 2.2
Total cholesterol (mmol/L)	4.46 ± 0.26	4.75 ± 0.4	4.06 ± 0.24
LDL cholesterol (mmol/L)	2.37 ± 0.20	2.53 ± 0.28	2.17 ± 0.29
Systolic BP (mmHg)	119.6 ± 13.9	111.3 ± 9.0	130.8 ± 11.6
Diastolic BP (mmHg)	74.4 ± 7.9	71.19 ± 61.7	78.6 ± 9.2

[1] All values are mean ±SEMs. BP, blood pressure.

Intervention product characteristics

Viscosity

At 10°C, control beverage and yoghurt products exhibited viscosity and flow behavior stability over time and replicate measurements, beverage c = 1 mPas, n = 1 (**Supplemental Figure 1**). For all samples, viscosity decreased with the magnitude of ~3 following a temperature increase from 10 to 37°C, which agrees well with our previous study on β-glucan viscosity temperature dependence (13). Oat and barley β-glucan black-currant beverages showed viscosity and flow behavior stability over time indicating that β-glucans were not susceptible to ascorbic acid-induced oxidative degradation in the drinks which has been suggested by Kivelä *et al*. (14). The β-glucan beverages differed in viscosity at 10°C in the following order: O (c = 180 mPas) > B (c = 80 mPas) > Bm (c = 30 mPas), whereas all preparations showed Newtonian flow behavior (n = 0.95-1). Yoghurts also showed viscosity and flow behavior stability over time, which indicate no adverse effect from the yoghurt starter cultures on β-glucan polymers as suggested by Gee *et al*. (15). Mixing of hydrated β-glucans into readymade yoghurt additionally prevented yoghurt phase separation seen in studies where β-glucan powder is mixed with milk prior to acidification (16). Both barley β-glucan yoghurts showed similar viscosities around 1000 mPas, whereas the oat β-glucan had higher viscosity, c = 3400 mPas. As expected, the yoghurts displayed shear thinning behavior

(n = 0.5-0.7). Compared to control products the addition of β-glucan solution to beverage increased the product viscosity, whereas the addition to yoghurt decreased the product viscosity.

Dietary intake

There was no overall effect of treatment on changes in energy and dietary fiber intake or E% from protein (P > 0.15) (**Table 3**). However, treatment affected changes E% from carbohydrates (P =0.04) and a tendency was observed for E% from fat (P = 0.08), where posthoc pairwise comparisons only revealed that the decrease in E% from fat during oat β-glucan consumption tended to differ from control (P = 0.08). Surprisingly, total fiber intake was not increased with any of the treatments compared to baseline despite the fiber supplementation, but tended to decrease with β-glucan consumption (23.2 ±2.7 g/d) compared to baseline (26.3 ± 2.9 g/d) (P =0.09).

Blood lipids

Changes in fasting total, LDL, and HDL cholesterol concentrations did not differ between treatments (P > 0.15) (**Figure 1; Table 4**). However, consumption of 3.3g oat β-glucans for three weeks resulted in reductions in total cholesterol (-0.29 ± 0.09; P < 0.01), LDL (-0.23 ± 0.07, P < 0.01), and HDL cholesterol (-0.05 ± 0.0.3, P <0.05) compared to baseline (Figure 1, Table 4), whereas this was not the case for either of the two barley β-glucans. It was examined if a relation between the β-glucan structural feature (DP3:DP4 ratio) and changes in LDL cholesterol after oat ß-glucan treatment was present, but the regression was very weak (R^2 = 0.039; P = 0.92). As mean total dietary fiber intake in the subjects exceeded that of the general population, it was examined if an inverse relationship between habitual fiber intake and LDL cholesterol lowering after oat treatment was present. In this case the regression was stronger (R^2 = 0.414; P = 0.02) (data not shown).

Table 3 Composition of subjects' diets[1]

	C		O[2]		B		Bm		*P*[3]
	Baseline	**Wk 3**	**Baseline**	**Wk 3**	**Baseline**	**Wk 3**	**Baseline**	**Wk 3**	
Energy (kJ/d)[4]	9749 ± 790	9780 ± 876	9869 ± 836	9718 ± 711	10117 ± 746	9630 ± 740	9326 ± 758	9510 ± 702	0.79
Protein (E%)[4]	15.5 ± 0.8	16.0 ± 0.7	15.1 ± 0.7	15.2 ± 0.6	16.2 ± 0.6	15.6 ± 0.6	16.0 ± 0.8	152 ± 0.8	0.17
Fat (E%)[4]	31.4 ± 1.4	29.3 ± 1.5	29.7 ± 1.1	31.7 ± 1.7	32.5 ± 1.2	29.3 ± 1.3[a]	32.1 ± 1.4	32.2 ± 14	0.08
Carbohydrates (E%)[4]	50.8 ± 1.7	50.9 ± 2.1	52.1 ± 1.2	49.0 ± 2.0[b]	48.7 ± 1.5	52.5 ± 1.8[b]	49.8 ± 1.5	50.3 ± 1.7	0.04
Dietary fiber (g/d)[4]	26.9 ± 2.4	26.6 ± 2.8	26.3 ± 2.9	23.2 ± 2.7	29.5 ± 3.0	28.7 ± 4.2	26.8 ± 3.6	24.2 ± 2.4	0.71

[1] All values are mean ±SEMs. C, non-fiber control; O, 3.3g oat β-glucan/d; B, 3.3g barley β-glucan/d; Bm, 3.3g barley mutant β-glucan/d.
[2] O: n = 13.
[3] Main effect of treatment by ANCOVA.
[4] Main effect of time by ANCOVA (i.e. baseline compared with wk 3). Values with different superscript letters within row and treatment are significantly different, a) compared to baseline $P < 0.01$; b) compared to baseline: $P < 0.05$.

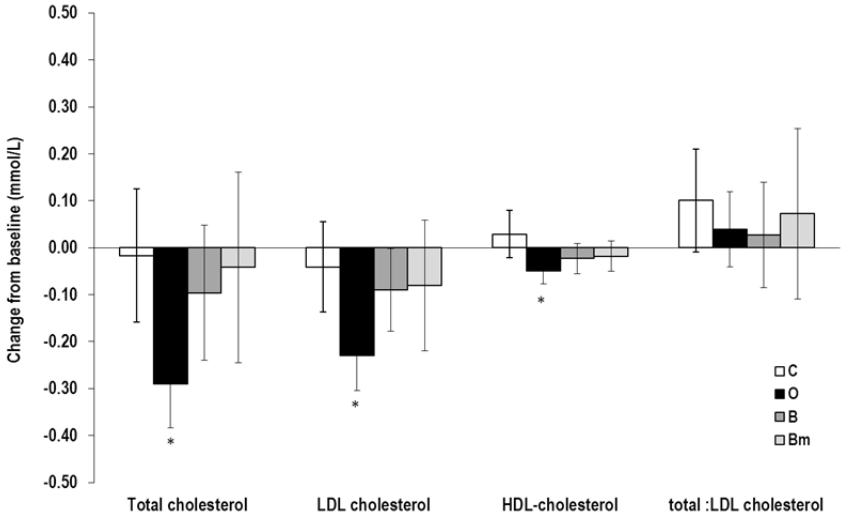

Figure 1 Mean (±SEMs) changes in total, LDL- and HDL-cholesterol (mmol/L) and total:LDL cholesterol ratio from baseline after 3-wk consumption of control (C) or 3.3g of oat ß-glucan (O), barley ß-glucan (B) and barley mutant ß-glucan (Bm) (n = 14; n=13 for O). ANCOVA showed no overall effect of

treatment for total cholesterol (P = 0.31), LDL cholesterol (P = 0.46) or HDL cholesterol (P = 0.19), but total, LDL and HDL-cholesterol decreased from baseline (P<0.05), indicated by an asterisk.

Table 4 Mean of endpoints before and after 3-wk intervention[1]

	C		O[2]		B		Bm		*P*[3]
	Baseline	**Wk 3**	**Baseline**	**Wk 3**	**Baseline**	**Wk 3**	**Baseline**	**Wk 3**	
Body weight (kg)	70.1 ± 3.4	70.2 ± 3.4	71.1 ± 3.5	71.0 ± 3.6	70.1 ± 3.5	70.1 ± 3.5	70.5 ± 3.5	70.1 ± 3.4	0.40
Sys BP (mmHg)	72.5 ± 2.0	70.1 ± 1.2	73.1 ± 1.4	71.1 ± 1.6	71.5 ± 1.6	69.3 ± 1.8	70.3 ± 1.4	69.3 ± 1.4	0.84
Dia BP(mmHg)	118.1 ± 3.5	116.9 ± 2.7	119.4 ± 2.9	120.3 ± 3.7	117.0 ± 3.2	115.5 ± 2.8	115.3 ± 2.7	115.3 ± 2.8	0.90
T-C (mmol/L)[4]	4.34 ± 0.22	4.33 ± 0.25	4.66 ± 0.21	4.38 ± 0.22[a]	4.43 ± 0.21	4.33 ± 0.24	4.39 ± 0.25	4.34 ± 0.26	0.31
LDL-C (mmol/L)[4]	2.39 ± 0.17	2.35 ± 0.21	2.56 ± 0.20	2.32 ± 0.17[a]	2.42 ± 0.18	2.33 ± 0.18	2.40 ± 0.18	2.32 ± 0.19	0.46
HDL-C (mmol/L)[4]	1.30 ± 0.07	1.33 ± 0.08	1.38 ± 0.06	1.33 ± 0.06[b]	1.37 ± 0.07	1.35 ± 0.07	1.31 ± 0.08	1.29 ± 0.07	0.19
TG (mmol/L)[5]	0.68 (0.52;0.84)	0.68 (0.53;83)	0.99 (0.74;1.24)	0.81 (0.53;1.11)*	0.64 (0.51;0.77)	0.71 (0.55;0.87)	0.82 (0.66;0.98)	0.83 (0.58;1.08)	0.05
TG (mmol/L) [5,6]	0.68 (0.52;0.84)	0.68 (0.53;83)	0.99 (0.80;1.16)	0.81 (0.67;0.98)	0.64 (0.51;0.77)	0.71 (0.55;0.87)	0.82 (0.66;0.98)	0.83 (0.58;1.08)	0.30
AUC TG	159 (114;203)	154 (115;192)	209 (137;281)	206 (111;302)	164 (128;200)	167 (116;218)	188 (1415;235)	197 (134;261)	0.91
AUC TG[6]	159 (114;203)	154 (115;192)	209 (161;257)	206 (94;319)	164 (128;200)	167 (116;218)	188 (141;235)	197 (134;261)	0.27
SCFA (µmol/L)	127.9 ± 9.8	137.8 ± 13.0	111.9 ± 7.4	132.5 ± 12.8	142.3 ± 17.2	119.1 ± 9.3	128.2 ± 9.9	141.4 ± 12.3	0.01
Ace (µmol/L)	115.8 ± 9.3	125.6 ± 12.2	100.4 ± 7.3	119.0 ± 11.8	130.0 ± 16.3	108.6 ± 9.1	114.6 ± 9.2	128.7 ± 11.6	0.01
Pro (µmol/L)	5.0 (4.2;5.7)	3.8 (2.9;4.7)	4.2 (3.4;5.0)	4.7 (1.9;7.5)	4.5 (3.3;5.8)	4.1 (3.6;4.6)	4.9 (3.3;6.4)	5.0 (3.9;6.2)	0.4
But (µmol/L)	1.8 (1.5;2.1)	1.7 (1.4;2.1)	1.4 (1.2;1.7)	1.4 (0.6;2.2)	1.5 (1.2;1.9)	1.6 (1.3;1.9)	2.0 (1.6;3.2.4)	1.7 (1.3;2.1)	0.77

[1] Values are mean ±SEMs (n = 14). BP, blood pressure; Sys, systolic; Dia, diastolic, T-C, total cholesterol; LDL-C, LDL cholesterol, HDL-C, HDL cholesterol, TG, triacylglycerol; SCFA, total short chain fatty acids; Ace, acetate; Pro, propionate; But, butyrate; C, non-fiber control; O, 3.3g oat β-glucan/d; B, 3.3g barley β-glucan/d; Bm, 3.3g barley mutant β-glucan/d.

[2] O: n = 13.

[3] Main effect of treatment by ANCOVA. Values with an asterisk indicate that change within group wad different compared from change in control; P <0.05.

[4] Main effect of time by ANCOVA (i.e. baseline compared with wk 3). Values with different superscript letters within row and treatment are significantly different, a) compared to baseline P < 0.01; b) compared to baseline: P <0.05.

[5] Presented as median (95% CI).

[6] triglycerides presented without two potential outliers; O: n = 11.

For changes in triacylglycerol, an overall effect of treatment was found when including all observations (P = 0.05), and posthoc pairwise comparisons showed that the change after oat β-glucan (-0.15 mmol/L; 95% CI -0.47, 0.17) differed from the change observed with control (-0.02 mmol/L; 95% CI -0.13, 0.09) (P = 0.03) (**Figure 2**, Table 4). Neither of the barley β-glucans differed from the control treatment. Despite fasting, two individuals had high fasting and postprandial triacylglycerol concentrations on one meal test day, which occurred either before or after oat β-glucan treatment. Thus the effect on triacylglycerol was repeated excluding these observations. No effect was observed when these two outlying observations were removed (P = 0.30), although the median change with oat treatment was unchanged (-0.15 mmol/L; 95% CI -0.30, 0.00). We also compared the effect of treatment on the acute postprandial triacylglycerol by testing the effect of treatment on day 1 and 22 separately. After adjustment for period, sex, baseline triacylglycerol and habitual fiber intake, differences in AUC were not affected by treatment on day 1 or 22, whether or not outlying observations were included.

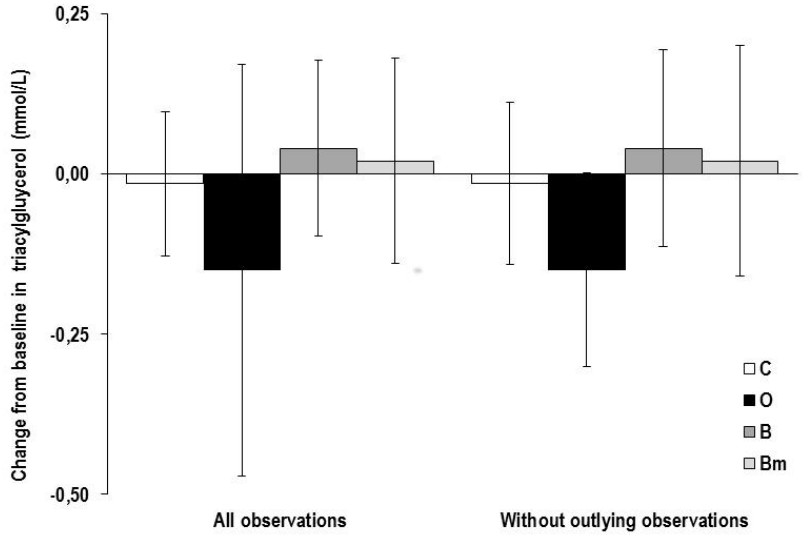

Figure 2 Median (± 95% CI) changes in triacylgycerol (mmol/L) from baseline after 3-wk consumption of control (C) or 3.3g of oat ß-glucan (O), barley ß-glucan (B) and barley mutant ß-glucan (Bm) with and without two outlying observations (n = 14 for C, B, Bm and n = 11 for O). ANCOVA showed an overall effect of treatment when including all observations (P = 0.05), and posthoc pairwise comparisons showed that O and C differed, as indicated by an asterisk. No effect was observed when two outlying observations were removed (P = 0.30).

An overall effect of treatment on changes in plasma total SCFA and acetate (P < 0.01) was observed; however, posthoc pairwise comparison did not show significant differences

between treatments (Table 4). Total SCFA and acetate tended to increase after oat β-glucan consumption (P = 0.08 and 0.09, respectively), whereas barley β-glucan treatment tended to lower concentrations of total SCFA (P = 0.10) after three weeks compared to control (Table 4). Changes in propionate and butyrate concentrations was unaffected by treatment (P > 0.40).

Fecal endpoints

Changes in total fecal output, fecal dry matter content, stool frequency, pH, energy excretion and energy digestibility did not differ between treatments in the oat, barley, and barley mutant treatments compared to control (P > 0.40) (**Table 5**).

DISCUSSSION

Our results do not fully support the hypothesis that three weeks daily intake of structurally different 3.3g β-glucans of similar MM, extracted from an oat, barley, and barley mutant breed, affect concentrations of total, LDL, and HDL cholesterol, changes from baseline did not differ from the change observed with control treatment. A reduction in total, LDL, and HDL cholesterol compared to baseline levels in the oat β-glucan treatment group does however suggest a potential of the oat ß-glucan. We propose that structural differences, mainly different DP3:DP4 ratios affecting solubility, between the β-glucans explain why changes from baseline were observed for the oat β-glucan.

The ability of β-glucans to lower plasma cholesterol is commonly ascribed to their ability to form highly viscous solutions (17). It is thought that an increased viscosity of the upper GIT content upon β-glucan consumption may entrap or encapsulate mixed bile acid micelles leading to a greater bile acid excretion (18;19). As a consequence, the feedback inhibition of the key enzyme of bile acid synthesis cholesterol 7α-hydroxylase is reduced and thus the *de novo* synthesis of bile acid increased, with plasma cholesterol as substrate (20).

Table 5 Mean of endpoint before and after 3-wk intervention[1]

	C		O[2]		B		Bm		*p*[3]
	Baseline	**Wk 3**	**Baseline**	**Wk 3**	**Baseline**	**Wk 3**	**Baseline**	**Wk 3**	
Fecal output (g/d)	185 ±22	160 ± 20	175 ± 29	175 ± 23	177 ± 15	179 ± 28	155 ± 21	147 ± 17	0.76
Fecal dry matter (g/d)	44.4 ± 5.4	36.2 ± 3.9	42.8 ± 6.7	41.8 ± 4.9	44.9 ± 4.4	42.4 ± 7.0	43.3 ± 5.6	40.0 ± 6.2	0.82
Stool frequency (n/d)	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.86
Fecal pH	6.61 ± 0.17	6.54 ± 0.14	6.66 ± 0.12	6.55 ± 0.14	6.78 ± 0.14	6.62 ± 0.16	6.54 ± 0.15	6.64 ± 10	0.44
EE (kJ/d)	968 ± 129	770 ± 86	931 ± 149	885 ± 108	965 ± 103	915 ± 158	958 ±139	865 ± 142	0.73
ED (% of intake)	89.7 ± 1.2	91.6 ± 0.9	90.6 ± 1.4	91.1± 0.8	89.9 ± 1.2	90.5 ± 1.5	89.6 ± 1.2	90.4 ± 1.8	0.93

[1] All values are mean ±SEMs (n = 14). EE, Energy excretion; ED, Energy digestibility; C, non-fiber control; O, 3.3g oat β-glucan/d; B, 3.3g barley β-glucan/d; Bm, 3.3g barley mutant β-glucan/d.

[2] n = 13 for oat treatment.

[3] Main effect of treatment by ANCOVA.

Viscosity measurements of the β-glucan beverages, at a concentration of 0.7%, showed a greater apparent viscosity for the oat β-glucan at both 10 and 37 °C than for both the barley and the barley mutant β-glucan most likely reflecting a higher solubility for oat ß-glucan. The viscous properties of β-glucans are determined by their MM, concentration, and molecular structure which in turn determines the solubility (17). The MM of the extracted β-glucans in our study ranged from 250 to 300 kDa (9), which is medium compared to other extracted β-glucans (21) but low in comparison to the average MM from 2,000 to 3,000 kDa reported for native cereal β-glucans (22). However, it has previously been shown in a large intervention study that 3g high (2,100 kDa) and medium (530 kDa) MM oat β-glucans lowered LDL cholesterol similarly, but that the efficacy was reduced by 50% when MM was reduced to 210 kDa (23). In this study the MM of the three β-glucans were similar, thus the observed viscosity differences cannot be attributed to varying MM.

Another influencing factor on the viscosity is the solubility of the β-glucan molecules, as only solubilized β-glucan polymers are able to entangle and form highly viscous solutions (17). Although cereal β-glucans are classified as soluble fibers, they are only partly soluble in water and the extent to which they are soluble is closely related to their molecular structure (8). Significant block structural differences were found for mutant barley, mother barley and oat β-glucans with DP3:DP4 ratios of 4.7 > 3.5 > 2.3, respectively, implying a greater relative amount of DP3 oligomers in the barley β-glucans relative to the oat β-glucan. The higher number of DP3 units of the barley β-glucans may result in lower solubility, as DP3 units are likely to be organized in longer, repetitive cellotriosyl sequences, which are prone to aggregate and form insoluble species (8;24). Therefore, the amount of insoluble β-glucan may determine viscosity and in turn physiological effects. The same solubilization procedure was applied for all three dry extracts, thus it is likely that a greater proportion of the oat β-glucan than of the two barley β-glucans was solubilized as indicated by the dietary fiber assay. We propose that this accounts for the cholesterol reductions compared to baseline observed for the oat β-glucan treatment and not for the two barley β-glucan treatments.

The importance of solubility was also highlighted by others. In a study by Beer *et al.* (1995) 9g/ d of an oat gum β-glucan incorporated into a milk-based instant whip did not affect total cholesterol concentrations despite a relatively high MM of 1000 kDa (5). In contrast, Braaten *et al.* (1994) effectively lowered serum cholesterol with a smaller dose of 5.6 g of an oat gum β-glucan of similar MM (1200 kDa) given as a beverage (25). Apparently the main difference

between the two studies was the solubility of the applied β-glucans, which was reported as high in the study by Braaten *et al*. and poor in the study by Beer *et al*. (5;25). Furthermore, low solubility is likely the reason why daily administration of 10g barley β-glucan in the form of a concentrated powder that was incorporated into various food products such as bread, waffles, and cookies exhibited poor cholesterol-lowering properties in a study by Keogh *et al.* (2003) (6). The pure β-glucan powder was described as largely insoluble in cold water and apparently it was not solubilized before incorporating it into food products or in the upper GIT.

In addition to MM and solubility, also β-glucan concentration strongly influences the viscosity of a solution(26). Thus, β-glucan intake needs to be sufficient to increase intestinal β-glucan concentration and hence viscosity of GIT content. In this study, a daily dose of 3.3g β-glucan was applied which corresponds to the dose used in a recent study by Wolever *et al*. (2010). However, they found that a daily dose of 3g of β-glucans with a MM ≤ 210kDa had a 50 % lower effectiveness in lowering LDL cholesterol than β-glucans with a MM ≥ 530kDa. They suggest that higher doses of soluble β-glucan might compensate for a low MM, although a significantly greater concentration is needed for compensation, as this relationship is not linear (23). In addition, it can be speculated whether the β-glucan content of a single load rather than the daily dose as such is pivotal. In the present study, the daily dose was spread over two servings, which may have resulted in too small intestinal β-glucan concentrations considering the low MM. A minimum daily dose of 3 g β-glucan is suggested by the European Food Safety Authority (EFSA) to obtain a beneficial effect on blood cholesterol with oat and barley β-glucans (27). Further, the EFSA opinion only refers to *'non-processed or minimally processed'* oat and barley products (27) which naturally contain β-glucans of high MM (28). Our results support that not only dose, but also physico-chemical properties, including MM, molecular structure and solubility should be considered.

Besides a higher bile acid excretion due to an increased intestinal viscosity, it is also suggested that β-glucans may directly bind to bile acids leading to excess fecal bile acid excretion (29). However, the present study does not allow conclusions in regard to the structural composition of the β-glucans and their potential bile acid binding capacity. Finally, it has been proposed that the fermentation of β-glucan to SCFA by the intestinal microflora may lower serum cholesterol, as the generated SCFA propionate was found to inhibit *de novo* cholesterol synthesis in rats (30). However, this needs to be supported by studies conducted in

humans. In the present study, no changes in peripheral plasma SCFA concentrations were observed following β-glucan consumption, which may be linked to a high habitual dietary fiber intake of the participants. Also, measuring peripheral SCFA is not optimal, as the majority of SCFA metabolism occurs in the liver, and thus before they reach the peripheral circulation (31). Nonetheless, others have been able to detect differences in fasting plasma SCFA following a dietary fiber intervention (32).

Normally, dietary fibers are recognized for their cholesterol-reducing properties rather than lowering of fasting triacylglycerol. This study, however, showed that oat β-glucan resulted in a greater decrease in fasting triacylglycerol compared to control. This appear to be in agreement with our previous finding that oat bran consumption lowered triacylglycerol by as much as 21% (11) and with other studies that have shown that oat bran can reduce postprandial plasma triacylglycerol responses (33;34). It is likely that a decreased postprandial lipemia, if occurring repeatedly over a 3-wk period, may reduce fasting triacylglycerol. However, in the present study, we did not observe a diminished postprandial triacylglycerol response, which is likely accountable to a too small fat load of the standardized breakfast meal (10 g).

During the course of the study subjects maintained their habitual diet. Dietary food records revealed that the average dietary fiber intake was high and meeting the Nordic nutrition recommendations of 25-30 g /day (35). Moreover, some individuals consumed quite large amounts of rye bread, which is another source of soluble fibes (36). As also indicated by the inverse relation between habitual dietary fiber intake and changes in LDL cholesterol with oat ß-glucan, it is possible that supplementing the diet with 3.3 g of extracted β-glucan may be more relevant in a population with a low habitual dietary fiber intake. We also found an inverse relation between baseline LDL cholesterol and changes in LDL cholesterol, which is in agreement with other studies where lack of hypo-cholesterolemic effects of β-glucan was observed in persons exhibiting low initial cholesterol concentrations (5;37). Most studies that report reduction in cholesterol levels were conducted in hypercholesterolemic individuals (25;38;39). Therefore it can be speculated that a similar β-glucan intervention might show more pronounced cholesterol-lowering effects if added to the diet of moderately hyper-cholesterolemic individuals consuming a typical Western diet that is low in dietary fibers. Finally, it should be noted that only 13 as opposed to 14 of the 16 subjects completed the oat

treatment, thus the study was sufficiently powered for the difference between treatments to become significant.

The current study does not provide strong support for the hypocholesterolemic effects of extracted oat, barley and barley mutant β-glucans with major structural differences in comparison to a control treatment. However, the higher solubility and viscosity of the oat β-glucan suggest a certain hypocholesterolemic potential, which is also indicated by the reductions in total and LDL cholesterol compared to baseline. Mildly hyper-cholesterolemic individuals, consuming a diet low in dietary fibers, might benefit by adding oat β-glucan to their diet. Further studies are required to investigate the hypocholesterolemic potential of structurally different barley and oat β-glucans in more susceptible groups of volunteers.

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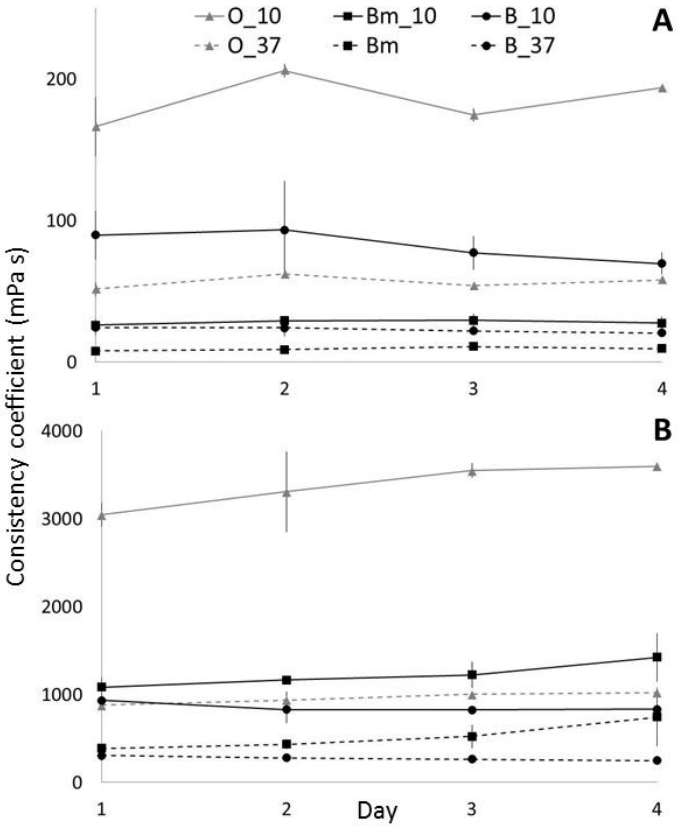
ONLINE SUPPLEMENTAL MATERIAL



Supplemental figure 1 Viscosity at 10 and 37º (expressed as consistency coefficient *c*) of β-glucan beverages (A) and yoghurts (B) over 4 days of storage at +5ºC.

Paper VI

Mikkelsen MS, Rasmussen MA, Savorani F, Jespersen BM,
Kristensen M, Engelsen SB

Observations from a non-confirmatory dietary fibre intervention study with barley and oat β-glucans using NMR metabolomics

Manuscript

Observations from a non-confirmatory dietary fibre intervention study with barley and oat β-glucans using NMR metabolomics

Mette Skau Mikkelsen[*1], Morten Arendt Rasmussen[1], Francesco Savorani[1], Birthe Møller Jespersen[1], Mette Kristensen[2], Søren Balling Engelsen[1]

[1]*Quality & Technology, Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark;* [2]*Department of Department of Nutrition, Exercise and Sports, Faculty of Science, University of Copenhagen, Rolighedsvej 30, 1958 Frederiksberg C, Denmark*

*Corresponding author: Mette Skau Mikkelsen; Tel: +45 35 33 35 07; Fax: +45 35 33 32 45; E-mail: skau@life.ku.dk

Abstract

Employing ^{1}H NMR spectroscopy and multivariate data analysis, this study investigate the plasma metabolic effectiveness from 3.3 g mixed linkage barley or oat β-glucan fibre per day. Three large-scale extracted, equally sized but structurally different β-glucans, were tested in a blinded randomised cross-over design using young healthy adults, where the β-glucans were compared to a non-fibre control during a 21 day dietary intervention period. It was shown that the plasma metabolic data correlated well with reference plasma glucose and plasma triglyceride measurements. Subject variance was found to significantly dominate data although participant age and BMI exhibited little variance. The second most influential variation was due to gender and characteristic lipoprotein profiles were found for male and female samples. No significant metabolic difference in plasma between 3-week β-glucan treatments and control was found. However, a tendency to postprandial effect of barley β-glucan compared to control was indicated and most pronounced in males. Two sets of measurements (0, 2 and 4 hour), one male and one female, appeared to represent fat boosted systems prior to β-glucan treatment and these systems might be more sensitive to β-glucan fibre exposure compared to average metabolomes of young healthy adults.

Keywords

Human study, NMR spectroscopy, metabolomics, β-glucan, barley, oat

Introduction

It is well established that viscous soluble dietary fibres like barley and oat mixed linkage β-glucans affect cholesterol metabolism (Behall et al., 1997; Brown et al., 1999; Gunness and Gidley, 2010; Theuwissen and Mensink, 2008; Wood, 2004), however, the underlying mechanisms of the hypocholesterolemic actions of β-glucans are still not fully understood. One proposed mechanism involves interference with lipid and/or bile acid metabolism as the viscous properties of β-glucans may prevent the bile acid reabsorption in the small intestine leading to increased excretion of bile acids via faeces (Gunness and Gidley, 2010; Theuwissen and Mensink, 2008).

The risk of cardiovascular disease is related to the distribution of cholesterol in different lipoprotein fractions (Castelli, 1996). Lipoprotein particles functions as transport vehicles for the water insoluble lipids in the human body and they are usually divided into five main classes: chylomicrons and very low, intermediate, low and high density lipoproteins (VLDL, IDL, LDL and HDL, respectively) (Ala-Korpela, 1995). This classification is related to the physiological and physical characteristics of the lipoproteins and their isolation by ultracentrifugation based on their density. Standard analytical measurements are traditionally used for monitoring single biomarkers like VLDL and LDL cholesterol in clinical medicine, but techniques aimed at assessing large numbers of metabolites that are substrates, intermediate or end products in various metabolic pathways are increasingly relevant in the risk assessment of metabolic conditions like cardiovascular disease (Ala-Korpela et al., 2008).

Metabolomics is an omics approach to identify and monitor metabolic characteristics, changes and phenotypes with respect to various synergetic factors such as environment, life style, diet and potential pathophysiological processes (Fiehn, 2002; Giovane et al., 2008). Recently, the concept of nutri-metabolomics has evoked with the purpose of relating the intake of a specific dietary component to specific metabolic fingerprints (Savorani et al. 2012). Mass spectrometry (MS) (Kristensen et al., 2012a) and proton nuclear magnetic resonance (NMR) spectroscopy (Kristensen et al., 2010; Savorani et al., 2010a) are the two key technological platforms in the field. Especially, NMR profiling of blood serum and plasma has shown to be promising in the characterisation and quantification of lipoprotein subclasses and in turn identification of early biomarkers associated with the risk of cardiovascular disease (Ala-Korpela, 2008; Otvos et al., 1992). Earlier we used multivariate calibration for NMR quantification of lipoprotein main- and subfractions in human plasma samples (Dyrby et al.,

2005; Petersen et al., 2005) and iPLS regression models were constructed from NMR spectra on rat plasma in order to predict the amount of cholesterol in HDL, LDL and VLDL as well as the total plasma cholesterol (Kristensen et al., 2010). In these studies reference lipoprotein measurements were available from ultracentrifugation of the plasma.

Recently, we investigated the traditional exposure and effect markers from flaxseed dietary fibre intake (Kristensen et al., 2012b) and found a relation between fibre viscosity and effect. Using the same experimental design, a full-scale dietary human intervention study on differently structured barley and oat β-glucan fibres was conducted and effects on blood lipids and faecal endpoints were reported (Ibrügger et al., 2012). The results did not fully support the hypocholesterolemic action of barley and oat β-glucans. However, a greater potential was indicated for oat β-glucan, presumably due to higher solubility and viscosity.

In this study, NMR spectroscopy in combination with multivariate data analysis was applied to investigate the full blood metabolic effects of daily supplementation of mixed linkage β-glucans from oat and barley. It is hypothesised that an increased dietary fibre intake manifests by perturbation in the blood metabolome and that by using nutri-metabolomics techniques, it may be possible to detect additional responses to those found in our parallel study with the traditional risk markers (Ibrügger et al., 2012).

Materials and methods

The intervention study

In a randomised, blinded, cross-over 3-week intervention study the hypocholesterolemic effects of 3.3g β-glucan/day from three equally sized but structurally different oat and barley β-glucans (Mikkelsen et al., 2013) were investigated in free-living normocholesterolemic individuals that maintained their habitual diet (Ibrügger et al., 2012). Fourteen (of 16) young adults (8F/6M) completed the 4 intervention treatment periods separated by 2-3 weeks wash-out. Treatments consisted of: oat (O), mutant barley (Bm), mother barley (B) β-glucan and control (C) without fibre. Barley β-glucans showed high ratios of cellotriosyl to cellotetraosyl oligomer units in the polysaccharide chain and low solubility compared to oat with less cellotriosyl units and high solubility. For further physico-chemical characterisation of β-glucans and intervention test products please refer to Mikkelsen et al. (2013) and Ibrügger et al. (2012), respectively.

Collection and preparation of plasma samples

Blood was collected as fasting (0 hour) and postprandial samples (2 and 4 hour) after a β-glucan or control test meal at the start (day 1) and end (day 22) of each treatment period. A schematic overview of the 4-armed cross-over study design and blood sampling performed in the treatment periods is presented in Fig. 1. With only 6 samples lost a total of 328 fasting and postprandial blood samples were collected from the 14 study participants.

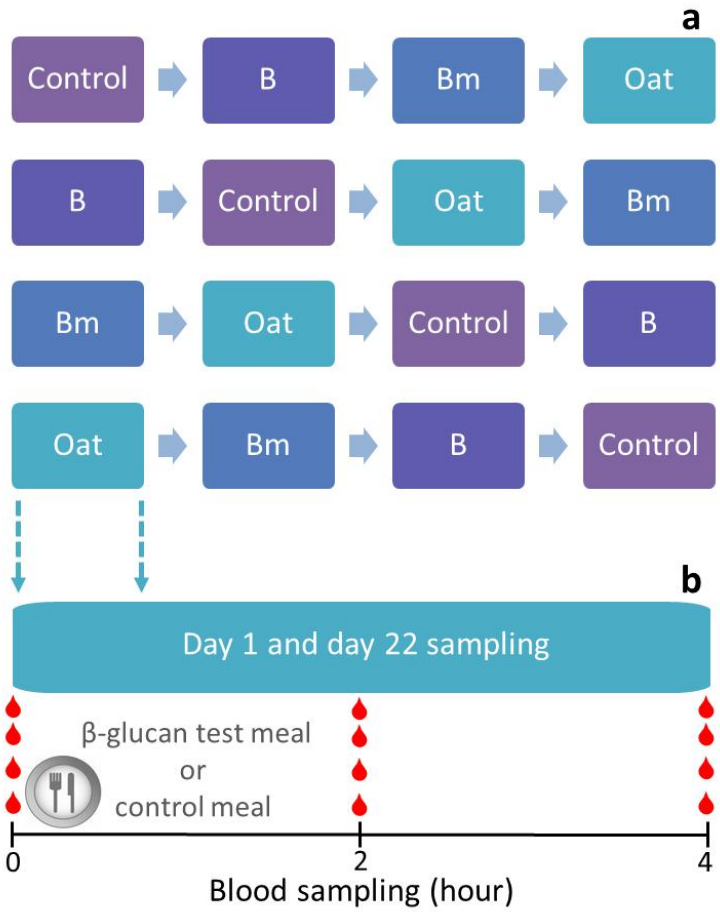


Fig. 1 (a) Schematic overview of the 4-armed cross-over study design with oat (O), mutant barley (Bm), mother barley (B) β-glucan or control (C) treatments and (b) blood sampling (0h, 2h and 4h) preformed at the beginning (day 1) and end (day 22) of each treatment period.

The blood was collected into 4 ml vials containing heparin as anticoagulant and centrifuged at 3000 g, 4°C for 10 min. The plasma fraction was stored at -80°C until NMR analysis. Fasting concentrations of serum total, and LDL cholesterol along with both fasting and postprandial plasma triglycerides (TG) and 30 additional body measures is reported by Ibrügger et al. (2012).

NMR data acquisition and preprocessing

Plasma samples were thawed on ice and 300 µl plasma was transferred to 5 mm NMR tubes together with 300 µl phosphate buffer (pH 7.4) containing trimethylsilyl propionate (TSP) as reference signal and D_2O for the lock signal. NMR profiles were acquired on a Bruker Avance III 600 spectrometer (Bruker Biospin Gmbh, Rheinstetten, Germany) operating at a Larmor frequency of 600.13 MHz for protons, equipped with a double tuned cryo-probe (TCI) set for 5 mm sample tubes and a cooled autosampler (SampleJet). Spectra were acquired from all plasma samples using the following two experiments: 1) CPMG edited spectra in which the short proton relaxation times related to the larger molecules are filtered away resulting in a flatter baseline and enhancing the contributions from smaller molecules and 2) 1D NOESY-Presat edited spectra which gives the best overview of all types of molecules present in plasma and assure a better suppression of the water signal (Beckonert et al., 2007). All experiments were performed at 310K with a fixed Receiver gain, which was found through initial tests. The data was collected using a total of 128 scans. Prior to Fourier transformation the data set was zerofilled to 64 K points and apodised by 0.3 Hz Lorentzian line broadening and thereafter baseline- and phase corrected automatically. In the following only the CPMG ^{1}H NMR spectra are analysed and discussed as the NOESY spectra for the time being have not yet been fully processed. The spectral area chosen for multivariate data analysis was 0-8 ppm with exclusion of the 4.4-4.9 ppm region dominated by the residual water signal (Fig. 2). To correct for spectral misalignment the entire dataset was globally aligned with respect to the α-$_D$-glucose signal around 5.25 ppm using the *i*coshift algorithm (Savorani et al., 2010b). Global and TSP area normalisation of data was evaluated, however, the aligned raw data was chosen as the most robust data block.

Data analysis

The general variance structure of the blood plasma spectral data was investigated by principal component analysis (PCA) (Wold et al., 1987) and Partial Least Squares regression (PLS) (Wold et al., 1983) was used to relate spectral data to reference biomarker measurements. Prior to PCA and PLS on spectra, data were mean centered. Test set validation using 250 samples for building the model and 78 samples for testing the model performance was used for development of the PLS calibration models and to determine the optimal number of components to be used. Data were orthogonalised (Smilde et al., 2005) according to subject for handling of inter-individual variation.

Initially, the numbers of variables were reduced by peak integration. The spectra were visually divided into intervals with optimally a single chemical signal. For each interval the spectra were either integrated by peak-height, peak-area or multivariate curve resolution (MCR) (Tauler, 1995). In case of overlapping peaks, MCR with several components was used to integrate the spectra. In total 235 intervals were identified representing 251 signals.

Multilevel PLS-DA (van Velzen et al., 2008) was used to analyse individual contrast between β-glucan interventions and control diet. Let for example $\mathbf{x}_{i\text{B-t0}}$ and $\mathbf{x}_{i\text{B-t2}}$ be samples from subject *i* at time 0 hours (baseline) and 2 hours (postprandial) acquired during barley (B) intervention. Further, let $\mathbf{x}_{i\text{C-t0}}$ and $\mathbf{x}_{i\text{C-t2}}$ be the corresponding samples during control diet intervention. The purpose is to investigate differences between the dietary response, defined as the difference between plasma composition at baseline and after intervention ($\mathbf{dx}_{i\text{B-t2-t0}} = \mathbf{x}_{i\text{B-t2}} - \mathbf{x}_{i\text{B-t0}}$), for treatment and control. This corresponds to compare $\mathbf{dx}_{i\text{B-t2-t0}}$ with $\mathbf{dx}_{i\text{C-t2-t0}}$ across all individuals ($i = 1,\ldots,14$). For univariate data, this corresponds to the paired t-test. For multivariate data, this can be formulated as a classification problem discriminating $\mathbf{DX}_{\text{B-t2-t0}}$ from $\mathbf{DX}_{\text{C-t2-t0}}$ and utilizing the subject information when validating the results. Pairwise comparison was conducted between control (C) and barley (B), barley mutant (Bm) and oat (O), respectively, for dietary metabolic changes between baseline and postprandial (2 and 4h) or between start and end of treatment period baseline samples (day 1 and 22). In total 9 two-class classification problems were solved. The results for each model were cross validated, segmenting the samples according to subject. The misclassification rate was translated into a probability of finding the observed discrimination under the assumption of no information. Multivariate data analysis was computed in MATLAB version 7.14.0.739 R2012a (Mathworks Inc., Natick, USA) equipped with the PLS toolbox v.6.1. (Eigenvector Research Inc., Manson, WA, USA).

Results and discussion

Three equally sized but differently structured barley and oat β-glucans were compared side-by-side in a cross-over human intervention study for their health promoting effectiveness (Ibrügger et al., 2012). In the present study, the blood metabolic effects of three weeks 3.3 g β-glucan/day were investigated and compared to a no fibre control treatment. The 14 participants were young healthy adults with normal BMI (19-27) on habitual diets meeting the Nordic nutrition recommendations of 25-30 g fibre/day and the change in fibre intake from an additional 3.3 g β-glucan/day supplement may be considered small in relation to the high

habitual dietary fibre intake. The parallel study on traditional blood lipid biomarkers did not provide strong support for the hypocholesterolemic effects of extracted barley and oat β-glucans and it was suggested that normocholesterolemic individuals are less susceptible to the health effects from dietary β-glucan fibre supplementation (Ibrügger et al., 2012).

Plasma NMR spectra

In the following, NMR metabolomics data of fasting and postprandial blood plasma samples from the barley and oat β-glucan human intervention study is presented. An example of the 600 MHz ^{1}H NMR spectra of the human plasma is displayed in Fig. 2.

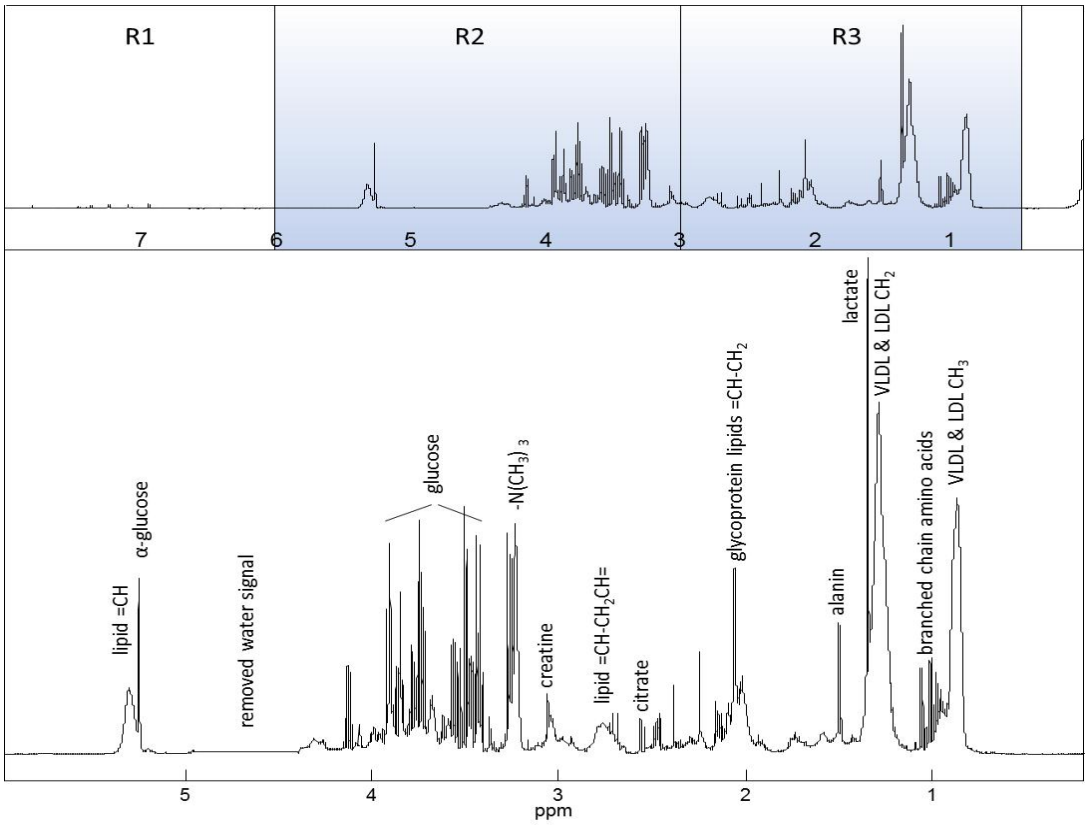


Fig. 2 Top: An average ^{1}H NMR Carr-Purcell Meiboom-Gill (cpmg) spectrum of human plasma (0-8 ppm) with spectral regions R1 (dominated by proteins), R2 (dominated by carbohydrates) and R3 (dominated by lipids). Bottom: R2 and R3 regions (0.5-6 ppm) including assignment of the most prominent peaks.

Assignment of the spectra was made according to previous investigations (Ala-Korpela, 2007; Nicholson et al., 1995) with the most important resonances for this study being the lipoprotein broad signals around 0.9 ppm (VLDL1 and LDL1) and 1.3 ppm (VLDL2 and LDL2), which refer to the $–CH_3$ and $–CH_2$ groups of triglycerides, cholesterol compounds and phospholipids, respectively. Initial spectral preprocessing revealed that global and TSP

normalisation generally disturbed the α-glucose spectral distribution according to reference blood glucose concentrations and the intensity differences of the lipid signals adjacent to the α-glucose signal were masked by the global normalisation (Fig. 3). Thus, raw spectra were chosen as the most robust data block for the subsequent metabolomics data analysis.

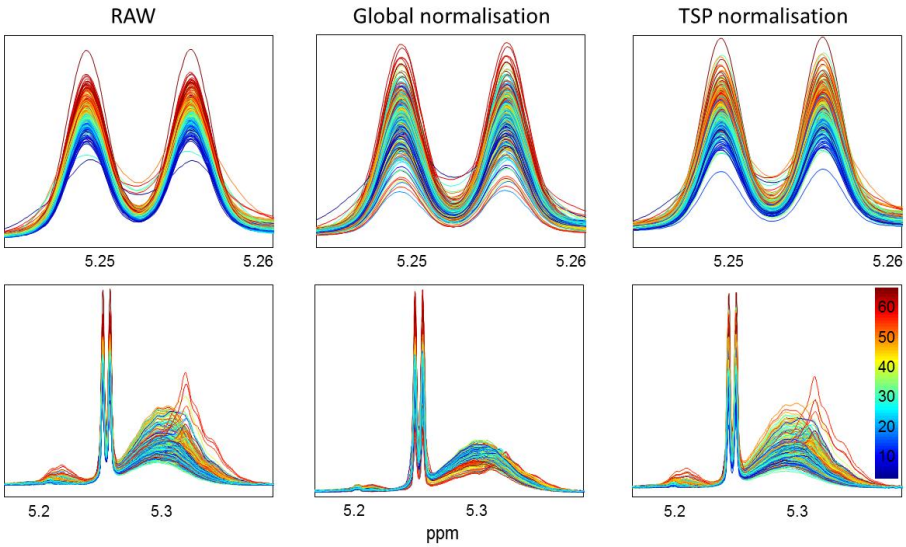


Fig. 3 Effect of global and TSP normalisation procedures on spectra as compared to raw data. Upper row: NMR α-glucose peak around 5.25 ppm. Lower row: NMR profile of α-glucose and adjacent lipid peaks (inverse presentation). All spectra are coloured according to reference blood glucose content.

Fig. 4 shows the measured versus predicted plot from the model based on NMR α-glucose spectral prediction of blood glucose (r^2=0.8). The model is based on 2 PLS components and the prediction error is 0.24 mmol/L, which demonstrate good agreement between spectral data and the reference measurement.

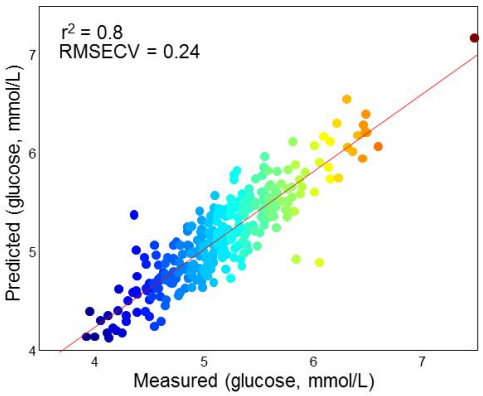


Fig. 4 PLS result for spectral (α-glucose peak at 5.24-5.26 ppm) prediction of blood glucose using a 2 latent variable model. Samples are coloured according to reference blood glucose content.

In order to investigate the main variance amongst the samples, PCA was performed on the entire spectral data set (0-8 ppm). Figure 5 shows the NMR spectra coloured according to gender (a) and the PCA score plot (b). The first two principal components (PCs) explain 89% of the data variation and reveal a clear separation of plasma samples according to gender. No apparent groupings at the individual level could be observed from treatments (O, Bm, B, control). In order to remove the large inter-individual variation between samples, the ^{1}H NMR spectra were orthogonalised. As observed in Fig. 5c, orthogonalisation according to subject variation also removed the dominating gender variation from data.

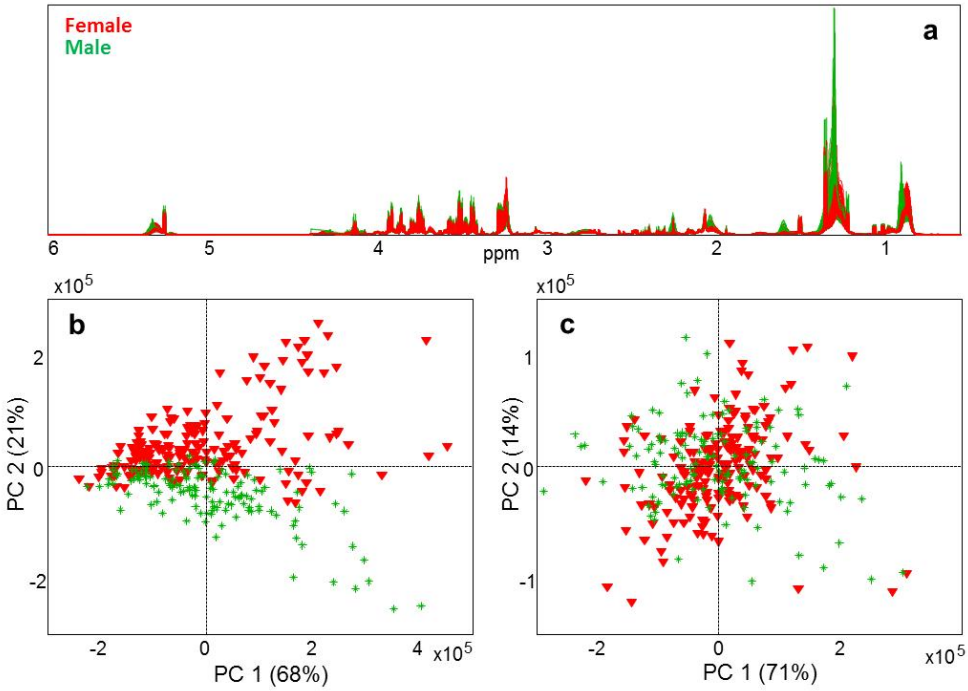


Fig. 5 (a) NMR spectra (0.5-6 ppm) coloured according to gender. (b) Score plot from PCA on all mean centered NMR spectra (0-8 ppm). (c) PCA score plot of subject orthogonalised NMR spectra.

To examine the effect of β-glucan consumption on the metabolic profiles, O, Bm and B samples were compared to the control by application of two class classification PLS-DA for the entire spectra and subsequently for the three spectral regions seen in Fig. 2. Figure 6 shows the discrimination power for different combinations of intervention, timely effect and spectral region. A tendency for a difference between B treatment and control (0-2h) in the lipid region is indicated.

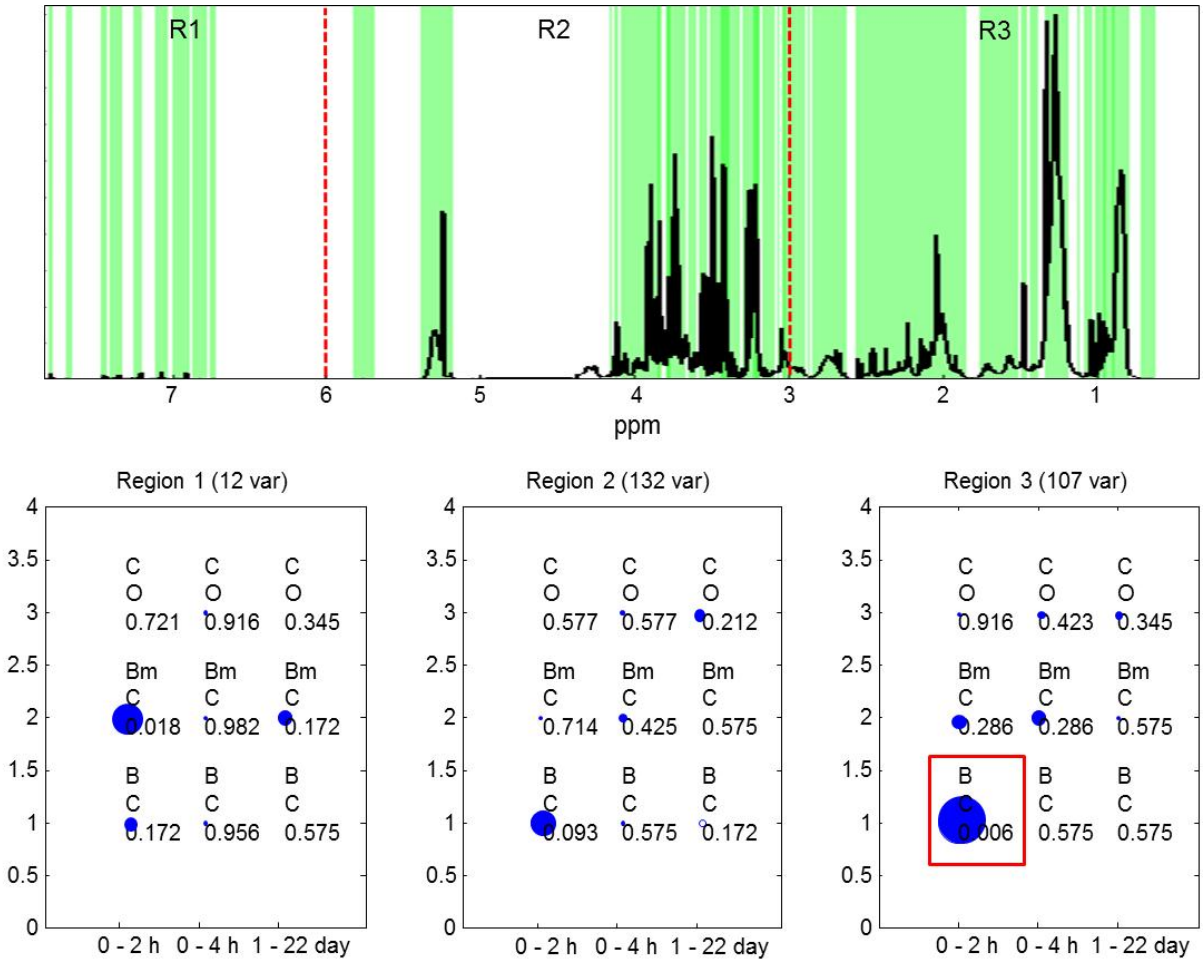


Fig 6 Top: Split of full NMR spectra into 251 intervals (green bins) in the R1 (protein), R2 (carbohydrate) and R3 (lipid) regions. Bottom: PLS-DA results for each region from two class discrimination testing of barley mutant, barley and oat treatments (Bm, B and O) against control (C). For each treatment, there are calculated contrasts between two different time points' e.g. day 1 baseline sample (0h) and postprandial sample (2 or 4h) or between start and end of treatment period baseline samples (day 1 and 22). Bullet sizes indicate the magnitude of treatment difference from control.

Figure 7a shows a score plot from a PLS-DA model discriminating barley (B) and control (C) for 0-2 hours. Bm and O samples are imposed on the estimated subspace. Interestingly, the effect from B treatment is largest for the males (Fig. 7c) whereas for the females there is a larger overlap (Fig. 7b).

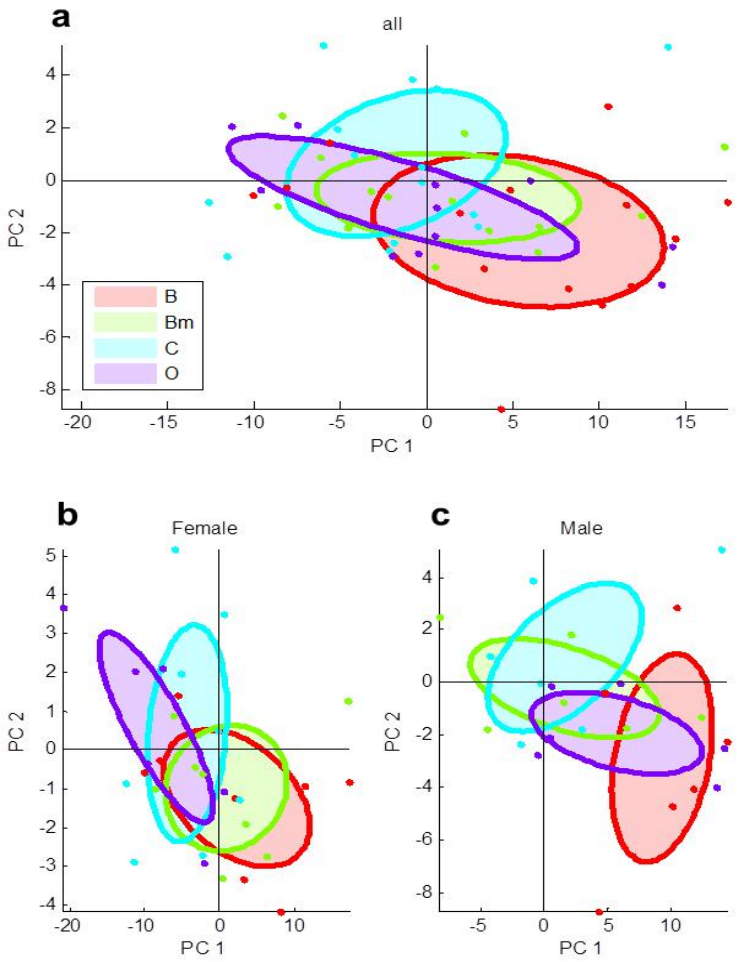


Fig. 7 (a) PLS-DA score plots based on 107 NMR intervals in the lipid region (R3) with presentation of female (b) and male (c) groups individually. The PLS-DA results are based on the 0 to 2 hour contrasts at day 1 from 14 subjects on barley mutant (Bm), barley (B), oat (O) and control (C) treatments.

Outlier's detection

Initial multivariate data mining revealed subject samples 4982_1-3 and 4993_10-12 to be clear outliers (Fig. 8). Both 4982_1-3 (a female at day 1) and 4993_10-12 (a male at day 22) were treated with oat β-glucan corresponding to the start and end of their treatment period, respectively.

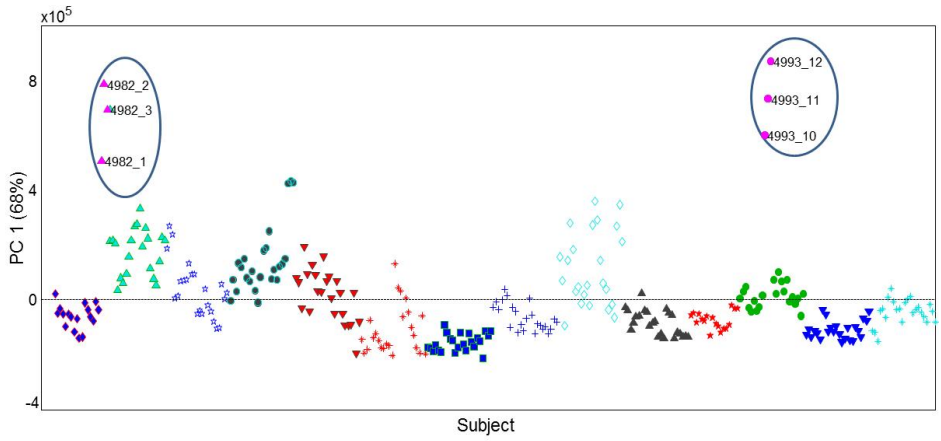


Fig. 8 PC1 from PCA on the full NMR spectra (0-8 ppm) explains 68% of the data variation and is highly dominated by subject variance. Subject samples 4982_1-3 (start of oat treatment) from a female and 4993_10-12 (end of oat treatment) from a male show clear outlier behaviour.

Even though the participants were instructed to fast at least 10 hours prior to blood sampling at the start and end of each period, an obvious reason for significant deviating blood metabolic levels, as observed for 4982_1-3 and 4993_10-12 samples, could be intake of food just before blood sampling or intake of a particularly fat rich food the night before the sampling day. PCA on three spectral regions: a) 1.2-1.23 ppm including the fasting signal of D-3-hydroxybutyrate (Wijeyesekera et al., 2012), b) 3.4-3.95 ppm containing glucose signals and b) 0.6-1.4 ppm containing lipoprotein signals was conducted to further examine the nature of the outlying behaviour.

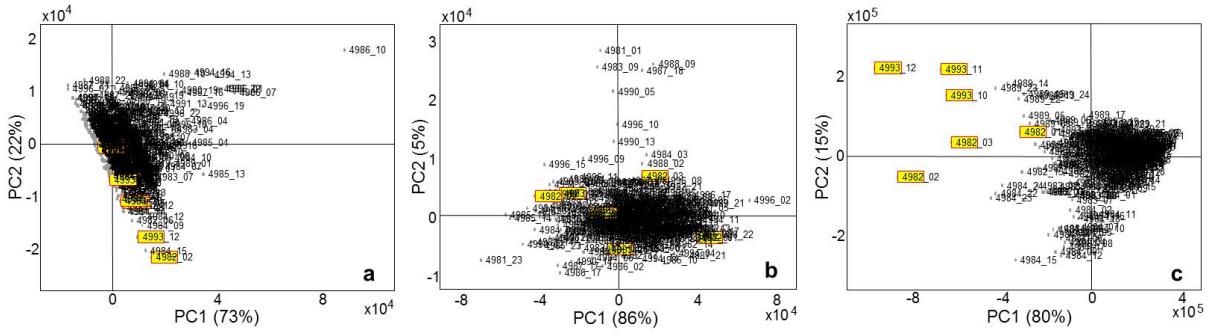


Fig. 9 PCA score plots on mean centered spectra. (a) 3-OH-butyrate signal at 1.2-1.23 ppm. (b) Glucose region at 3.4-3.95 ppm. (c) Lipoprotein region at 0.6-1.4 ppm. Outlying samples 4982_1-3 and 4993_10-12 are highlighted.

As observed in Fig. 9, subject samples 4982_1-3 and 4993_10-12 are not outliers for 3-OH-butyrate (a) and glucose (b) signals, however, they are clearly different from the other samples in the lipoprotein region (c). The average blood glucose levels of samples 4982_1-3

and 4993_10-12 indicate an apparent clearance of their metabolic systems (fasting) prior to blood sampling, whereas the extreme signals in the lipoprotein region indicate fat boosted systems. This is in accordance with the reference data and diet registrations (Ibrügger et al., 2012) showing extreme plasma triglyceride concentrations in addition to 4982 mentioning a high intake (3L) of popcorn the evening before blood sampling. As compared to subjects 4982 and 4993 otherwise showing average metabolic patterns, the outlying behaviour of 4982_1-3 and 4993_10-12 samples therefore is believed to stem from high fat intake the day before sampling. In addition, a potentially lowered capacity to clear fat from their metabolic systems could in fact refer to both individuals showing high BMI (26-27).

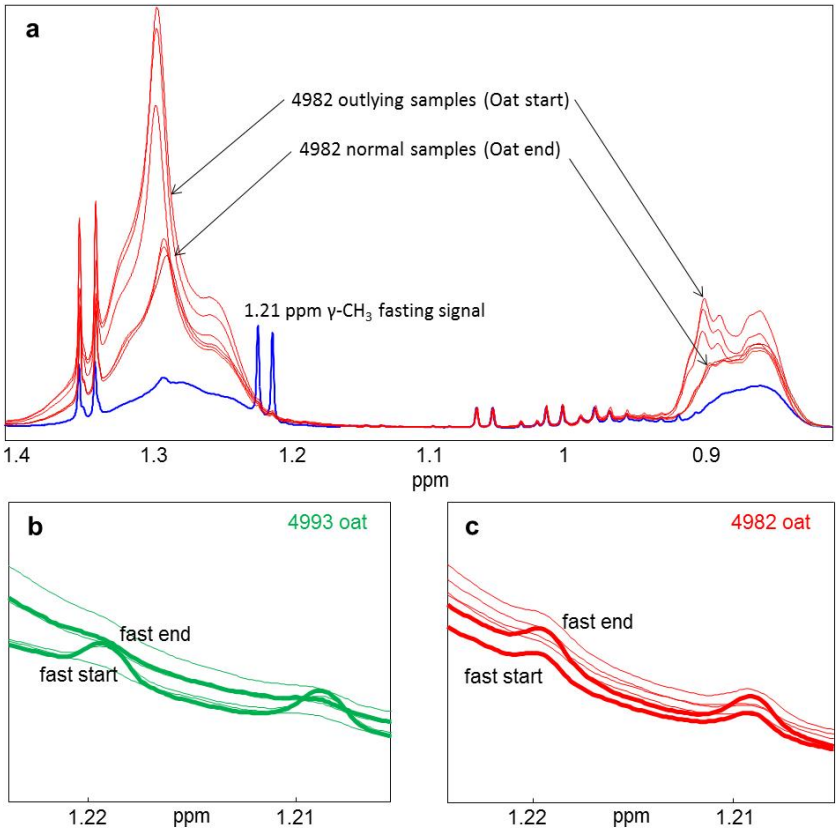


Fig. 10 (a) Lipoprotein region (0.6-1.4 ppm) including the γ-CH_3 fasting signal from 3-OH-butyrate at 1.21 ppm. Blue spectrum is from sample 4986_10 extreme in 3-OH-butyrate. Red spectra are 4982 start and end samples from oat treatment with outlying samples (oat start) showing increased lipoprotein intensities as compared to normal samples (oat end). (b) Oat samples from male 4993 with normal sample (fast start) showing the γ-CH_3 duplet fasting signal in contrast to the outlier sample (fast end). (c) Oat samples from female 4982 with outlier (fast start) and normal (fast end) samples both showing the γ-CH_3 duplet fasting signal at 1.21-1.22 ppm.

Presence of ketone bodies like 3-OH-butyrate among blood metabolites indicate initiation of the fat oxidation (Wijeyesekera et al., 2012) and in turn fasting condition. The γ-CH_3 fasting signal from 3-OH-butyrate at 1.21 ppm was further investigated for the 4982 and 4993 outlying samples with exaggerated lipoprotein profiles (Fig. 10).

As observed in Fig. 10a, the reference 4986_10 sample high in 3-OH-butyrate (blue spectrum) shows low intensities of the 0.9 and 1.3 ppm lipoprotein signals in accordance with a normal fasting metabolic system where fat oxidation from the adipose tissue is initiated. In the 4993 outlying system observed in Fig. 10b (fast end), no γ-CH_3 signal at 1.21 ppm is present as compared to the normal sample (fast start), which indicate a less cleared metabolic system. Interestingly, both outlying and normal fasting samples of 4982 showed the γ-CH_3 signal meaning the onset of fat oxidation in spite of high blood lipoprotein levels (Fig. 10c). Thus, onset of fat oxidation exhibit great inter-individual variation.

Barley and oat β-glucans are recognised for their fibre satiety-, fattening of blood glucose- and hypocholesterolemic effects (Wood, 2007). The 3-OH-butyrate fasting signal may be considered as an approximate hunger and/or satiety biomarker whereas glucose and lipoprotein signals are valid biomarkers for the blood glucose and cholesterol effects, respectively. Time trajectories compressing the 4-armed 3-week intervention study design for 3-OH-butyrate, glucose and lipoprotein signals, respectively, are shown in Fig. 11.

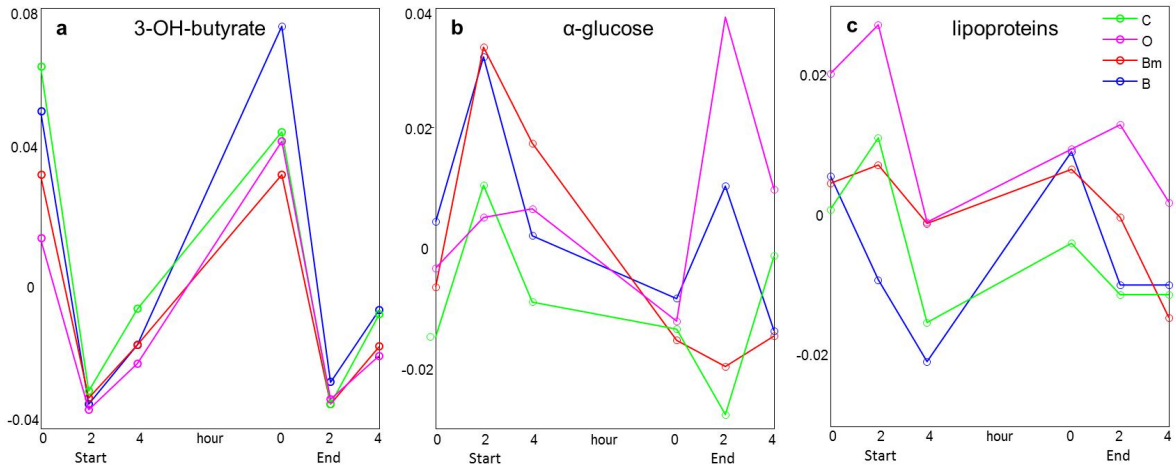


Fig. 11 Time trajectories without outlying samples for: (a) 3-OH-butyrate (1.2-1.23 ppm), (b) α-glucose and glucose region (5.24-5.26, 3.4-3.95 ppm) and (c) lipoproteins (0.6-1.4 ppm). Data are presented as averages across the 14 subjects for the barley mutant (Bm), barley (B), oat (O) and control (C) treatments.

The 3-OH-butyrate high fasting (0h) levels followed by a postprandial drop (2h) and subsequent increases (4h) was a general pattern across all treatments at the start and end of the intervention (Fig. 11a). Thus, no approximate fibre satiety effect was observed from the NMR spectral data. Glucose signals obviously showed the opposite pattern (Fig. 11b), with low fasting levels, postprandial rises (2h) followed by decreasing levels (4h), and no significant differences were seen between treatments. Lipoprotein patterns (Fig. 11c) seemed more promiscuous, however, indicating a slight difference between the control (C) and barley (B) treatment as also seen in the PLS-DA analysis in Fig. 6. Because different metabolomes display different response kinetics upon a nutritional challenge, it is obvious that more frequent postprandial blood sampling would have strengthened the analysis of the glucose and lipoprotein postprandial response patterns.

The lipoprotein region

After initial data analysis, the lipoprotein region was selected for subsequent analysis. Lipoprotein signals referred to plasma triglyceride measures as seen in Fig. 12a and NMR spectral (0.82-0.93 ppm, Fig.12b) prediction of triglyceride content was generally good (Fig. 12c) with r^2=0.9 and RMSECV=0.11 mmol/L.

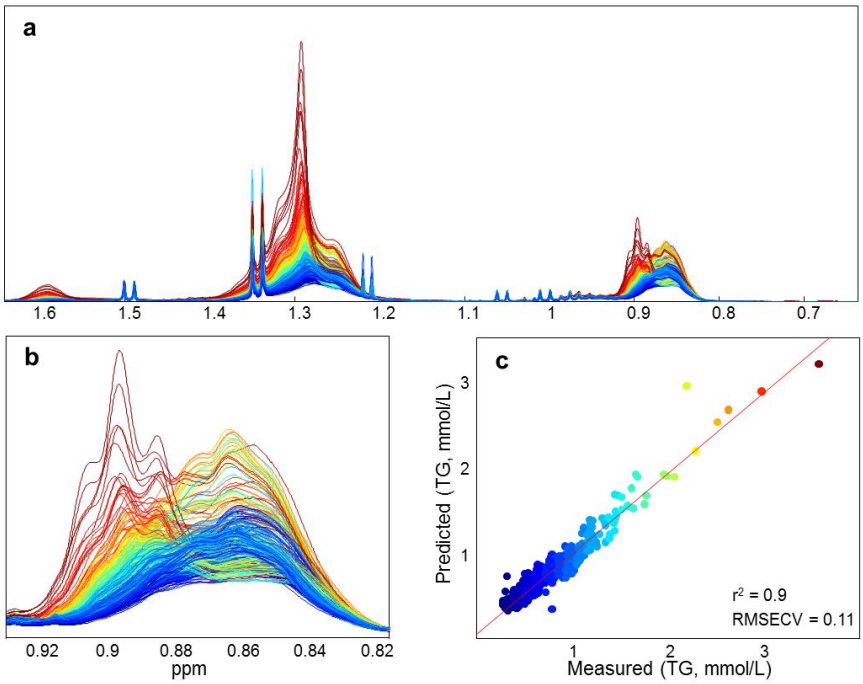


Fig. 12 (a) NMR spectral cholesterol, triglyceride and lipoprotein region (0.6-1.7 ppm) coloured according to reference plasma triglyceride data. (b) NMR lineplot of lipoprotein peaks around 0.9 ppm including outlying samples. (c) PLS result for spectral (0.82-0.93 ppm) prediction of plasma triglyceride (TG) using a 2 latent variable model.

Subject BMI values, spanned equally by males and females, ranged from 19 to 27 and NMR lipid signals seemed to be ranked according to BMI (Fig. 13a). Further investigation of the 0.9 ppm lipoprotein signals (Fig. 13b) indicated some arrangement of the fractions according to BMI but the sample distribution in the PCA score plot (Fig. 13c) cannot be explained by BMI.

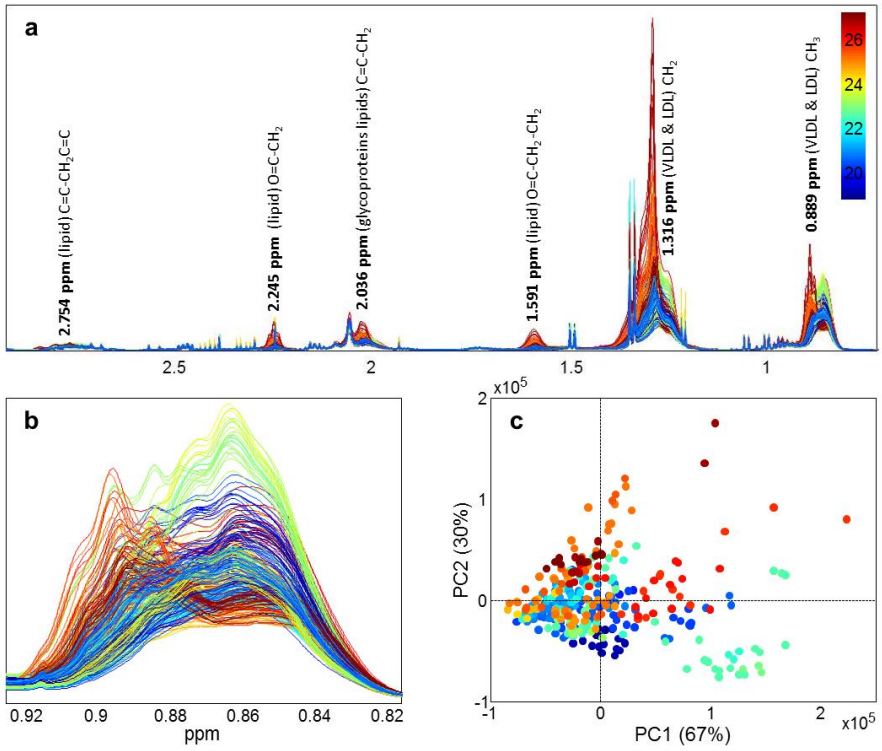


Fig. 13 (a) NMR spectra (0.6-3 ppm) including assignment of the lipid signals. (b) Close up of the 0.9 ppm lipoprotein signals without outlying samples. (c) PCA score plot of the 0.9 ppm lipoprotein signals showing the first two principal components which explain 97% of the data variation. All samples are coloured according to BMI.

Lipoprotein profiles

As previously mentioned, gender constituted the main variance amongst samples and this variance was particularly confounded to the lipoprotein region (Fig. 14).

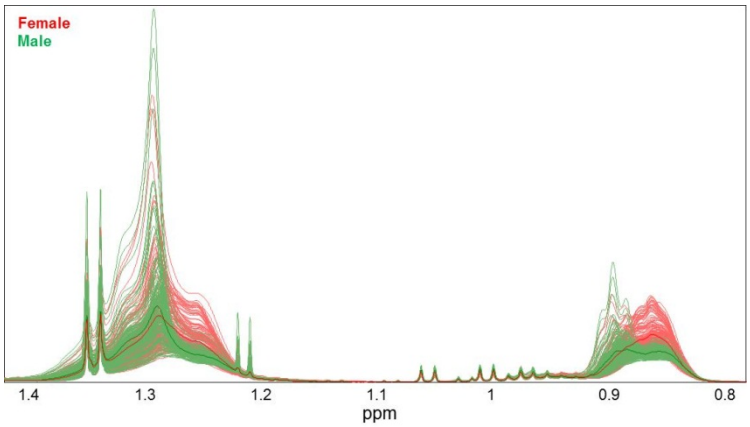


Fig. 14 Plasma NMR profiles of the lipoprotein region (0.6-1.4 ppm). Male and female average profiles are highlighted.

As observed in Fig. 14, clear differences exist for male and female lipoprotein profiles, which is in accordance with earlier findings (Heitmann, 1992). The general male 0.9 ppm lipoprotein profile is oriented to the left whereas the female profile is oriented to the right. To investigate dependence of gender and BMI upon the lipoprotein profile profiles of male and female subjects with equal BMI were compared. Apparently, the profiles around 0.9 ppm are sharpened with increasing BMI, as illustrated in Fig. 15a-c. This indicates the existence of subject unique lipoprotein profiles dependent on both gender and BMI.

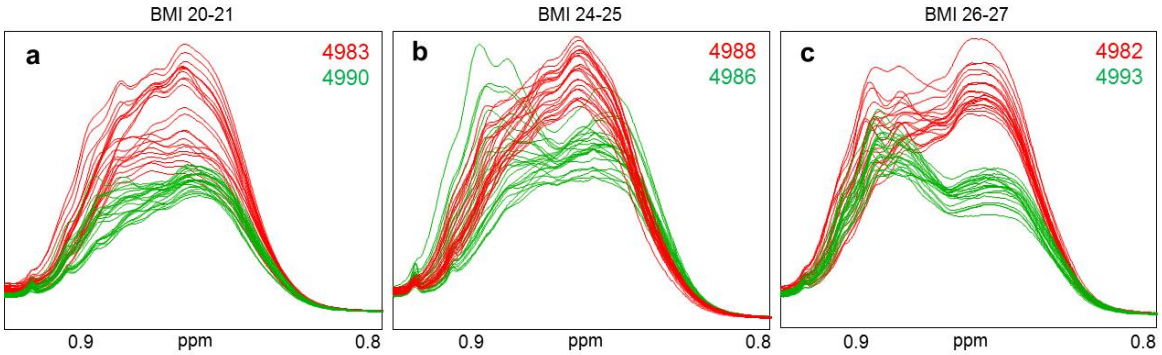


Fig. 15 Lipoprotein profiles around 0.9 ppm of male and female subjects with BMI 20-21 (a), 24-25 (b) and 26-27 (c).

The 0.9 ppm profiles of subject samples 4982_1-3 and 4993_10-12 were compared to their counterpart normal samples to examine the effect from high fat diets on the subject lipoprotein fingerprint.

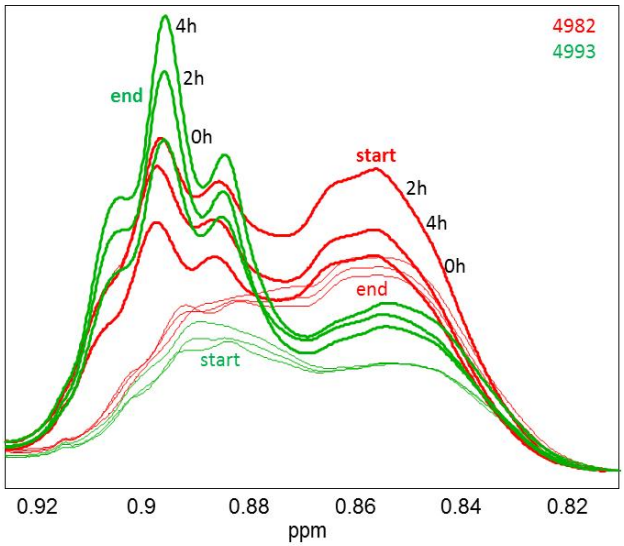


Fig. 16 Lineplot of the 0.9 ppm lipoprotein peaks for 4982 and 4993 on oat treatment (outlier samples 4982_1-3 and 4992_10-12 are marked with bold).

Figure 16 shows the highly elevated intensities for the fat boosted samples as compared to normal and it is observed that the 0.9 ppm lipoprotein landscape is significantly sharpened. Thus, the subject dependent lipoprotein profile is further magnified by high dietary fat intake. Presumably, such systems might be more prone to respond to the hypocholesterolemic actions of β-glucan dietary fibres. Clearly, further human studies using more susceptible volunteers, longer intervention period or extreme doses are required to explore the plasma metabolic effectiveness of barley and oat β-glucans.

Conclusions

No significant blood metabolic exposure and effect markers were identified for 3-week 3.3 g/day consumption of extracted barley and oat β-glucan as investigated by NMR spectroscopy. However, a tendency to postprandial effect of barley β-glucan compared to control was indicated and most pronounced in males. The results of this study reveal the existence of subject unique lipoprotein profiles, which are dependent on gender, BMI and diet. Finally, on the basis of fat boosted outliers it is hypothesised, that β-glucan driven health effects from 3.3 g/day may only be detectable in young healthy adults when being fat boosted.

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