Extractable Cell-Wall Polysaccharides in Cereals, with Emphasis on β-Glucan in Steeped and Germinated Barley

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Abstract

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Barley is a valuable cereal with an old tradition in malt, food and feed. With new food applications this cereal can be re-discovered. Addition of a malting step in a food process can change the texture and taste of a product. It is also a process with high potential for improvement of nutrient availability.

Extractable cell-wall polysaccharides have been linked to some positive health effects in humans. In cereals, these polysaccharides are mainly β-glucan and arabinoxylan. The structure of extractable arabinoxylan and the content of bound ferulic acid dimers were studied in different cereals. For determination of amount and molecular weight of β-glucan a high performance size-exclusion chromatography system (HPSEC) was set up with detection based on the specific binding of Calcofluor to β-glucan. This system was calibrated using a purified β-glucan fractionated into known narrow molecular weight ranges.

A naked and a covered barley steeped and germinated at different temperatures and with different additions in the steeping water were studied. The total content and the average molecular weight of β-glucan in the treated samples showed small changes after steeping at high temperature (48 °C), while steeping at lower temperature (15 °C) gave a significantly lower content. This was correlated with a strong increase of β-glucanase activity over the time of germination. Addition of lactic acid to the steeping water at 48 °C decreased the β-glucanase activity.

Porridges made from two malts with a low phytate content and intact β-glucan were introduced into a dynamic gastrointestinal model. Yield of β-glucan was found to increase with time spent in the model, while average molecular weight of β-glucan decreased.

Key words: β-Glucan, steeping, germination, barley, average molecular weight, size exclusion chromatography, Calcofluor and arabinoxylan.

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Sammanfattning

Korn är ett spannmål med en lång tradition inom malt, mat och foder. Korn har ett högt kostfiberinnehåll, vilket till största delen är β-glukaner men en del är också arabinoxylaner. Korn har också hög koncentration av stärkelse, proteiner, vitaminer och andra nutritionellt viktiga komponenter. För att korn ska återupptäckas som en del i vår kost behövs nya tillämpningar. Mältning ger en annorlunda textur och smak vilket kan utnyttjas vid tillverkning av produkter. Det är också en process som har en hög potential att öka tillgängligheten av näringsämnen. Detta på grund av att enzymer aktiveras under processen vilka bryter ner cellväggsstrukturen.

Mältningsprocessen är en trestegsprocess där man först blötlägger kornet för att sedan låta det gro. Groningen stoppas av ett torkningssteg. Processen kontrolleras bland annat av temperatur och vattenhalt. En viktig grupp av enzymer som aktiveras under mältning är de som bryter ner β-glukaner. Att β-glukanerna bryts ner är viktigt då man använder malt för att brygga öl, eftersom β-glukaner med hög molekylvikt skapar problem vid filtrering av ölet. Att bibehålla β-glukanerna under en process kan dock vara av intresse eftersom vissa positiva hälsoeffekter, såsom sänkt kolesterolhalt, har kopplats till dem.

Ett naket och ett täckt korn blötlades och groddes vid två olika temperaturer och med olika tillsatser i svattnet. Förändringen i total halt β-glukan och molekylvikt var liten då kornet blötlades vid den högre temperaturen (48 °C), den lägre temperaturen (15 °C) gav en mycket lägre halt och molekylvikt. Samtidigt noterades en kraftig ökning av β-glukan nedbrytande enzym för korn blötlagt vid den lägre temperaturen. Tillsats av mjölksyra vattnet vid blötläggningen minskade enzymaktiviteten vilket bevarade β-glukanet. Den höga temperaturen valdes för att den visats vara optimal för nedbrytning av fytat, en substans som finns i alla spannmål. I kroppen hindrar fytat absorptionen av viktiga mineral genom att göra dem olösliga.

Två malter, gjorda för att ha en låg fytathalt och en oförändrad β-glukan, användes för att koka gröt på. Dessa introducerades till en dynamisk *in vitro* mag-tarm modell och prover samlades. Förändring i extraktionsutbyte och molekylvikt av β-glukan bestämdes med hjälp av ett instrument där β-glukaner detekteras med hjälp av calcofluor. Detta är ett fluorescerande ämne som binder specifikt till β-glukan, vilket gör att proverna inte behöver upprenas innan analys. Längre tid i mag-tarm modellen gav ett högre extraktionsutbytet av β-glukan, medan medel molekylvikten av β-glukan minskade. Storleken på arabinoxylan minskade vid längre tid i mag-tarm modellen för det ena maltprovet, medan i det andra var de i stort sett oförändrade.

Contents

Appendix

Papers I-VI

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Dervilly-Pinel, G., Rimsten, L., Sauliner, L., Andersson, R., and Åman P. 2001. Water-extractable arabinoxylan from pearled flours of wheat, barley, rye and triticale. Evidence for the presence of ferulic acid dimers and their involvement in gel formation. *Journal of Cereal Science* 34(2), 207-214.

II. Rimsten, L., Stenberg, T., Andersson, R., Andersson A., and Åman P. 2003. Determination of β-glucan molecular weight using SEC with Calcofluor detection in cereal extracts. *Accepted for publication in Cereal Chemistry.*

III. Rimsten, L., Haraldsson, A-K., Andersson, R., Alminger, M., Sandberg, A-S., and Åman, P. 2002. Effects of malting on β-glucanase and phytase activity in barley grain. *Journal of the Science of Food and Agriculture*, 82(8), 904-912.

IV. Haraldsson, A-K., Rimsten, L., Alminger, M., Andlid, T., Andersson, R., Sandberg, A-S., and Åman, P. Effects of different steeping conditions on phytase and β-glucanase activity and microbiological quality in barley during malting. *Submitted for publication.*

V. Rimsten, L. and Andersson, R. Molecular weight distribution and structure of β-glucan in germinated barley. *Submitted for publication.*

VI. Rimsten, L., Andersson, R., Haraldsson, A-K., Alminger, M., Sandberg, A-S., and Åman, P. β-Glucan from barley is degraded during digestion in an *in vitro* gastrointestinal model. *Manuscript.*

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Objectives

- To characterise arabinoxylans and confirm the presence of ferulic acid dimers in water-extractable arabinoxylan from common cereals (**Paper I**)

- To set up a method for molecular weight determination of β-glucan using size exclusion chromatography and Calcofluor detection with a novel calibration (**Paper II**)

- To investigate the potential to decrease the activity of endogenous β-glucanase in barley malt, while the activity of phytase is high (**Papers III & IV**)

- To investigate changes in content of total and insoluble β-glucan over the time of germination for malted barley samples with different β-glucanase activities (**Papers III &IV**)

- To isolate and characterise extractable β-glucan from germinated barley with different β-glucanase activity (**Papers III & V**)

- To study the molecular weight distribution of β-glucan extractable in a specific temperature range from germinated barley with different β-glucanase activities (**Paper V**)

- To investigate whether molecular weight of β-glucan and size of arabinoxylan change during the digestion in a dynamic *in vitro* gastrointestinal model (**Paper VI**)

Introduction

Importance of barley and its major dietary fibre components

Barley is one of the major cereal grains, grown all over the world. One reason for this is its ability to grow in a wide diversity of environments (McIntosh *et al*., 1993). There are three distinct end-uses for barley: alcoholic beverages, human foods and feed, the latter being the main use. In Western countries only a small quantity of barley is used for food production (Edney, 1996), although in recent years there has been a demand to increase the intake of barley in humans. This interest stems from the many health benefits associated with whole grain cereals and dietary fibre, such as lower plasma cholesterol (Newman *et al*., 1989; Davidsson, Lynn & Gould, 1991), reduced glycaemic index (Jenkins *et al*., 2002; Cavallero *et al*., 2002). These effects are at least partly related to soluble dietary fibre and the viscosity that they bring to a solution. It is primarily β-glucan that is associated with these effects but arabinoxylan may also be involved since both fibre types have been shown to increase viscosity in a solution (Newman & Newman, 1992). Some important factors that influence solution viscosity are molecular size, structure and concentration.

Barley has a high content of β-glucan and also a high concentration of starch, protein, vitamins and other components of nutritional importance. This in all gives a cereal with a good composition of nutrients. However, like other cereals, barley contains large quantities of phytate, which is the major storage form of phosphorus in plants. Phytate negatively affects the bioavailability of essential minerals by forming insoluble mineral-phytate complexes at physiological pH and thereby inhibiting absorption. During food bioprocessing, such as bread baking and malting, phytate can be degraded by enzymatic hydrolysis (Larsson & Sandberg, 1992) giving an improved absorption of iron and zinc (Nävert, Sandström & Cederblad, 1985, Brune *et al*., 1992).

The malting process has been used for centuries to soften kernels and to impart distinctive flavours and colours. The most common area of use is brewing, where the malting process is used to get a good source of fermentable sugars for alcoholic fermentation. However, the process is also of interest for other purposes because it develops distinctive flavours and has the potential to increase the bioavailability of nutrients (Bamforth & Barclay, 1993). These are all effects that arise from the activation of enzymes during the malting process, which degrade the cell wall structure. One group of enzymes activated is β-glucan degrading enzymes, which are desirable in the brewing industry since high molecular weight β-glucan may cause problems when filtering beer. However, to keep the molecular weight of β-glucan high during processing may be of interest, because of the physiological effects connected with β-glucan.

The overall goal of this project was to be able to produce a product with a high nutritional value based on malted barley with intact β-glucan and extensively degraded phytate. To achieve this, the parameters during malting were chosen to achieve a low β-glucanase activity but high activity of phytase during the process.

The cereal grain

Structure of the grain with focus on barley

Cereal grains produce one seeded dry fruit called a caryopsis, more commonly named kernel or grain. The barley (*Hordeum vulgare L.*) kernel, like other cereal grains, contains carbohydrates, proteins, lipids, minerals, vitamins and other minor components (Evers & Millart, 2002). The outermost part of the grain, the husk (lemma and palea), contains almost all the lignin of the grain but hemicellulose and cellulose fibres are also present (Munck, 1981). Next is the pericarp (fruit coat), which has a chemical composition that resembles the husk except for the lack of lignin. The pericarp is closely attached to the testa, which surrounds the endosperm. The endosperm is the dominant component in grains and also the component with the most value. It consists of an aleurone layer and starchy endosperm. The aleurone layer of barley is two or three cells thick, while for wheat, oats and rye it is only one cell thick, and constitutes about 5 % of the grain (Stone, 1985). Aleurone cells are block-like, with thick walls and large nuclei occurring in layers surrounding the starchy endosperm (Evers & Millart, 2002). The aleurone cell walls are built up mainly of arabinoxylan and β-glucan (Bacic and Stone 1981). In barley, the aleurone cell walls contain about 67 % arabinoxylan and 26 % β-glucan, in contrast with the starchy endosperm cell walls of barley, which contain mainly β-glucan (75 %) and only 20 % arabinoxylan (Fincher & Stone, 1986). The starchy endosperm cell walls enclose starch granules embedded in a protein matrix. The germ, which consists of scutellum and embryo, comprises about 3 % of the grain and is rich in protein and fat.

Covered versus naked cultivars

The most common cultivars of barley and oats are covered, while most wheat and rye cultivars are naked. The hulls of a covered cereal are strongly attached to the seed after threshing, while in a naked type the hulls are loose and therefore lost during this process (Bhatty, 1986). The weight of a kernel from a naked type of barley is about 10 % less than that of a covered one (Munck, 1981). Most of the barley used in the Western world today is covered, for example covered barley is preferred in brewing. This is since the husk protects the acrospire during germination and serves as an aid for filtration (Bamforth & Barclay, 1993). For production of foods from covered barley, a prior dehulling step needs to be included. This is done by pearling, which is a gradual removal of the outer part of the kernel leaving a central part of the endosperm. The final product of fine pearl may not constitute more than 60-70 % of the grain (Bamforth & Barclay, 1993), which leads to a loss of essential amino acids and vitamins (Newman & Newman, 1991) as well as other bioactive components. Naked barley is therefore advantageous to use in food production since no hull needs to be removed and thus all nutrients are retained. In addition, using naked barley for malting has previously been shown to produce a malt with a composition and enzyme activities comparable to that of normal malts (Bhatty, 1996).

Non-starch polysaccharides

Dietary fibre consists of a mixture of components with a varying degree of solubility. The definition and methodology for measuring dietary fibre have been extensively debated, mainly regarding whether dietary fibre should be defined by its physiological attributes or by its chemical composition (Prosky, 2000). A general physiological definition states that dietary fibre consists of plant polysaccharides and lignin resistant to hydrolysis by the digestive enzymes of humans (Trowell, 1976). There are also more chemical definitions. One, which is used in the so-called "Uppsala-method", states that dietary fibre is the sum of non-starch polysaccharide residues, amylase-resistant starch and Klason lignin (Theander *et al*., 1995). Different countries and research groups have adopted different definitions for dietary fibre, which has led to inconsistent results. Therefore a committee was appointed by the American Association of Cereal Chemists (AACC) to evaluate the definitions and methodologies used. An updated definition was presented by this committee in 2001 which concluded that "Dietary fibre is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (DeVries, 2001). The committee also concluded that dietary fibre includes polysaccharides, oligosaccharides, lignin and associated substances.

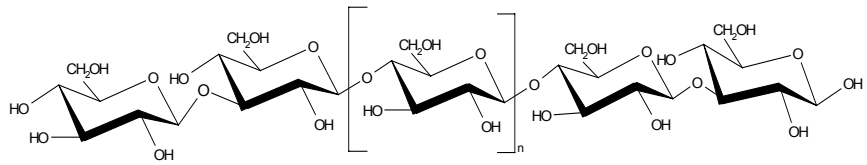
Dietary fibre polysaccharides are generally composed of ten different monosaccharides; arabinose, xylose, glucose, mannose, galactose, fucose, rhamnose, galaturonic acid, glucuronic acid and 4-O-methyl-glucoronic acid linked together to form different types of polymers. Polysaccharides contain ten or more sugar residues linked together by glycosidic linkages. The most common dietary fibre polysaccharides in cereals are cellulose, β-glucan and arabinoxylan.

Cellulose is the major structural polysaccharide of plants, in cereal grains it is the major component in the husk and outer layers. It is an unbranched linear molecule composed of (1→4) linked β-D-glucose residues, which easily associates with itself. This association and its high order make the molecule insoluble and therefore resistant to pathological attacks.

β-Glucan

The main component of the starchy endosperm cell walls of barley is β-glucan (Fig. 1), which is a family of polysaccharides that are heterogeneous in size, solubility and molecular structure (Bacic and Stone, 1981). The highest content of β-glucan is found in barley (2 - 11 %) and oats (2 - 7.5 %), while for wheat and rye it is a less abundant constituent (0.5-1 % and 1.4 –2.6 %, respectively) (Henry, 1987; Bhatty, 1992; Oscarsson *et al*., 1996; Nilsson *et al*., 1997)

β-Glucan consists of long linear chains of glucose residues linked through both β-(1→3) and β-(1→4)-linkages. The distribution of these linkages is neither random nor in a strictly repeating manner (Staudte *et al*., 1983). The (1→3)-linkages occur singly, while most of the (1→4)-linkages occur in groups of two or



n= 1 or 2 (90 %)
n=3 –13 (10 %)

Figure 1. Structure of β–glucan

three. This leads to a structure of predominantly (about 90 %) β-(1→3)-linked cellotriosyl and cellotetraosyl units (Woodward, Fincher & Stone, 1983; Wood, Weisz & Blackwell, 1994) (Fig. 1). The rest is said to contain longer blocks of 4-15 adjacent (1→4)-linked β-D-glucopyranosyl units (Wood, Weisz & Blackwell, 1994).

The molar ratio of cellotriosyl to cellotetraosyl units has been shown to vary depending on the source. Wheat, barley and rye have a consistently higher ratio of these components than oats (Wood, Weisz & Blackwell, 1994). The ratio of cellotriosyl to cellotetraosyl units is important for the differences in physical properties, such as solubility and viscosity.

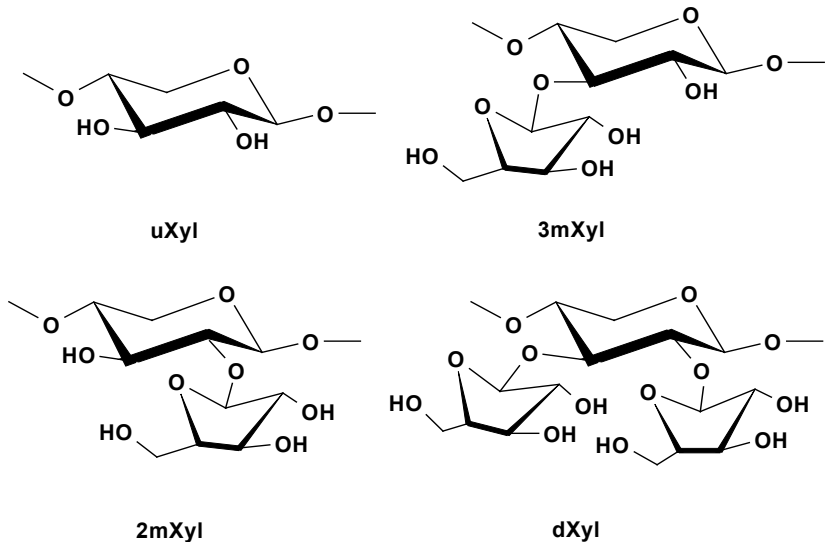


Figure 2. Main structural elements of arabinoxylan

Arabinoxylan

In recent years, wheat arabinoxylan has attracted attention since it has proven to have a significant influence on the water balance and rheological properties of dough, retrogradation of starch and bread quality. Arabinoxylan is a minor component of entire cereal grain, but constitutes an important part of plant cell walls. Together with other polysaccharides it builds up the cell walls of grain tissue and thus becomes a part of the skeletal framework (Fincher & Stone, 1986). It is a part of the so-called hemicellulose that is generally believed to act as cement between cellulose fibres.

Cereal arabinoxylans are a heterogeneous group in which substitution patterns and degree of polymerisation vary (Vinkx & Delcour, 1996). They have a common (1→4)-β-D-xylopyranosyl backbone that carries different substituents depending on the source of plant cell wall material. The major substituent is α-L-arabinofuranose residues attached by (1→3)- and/or (1→2)-glycosidic linkages. This gives four differently linked xylose residues, one unsubstituted (uXyl), two monosubstituted at either C2 (2mXyl) or C3 (3mXyl) and one disubstituted (dXyl) (Fig. 2) (Bengtsson, Åman & Andersson, 1991; Hoffmann *et al*., 1991; Gruppen, Hamer & Voragen, 1992; Gruppen, Kormelink & Voragen, 1993). Other minor, but common, substituents bound to the xylan backbone are 4-O-methyl-glucuronic acid and glucuronic acid. These uronic acids are bound to the C2 atom of the xylose residue (Fincher, 1975). The side groups are responsible for the solubility of arabinoxylan (Amado & Neukom, 1985). The relationship between the differently linked xylose residues in different cereals can be determined by NMR-spectroscopy. Wheat, barley and triticale have been found to contain similar proportions of uXyl, mXyl and dXyl in water-extractable arabinoxylan (Anderssson, Westerlund & Åman, 1994; Oscarsson *et al*., 1996; **Paper I**). However, barley was the only cereal studied that contained significant amounts of monosubstituted arabinoxylan at C2. A higher proportion of 3mXyl was found for rye, while uXyl and dXyl were generally in the same range for all cereals studied.

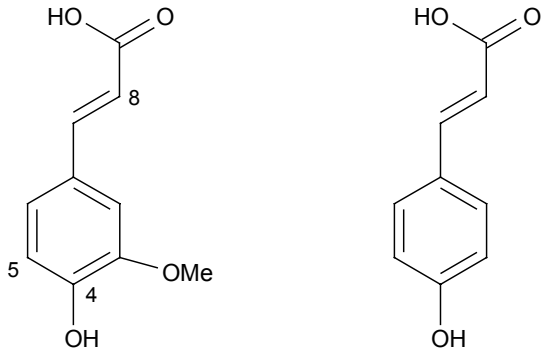


Figure 3. Structure of ferulic acid and *p*-Coumaric acid. Atom numbers depict the binding positions of the ferulic acid dimers.

Phenolic components bound to arabinoxylan

Plant cell walls contain bound phenolic acids, such as ferulic acid and *p*-coumaric acids (Ishii, 1997) (Fig. 3). In cereal heteroxylans, they are generally linked by ester linkage to the O-5 position of the arabinofuranose in the heteroxylans (Ishii, 1997; Sauliner & Thibault, 1999) and to the O-4 position of xylose in xyloglucans (Ishii & Hiroi, 1990). In the cell wall, ferulic acid has been found to be present as dimers and this leads to cross-linkage of cell wall polysaccharides (Geissmann & Neukom, 1973; Ralph, Grabber & Hatfield, 1995; Sauliner & Thibault, 1999). Dimers of hydroxycinnamates can be formed through oxidative cross-linking of the phenolic moieties by the action of peroxidase (Ishii, 1997, Ralph *et al*., 1994) or through photoisomerism by UV-light (Morrison, Hartely & Himmelbach, 1992). This cross-linking modifies the structure and is believed to play a very important role in the plant, since it influences the mechanical properties of the cell wall such as accessibility, extensibility, digestibility (Ishii, 1997).

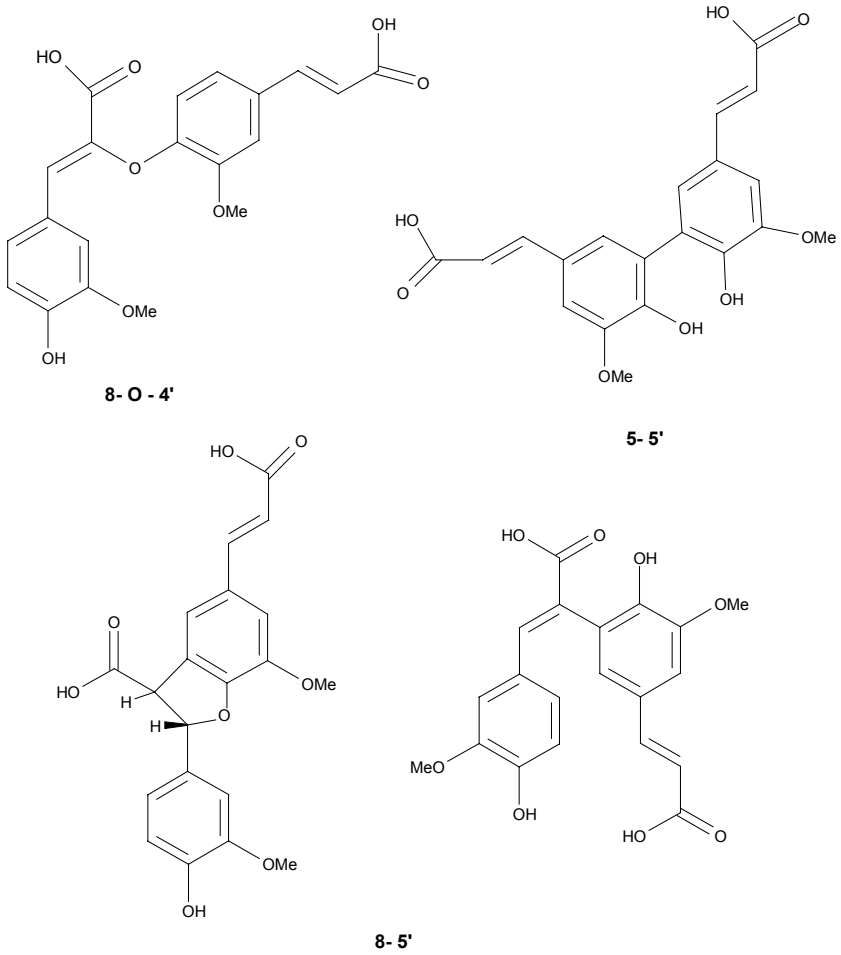


Figure 4. Structure of the dimers of ferulic acid found in barley, wheat, oats and rye

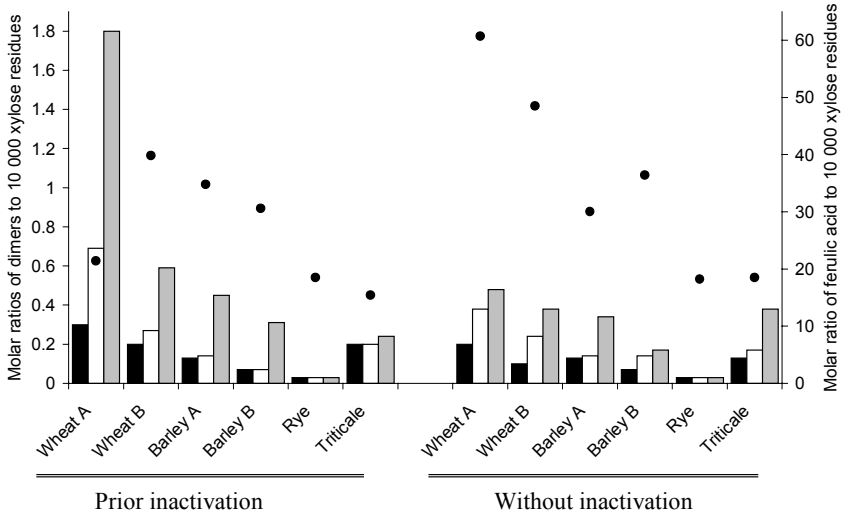


Figure 5. Amount of ferulic acid (●) and its dimers 5-5' (black bars), 8-0-4' (white bars) and 8-5' (grey bars) in wheat A (Soissons), wheat B (Sideral), barley A (Cindy), barley B (SW 8775), rye (Amando) and triticale (Trimaran) with and without enzyme inactivation prior to extraction.

Ralph *et al*. (1994) found five different dimers of ferulic acid in grass cell walls. Three of these types, 8-0-4′, 8-5′ and 5-5′ (Fig. 4) have been detected in water-extractable arabinoxylan from wheat (Figueroa-Espinoza & Rouau, 1998; Dervilly *et al*., 2000) We wanted to confirm the presence of ferulic dimers in other common cereals (**Paper I**). The three dimers, 8-0-4′, 8-5′ and 5-5′, were found in all cereals investigated (Fig. 5). Dimer formation has sometimes been claimed to be partly an extraction artefact arising from, for example, active endogenous peroxidases. Another extraction was therefore performed with a prior inactivation, by boiling in 80 % EtOH for 30 min with constant stirring. The same three dimers were also found in these extracts (Fig. 5). In wheat, the content of dimers was higher than without inactivation, while in barley the content was lower. This indicates that dimers are present *in vivo*, but during extraction additional dimers may be formed for some cereals, possibly for cereals with a higher activity of endogenous peroxidases (**Paper I**).

Soluble fractions of arabinoxylan have an ability to form gels under the influence of oxidative substances (Geissmann & Neukom, 1973; Markwalder & Neukom, 1976; Izydorzyck, Biliaderis & Bushuk, 1991) in both aqueous solutions and in concentrated flour suspensions (Meuser & Sukow, 1986). The mechanism involves the formation of a network by cross-linking of adjacent ferulic acids on the arabinoxylan. That ferulic acid is involved in the mechanism has been shown by the decrease of bound ferulic acid monomers in oxidised arabinoxylan, at the same time as ferulic acid dimers are increased (Izydorzyck & Biliaderis, 1992; Figueroa-

Espinoza & Rouau, 1998; **Paper I**). However, Izydorczyck & Biliaderis (1992) concluded that only arabinoxylan fractions of high molecular weight and with a relatively unsubstituted xylan back bone structure were able to form extensive cross-linking when high concentrations of ferulic acid were present.

Arabinogalactan

Arabinogalactans are predominantly found in the endosperm. They consist of a highly branched structure in which the galactopyranosyl units are bound through (1→3) and (1→6) glycosidic linkages to each other. Only single arabinose units are linked β-glycosidically to the galatose chain (Neukom & Markwalder, 1975; Amado & Neukom, 1985). This polymer is covalently bound to a peptide containing amino-acid hydroxyproline, which forms part of the link to the polymer (Fincher & Stone, 1974; Fincher, Sawyer & Stone, 1974).

Extraction and analysis of β-glucan

The extractability

The structure of β-glucan is the same as for cellulose except for the β-(1→3)-linkages, which introduce a kink to the chain. This gives molecules with less order and with a reduced tendency to aggregate and β-glucan is therefore a molecule that is partially soluble in water. How solubility of β-glucan is influenced by the order and frequency of these linkages has been under a lot of investigation. Some studies have shown that longer sequences of (1→4)-linkages give less soluble β-glucans because of intermolecular associations (Fincher & Stone, 1986; Woodward, Fincher & Stone, 1983). However, Izawa, Kano & Koshino (1993) 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 (Böhme & Kulicke, 1999; Cui & Wood, 2000).

The solubility or extractability of β-glucan is not only influenced by the structure of β-glucan but also by, for example, properties of the solute, such as temperature and pH. Wood, Paton & Siddiqui (1977) stated that the amount of β-glucan dissolved depends on fineness of grind, temperature, ionic strength and pH of the solvent. Therefore is it important to also state which conditions are used when talking about extractability of β-glucan. Generally it seems that the extractability of β-glucan increases with elevated temperatures and pH (Knuckles, Yokoyama & Chiu, 1997). The extractability also seems to depend on pre-treatments such as drying and heating, for example a prior boiling step with ethanol might increase extractability of β-glucan (Knuckles, Yokoyama, & Chiu, 1997; **Paper II**). A strong base like NaOH alone gives a higher yield than water at any temperature (Knuckles, Yokoyama & Chiu, 1997; Beer, Wood & Weisz, 1997; **Paper II**)

A complete extraction (solubilisation) of β-glucan can be accomplished by adding enzymes when extracting or by consecutive extractions from the same material. For example, complete extraction of β-glucan from oats or barley was found for consecutive extractions with water at different temperatures and a final extraction with NaOH (Knuckles, Yokoyama & Chiu; 1997; Beer, Wood & Weisz, 1997). Kanauchii & Bamforth (2001) studied the ability of a range of enzymes to solubilise β-glucan from the cell walls of barley. They found that lichenase, endo-1,4-β-xylanases, α-L-arabinofuranosidase and esterase all released β-glucan to a greater or lesser extent. It has been speculated that an arabinoxylan coating restricts the access of solvating water to β-glucan (Palmer, 1989), which was supported by Kanauchii & Bamforth (2001) since all enzymes tested except for lichenase were associated with arabinoxylan rather than β-glucan degradation. However, since lichenase is able to access on its own, this suggests that the covering of β-glucan is incomplete (Kanauchii & Bamforth, 2001). Some of the solubilising effect from xylanase might also arise from contaminating β-glucanase. For example, a rather large contamination of β-glucanase activity was found in Grindamyl H640 (Danisco, Copenhagen, Denmark) and Bio-Feed Wheat L (Novoenzymes A/S, Bagsvaerd, Denmark) and to a smaller extent in Xylanase M1 (Megazyme, Wicklow, Ireland) when these were used for β-glucan extraction (**Paper II**). Lichenase gives an almost complete extraction of β-glucan and is used for the extraction in the analysis of total content of β-glucan (McCleary & Glennie-Holmes, 1985). However, since the β-glucan is degraded, it is not a possible method for determination of molecular weight. Therefore molecular weight is determined most often on the β-glucan that is extractable under non-degenerative conditions.

Molecular weight determination

The molecular weight of β-glucan has been studied extensively since the physiological effects coupled to β-glucan seem to be related to its size. β-Glucan, as well as other polymers, have molecules covering a fairly wide range of molecular weights, a molecular weight gives an average of this distribution. However, the reported molecular weights of β-glucan vary significantly between studies. The main reasons for these discrepancies are probably the isolation procedures and the methods of determination.

HPSEC-RI-MALLS or HPSEC-RI-RALLS-Visc

A common way to determine the molecular weight and size (radius of gyration) of β-glucan is by using high performance size exclusion chromatography with refractive index detection and multi angle laser light scattering (HPSEC-RI-MALLS) or with right angle light scattering combined with a viscosity detector (HPSEC-RI-RALLS-Visc). The light scattering (LS) technique is said to be absolute, as it can determine molecular weights and mean square radius independently of any calibration or reference standards (Wyatt, 1993). However, for accurate determinations, the concentration of each eluting fraction given by the specific refractive index in the mobile phase used (*dn/dc*) is needed (Jackson &

Barth, 1995). For most polymers this value remains essentially constant over the range of masses measured (Wyatt, 1993). The LS-detectors and viscometer are non-specific and samples need therefore to be purified prior to the analysis in-order to get rid of other molecules that might co-elute.

The molecular weight is determined by illuminating the molecule with a beam of laser light at a certain wavelength and measuring the light scattered by the molecule, which is in direct proportion to molecular weight and concentration (White, 1999). Light scattering from multiple angles (MALLS) yields the weight average molecular weight ($\overline{M}_w$) after extrapolating the scattering data to zero angle. The variation in the scattered light with angle also depends on the mean square radius of the molecule. The primary result obtained by RI-Visc is the intrinsic viscosity, if the concentration in the sample is considered to be low enough. The $\overline{M}_w$ is considered to provide secondary data, when calculated from the intrinsic viscosity assuming an idealized chain conformation and using the 90° light scattering detector signal to generate a particle scattering function. The values of $\overline{M}_w$ obtained by SEC-RI-MALLS are of primary origin. In size exclusion chromatography, it is assumed that each slice of a chromatogram contains molecules of a very narrow molecular weight distribution.

Most of the cell wall polysaccharides are polydisperse, which means that they consist of molecules with a variety of chain lengths. An estimate of the polydispersity of a polymer is obtained from the ratio of $\overline{M}_w$ to number average molecular weight ($\overline{M}_n$). The $\overline{M}_w$ is influenced by the presence of large molecules, while $\overline{M}_n$ is strongly influenced by the presence of small molecules. For a monodisperse polymer the $\overline{M}_w$ equals $\overline{M}_n$ giving a polydispersity of 1, all molecules thus having identical molecular weights.

HPSEC-FD

Another way to determine molecular weight of β-glucan involves the specific binding of Calcofluor (Wood & Fulcher, 1978; Wood, 1980). This binding results in an increase in fluorescence intensity that is proportional to the concentration of β-glucan in solutions. It was first developed as a method to quantify β-glucan (Wood and Weisz, 1984; Mekis, Pinter & Bendek, 1987; Jørgensen, 1988), and led to the Calcofluor-flow injection analysis (FIA) system. The method started as an off-line process (Foldager and Jørgensen, 1984; Anderson, 1990; Manzanares *et al*., 1993), but has now been developed to an on-line post-column detection method (Wood, Weisz & Mahn, 1991; Suortti, 1993). In this method, extracts of β-glucan are first separated by size-exclusion chromatography (SEC) and then mixed with Calcofluor. The β-glucan-Calcofluor complex results in an increase in fluorescence intensity that can be detected by a fluorescence detector. The presence of other polysaccharides does not disturb the detection, since Calcofluor is selective for β-glucan. Therefore this method provides a simple way to determine molecular weights of β-glucan in extracts, without any prior purification steps. A high performance size exclusion chromatography system with fluorescence detection (HPSEC-FD) was set up in our lab (**Paper II**, Fig. 6).

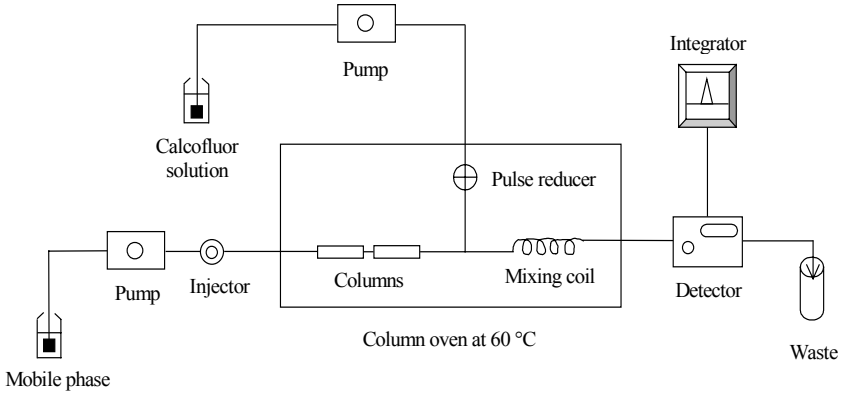


Figure 6. The set up of the high performance size exclusion chromatography-system with fluorescence detection

This type of SEC is a very precise method as only the elution volume and the relative detector signal are measured to determine molecular weight, though an accurate measurement is dependent on the accuracy of the calibration curve. Calibration of the columns is therefore an important step when setting up a HPSEC-system. Previously, pullulan standards have been used for calibration, but that has been shown to lead to overestimation of the molecular Wood, Weisz & Mahn, 1991; Vårum, Martinsen, & Smidsröd, 1991). Another approach has been to use purified β-glucan of known average molecular weight for the calibration (Wood, Weisz & Mahn, 1991; Suortti 1993). This is possible if each fraction covers a narrow molecular weight range while at the same time all fractions together cover the wide range of molecular weights. So far there are no β-glucan standards available to fulfil these criteria. Therefore we fractionated a purified β-glucan from germinated barley with a wide molecular weight range using a HPSEC-RI-MALLS-system with three serially connected columns (**Paper II**). The average molecular weight was determined for each 0.5 ml fraction with the HPSEC-RI-MALLS-system before injection on the HPSEC-FD. The peaks from each fraction were symmetrically distributed and covered a narrow molecular weight range, and their retention time in the HPSEC-FD-system was plotted against the molecular weight from the HPSEC-RI-MALLS-system for each fraction (Fig. 7). The curve showed a linear (r^2 = 0.995) relationship between log average molecular weight and retention time and it was used to calculate Calcofluor average molecular weight ($\overline{M}_{cf}$). This average includes only β-glucans large enough to be detected by Calcofluor, which is claimed to be β-glucans with a molecular weight over 10 000 D (Jørgensen & Aastrup, 1988). The molecular weight has previously been determined by the peak retention time, but our average is calculated by taking the whole area under the curve into account. This is to give a more true value for peaks not normally distributed or that are polymodal and cannot be described by a single retention time. Percentiles were also calculated, describing the molecular weight at which, for example, 10, 50 and 90 % of the

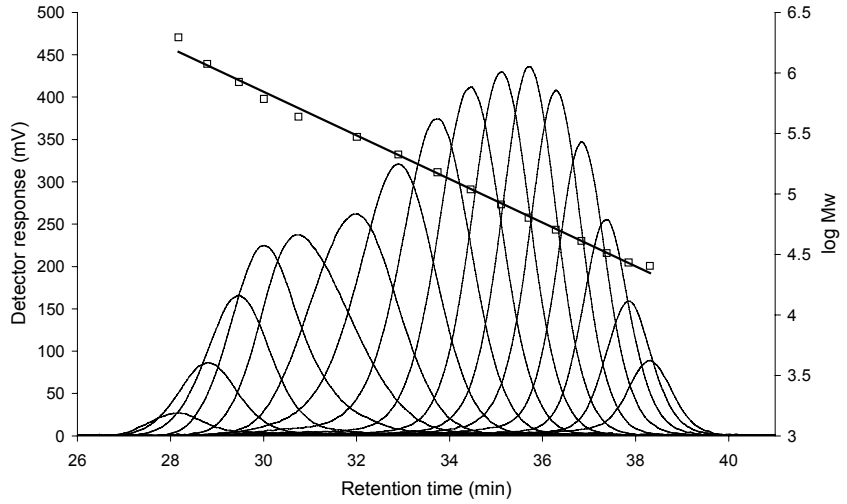


Figure 7. HPSEC-FD-chromatograms of the isolated β-glucan from germinated barley and their retention time in the HPSEC-FD-system plotted against log $\overline{Mw}$ from HPSEC-MALLS-RI.

distribution fall below that value. This gives a picture of how the molecular weights of β-glucans are distributed.

Calibration of the concentration for HPSEC is usually not required because only relative concentrations are needed. However, if this is done the injection on the HPSEC-FD will not only give the average molecular weight but also the amount of β-glucan on which it is determined. This of importance since, as said before, complete extraction of β-glucan is not easily accomplished and different samples give different extraction yields. The response in our system was tested by making dilutions (0.1-1 mg/mL) of four purified β-glucans of different molecular weights, three from barley and one from oats (**Paper II**). Barley and oats were chosen since they are structurally different with regard to their ratio of cellotriosyl to cellotetraosyl units (Wood, Weisz & Blackwell, 1991). Two dilutions of crude extracts were also included, these had molecular weights 10 times higher than the purified β-glucans. All β-glucans had similar linear relationships over increasing concentration with no difference in response between β-glucan from oats or barley. This would indicate that structural differences like the ratio between cellotriosyl and cellotetraosyl units do not influence the binding of Calcofluor. It also means that there is no difference in response between the β-glucans of low average molecular weight compared to those with high in the range tested or between purified β-glucans and β-glucans in a crude extract. Thus this method can be used as a fast and simple way to determine average molecular weight and extraction yield of β-glucan.

To get an accurate result an inactivation step prior to extraction is of importance. This is since we found that even a low β-glucanase activity in a sample will decrease the molecular weight of β-glucan significantly during 24 h. Complete inactivation of β-glucanase activity has previously been shown to be difficult, for example boiling in water or ethanol did not lead to a sufficient inactivation. However, these are ways to inhibit activity to increase stability of samples (Forrest and Wainwright, 1979; Wood, Weisz & Mahn, 1991; Knuckles, Yokoyama & Chiu, 1997; Knuckles and Chiu 1999; **Paper II**). It has also been found that high pH reduces activity of β-glucanase (Bamforth, Martin & Wainwright, 1979; Knuckles and Chiu, 1999).

Determination of β-glucan structure

To determine the primary structure of a complex carbohydrate, several characteristics have to be determined, including for example the glycosyl residue composition, their absolute configuration and type of glycosidic linkages between residues. It also includes linkages to other polymers, such as proteins, polyphenols or other polysaccharides (McNeil *et al*., 1982). Two techniques commonly used to determine part of the primary structure of β-glucan are NMR-spectroscopy and analysis of fragments released by lichenase with high performance anion-exchange chromatography with pulsed amperiometric detection (HPAEC-PAD).

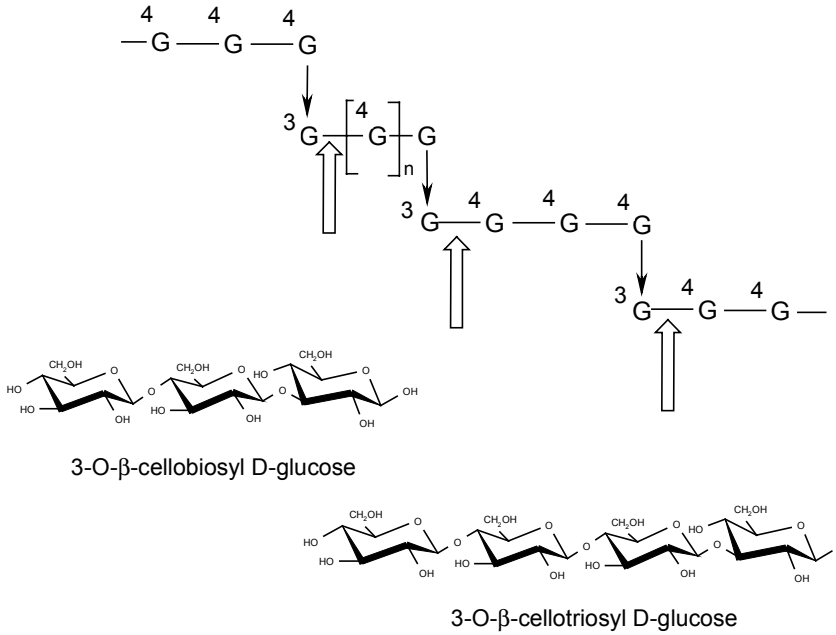


Figure 8. Structure of barely β-glucan (n ≥1) with the hydrolysing sites for lichenase (white arrows) and its major degradation products.

NMR can be used as a direct identification tool or to determine the purity of an isolated polysaccharide fraction. It can also provide information about glycosyl linkage composition and anomeric configuration of each residue. The number of anomeric signals in a NMR-spectrum reflects the number of differently linked sugar residues, the molar proportion of which can be calculated from the corresponding integrals (Åman & Westerlund, 1996).

Determination of oligosaccharides after lichenase digestion

Lichenase cleaves the (1→4)-linkages of the 3-O-substituted glucose residues (Fig. 8), from which fragments have been analysed by several techniques, for example by various chromatographic analyses (Woodward, Fincher & Stone, 1983; Wood, Weisz & Blackwell, 1991). However, these methods do not have an adequate resolution for peaks from oligosaccharides with a degree of polymerization (DP) above 4. Wood, Weisz & Blackwell (1994) successfully applied HPAEC-PAD to analyse these oligosaccharides. This system had previously been shown to sensitively and selectively identify oligosaccharides of a wide DP range as anions at elevated pH (Koizumi *et al.,* 1989).

In general, the sensitivity of PAD decreases rapidly from DP 2 to DP 6, while for higher oligosaccharides the decrease in the sensitivity is more limited (Timmermans *et al*., 1994). There are no standards available to test this response, but they can be isolated from a lichenase-hydrolysed purified β-glucan. Wood, Weisz & Blackwell (1994) concluded that the response factors for DP 3 and DP 4 were 0.49 and 0.42 respectively but could vary depending on electrode used and the difference was therefore negligible. The response from oligosaccharides with a degree of polymerisation (DP) 3, 4, 5 and 6 was tested in our HPAEC-PAD-system and it was found that the response of DP 4 was 85 %, DP 5 52 % and DP 6 43 % of the response of DP 3. Therefore these response factors were used to calculate the weight percentage of DP 3, 4, 5 and 6 of β-glucan from barley that had been steeped and germinated.

Malting

All cereal grains are potentially sources of nutrients for humans and livestock and serve mainly as a source of energy. However, processing is needed to make this energy available in feed or food. The malting process has been used for centuries to soften kernels and to impart distinctive flavours and colours. The most common area of use is brewing, where the malting process is used to get a good source of fermentable sugars for alcoholic fermentation. However, the process is also of interest for other purposes with respect to its potential to increase the bioavailability of nutrients, impart distinctive flavours and change flour texture (Bamforth & Barclay, 1993). The endosperm of cereals stores starch and protein that can later be degraded by enzymes to provide energy and nitrogen, for example during germination. The enzymes that degrade starch and protein are compartmentalised by the walls of the starchy endosperm. An important event in the germination process is thus to degrade the starchy endosperm cell wall. The

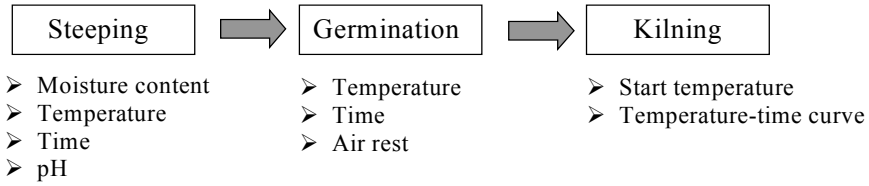


Figure 9. The three steps included in the malting process and the parameters for each step that can be varied to obtain desired result

malting process consists of three steps: steeping, germination and kilning, which all include parameters that can be varied to obtain the desired result (Fig. 9).

Steeping

During steeping, the barley is soaked in water to reach a defined moisture content, which is usually around 42-46 % moisture. Temperatures used are normally around 14-18 °C for up to 48 h (Bamforth, 2002). Steeping is the most important step in malting and the most critical one since the uptake and distribution of moisture in the kernels influences the quality of the malt. At the beginning of the steeping, the embryo and husk absorb water more rapidly than the starchy endosperm. This water uptake seems to be regulated by the embryo, by an unknown mechanism (Bamforth & Barclay, 1993).

Various factors influence the uptake of water, such as character of the barley, which includes for example variety of the barley, kernel size and initial moisture content (Axcell, Jankovsky & Morall, 1983). The steeping process of course also influences the water uptake. Parameters that can be changed here are, for example, temperature of the steeping water and the supply of oxygen to support respiration. Oxygen is supplied in the malting process by an air rest or by oxygenating the water. This removes carbon dioxide and ethanol, which are produced as a result of the respiratory metabolism in the embryo and aleurone tissues and from the action of microorganisms populating the surface tissues (Bamforth & Barclay, 1993). The combinations of air and water are innumerable, but all have the same goal, to activate the embryo and evenly hydrate the endosperm.

Germination

Germination is controlled by several different factors, with the most important being the supply of oxygen, removal of carbon dioxide and elimination of heat formed by respiration. Germination is usually performed at 16-20 °C (Bamforth, 2000). At a higher temperature the grains germinate faster and thus produce enzymes at an earlier stage. However, the rate of the formation of enzymes is stopped after some time, thus in the end grains germinated at lower temperatures contain higher levels of enzyme than grains germinated at higher temperatures (Palmer, 1989). Long cool germination cycles maximise fermentability and minimise malting losses. During germination, moisture is transferred from the malt

to the surrounding air. To sustain its growth, the embryo withdraws moisture from the starchy endosperm, causing a progressive drying (0.5% per day) of the endosperm. This is helped by spraying with water to retain the moisture content. About 4 % of the initial barley dry matter is lost to embryo respiration (Bamforth & Barclay, 1993).

Germination is initiated by the embryo releasing gibberellins, which are believed to be responsible for the production of hydrolytic enzymes in aleurone cells and scutellum (Palmer, 1989). The hydrolytic enzymes are released into the starchy endosperm, where starch and protein reserves are contained within the cell walls forming a network throughout the tissue. These walls contain mainly β-glucans and therefore β-glucan-degrading enzymes are important in providing access to the starch reserve for the amylolytic enzymes. Starch is an important energy source for the embryo (Fincher & Stone, 1986). An extensive break-down of β-glucan is desirable since a high content of β-glucan in malt affects the quality of the beer negatively (Bamforth & Barclay, 1993).

Kilning

The objectives of kilning are to stop botanical growth and internal modification and to reduce moisture content in the kernel. Chemical changes during kilning, mostly due to Maillard reactions, result in colour and flavour compounds that differ depending on the temperature used for this drying process (Bamforth & Martin, 1983).

To maximise survival of β-glucanase it is important to begin drying at a relatively mild temperature (40-50 °C) and to progressively increase the temperature, since enzymes withstand heat better at lower moisture content (Bamforth, 1994). For example, Bamforth & Martin (1983) showed that after drying germinated barley from 39.8 % to 25.9 % at 45 °C for 2 h, only 34 % of β-glucanase remains. However, continued drying at the same temperature gives little further loss. Drying at higher temperatures (65 °C and 85 °C) for the same time leads to losses of more than 95 %.

Solubilisation and hydrolysis of β-glucans by endogenous β-glucanase during malting

β-glucanases activated during malting

The β-glucan can be hydrolysed by three classes of β-glucanases detected in germinating barley. These are (1→4)-β-glucan 4-glucanohydrolase (cellulase), (1→3)(1→4)-β-glucan 4-glucanohydrolase (lichenase or barley β-glucanase) and to a lesser extent by), (1→3)-β-glucan glucanohydrolase (endo-(1→3)-glucanase) (Bamforth, 1982). These three classes of β-glucanase are able to hydrolyse most of the endosperm β-glucans to a mixture of β-linked oligosaccharides. To further degrade the oligosaccharides to glucose it is believed that β-glucosidase activity is required (Leah *et al*., 1995).

During the malting process cellulase has been shown to be secreted by microorganisms derived from fungal populations on the surface of the barley kernel (Yin & MacGregor, 1988 & 1989). This enzyme cleaves β-(1→4)-linkages within a long series of β-(1→4)-linked glucose units. Its role is rather unclear, since it seems unlikely that enzymes produced by microorganisms have a function in cell wall modification during germination (Bamforth & Barclay, 1993). However, ungerminated grains have been shown to also contain some cellulase activity (Bamforth, 1982).

The function of endo-(1→3)-β-glucanase in the germinating grain is difficult to ascertain, since it only catalyses the hydrolysis of several contiguous (1→3)-β-glucosyl linkages (Bathgate, Palmer & Wilson, 1974; Høj *et al.*, 1988) which are not present in β-glucans from barley endosperm cell walls (Woodward, JR., Phillips & Fincher, 1988). However, endo-(1→3)-β-glucanase is widely distributed in plants and increases markedly in barley grains during germination (Ballance, Meredith & Laberge, 1976). It has therefore been suggested that it may protect the grain against possible microbial attacks during malting (Fincher, 1989).

Barley β-glucanase seems to be the most important of the three classes since it is abundant in the endosperm of germinating grain and rapidly depolymerises (1→3)(1→4)-β-glucans (Woodward & Fincher, 1982b). It hydrolyses the (1→4)-β-glucosyl linkages in β-glucan that are adjacent to a (1→3)-β-glucosyl linkage. The major products released are 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellotriosyl-D-glucose (Fig. 8)(Woodward & Fincher, 1982b). There are principally two types of barley β-glucanase, designated isoenzyme I and II, which were separated by Woodward & Fincher (1982a & b). Both enzymes are basic and have similar kinetic properties and substrate specificities. However, they differ in size, isoelectric point and carbohydrate content (Woodward & Fincher, 1982a). Isoenzyme I is synthesised mainly in the scutellum, whereas isoenzyme II is produced exclusively in the aleurone layer (Stuart, Loi & Fincher, 1986). Both purified enzymes have the same pH optimum, at 4.7, and both lose approximately 90 % of their activity at 1.5 pH units from the optimum (Woodward & Fincher, 1982b).

β-Glucanase activity in malt

Several methods have been used to measure the activity of β-glucanase in malt, for example the rate of decrease in viscosity of a β-glucan solution (Bamforth, 1982) and rate of increase in reducing sugar equivalents have been used. However, the most common and accepted method today is that by McCleary & Shameer (1987). The principle is that azo-barley glucan substrate is depolymerised by the β-glucanase from the malt extract, fragments of which can be measured on the spectrophotometer. This method measures the activity of both barley β-glucanase and cellulase because both enzymes hydrolyse azo-barley glucan. It was developed to measure β-glucanase activity in malt, which usually contains high activities. To differentiate between two samples containing very low activities with this method is though more difficult. Therefore we used the HPSEC-FD-system to determine whether samples contained any β-glucanase activity (**Papers II & VI**). Sample

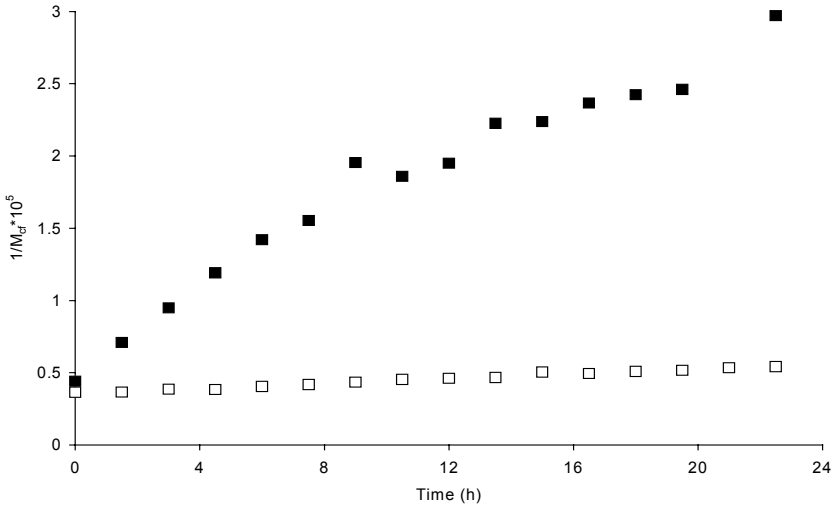


Figure 10. The inverse average molecular weight of a purified β-glucan mixed with two different enzyme extracts as a function of enzyme reaction time.

extracts were mixed with a solution of a purified β-glucan and injected repeatedly on the HPSEC-FD-system over 24 h. The inverse average molecular weight for each injection and sample was plotted against time after mixing (Fig. 10). By comparing these curves from each sample we can identify which sample contains the highest activity of β-glucanase. Thus no absolute values are determined, only relative levels.

The activity of β-glucanase in the malting process has been extensively studied, since this breakdown of β-glucan is important for the brewing industry. High molecular weight β-glucan may cause problems when filtering beer. Most studies have therefore had the overall goal to increase β-glucanase activity during malting. However, since β-glucan of high molecular weight may have some positive physiological effects, the overall goal in our experiments was the opposite (**Papers III & IV**). Thus we wanted to study how to stop or minimise the activity of β-glucanase during malting.

In the first malting trial (**Paper III**) two different steeping temperatures (15 and 48 °C), two moisture contents (38 and 42 %) and two germination temperatures (15 and 18 °C) were used (Table 1). The trial was set up as a full factorial design with these three variables and two levels, giving a total of eight experiments per barley genotype. The barley cultivars tested were one naked (SW 8775) and one covered barley (Cindy). The experiment showed that the steeping temperature was overall the most important factor in inactivation of β-glucanase activity (Fig. 11) while the other factors influenced the outcome very slightly. During germination of samples steeped at 48 °C, β-glucanase was activated in a slower mode than in samples steeped at 15 °C over the time of germination. Moisture content and germination temperatures had no or low effect on the activity of β-glucanase.

Table 1. *Experimental design for malting trial 1*

Experiment number	Steeping temperature (°C)	Moisture (%)	Germination temperature (°C)
1	15	38	15
2	15	42	15
3	48	38	15
4	48	42	15
5	15	38	18
6	15	42	18
7	48	38	18
8	48	42	18

A temperature of 15 °C is a common temperature for steeping and the activity of β-glucanase developed during the following germination was as previously reported (Barber, Jackson & Smith, 1994; Ellis *et al*., 1997). The temperature of 48 °C was instead a rather extreme temperature for steeping that, to our knowledge, has not been used before. This temperature was chosen to give optimal conditions for the development of phytate degradation (Bergman, Autio & Sandberg, 2000), since we wanted to have a high degradation rate of phytate.

A second malting trial was carried out (**Paper IV**) in which lactic acid or lactic acid bacteria were added to the steeping water (at 15 or 48 °C). Lactic acid was added to see whether a change in pH increased the activity of phytase. Another reason was that lactic acid during steeping helps to control microbial quality during malting. Addition of lactic acid bacteria in the steeping water has also been found to prevent undesired bacterial growth during malting (Haikara & Laitila, 1995). In this experiment, moisture content and germination temperature were set at 42 % and 15 °C, respectively, since these had previously been shown to have a minor effect on β-glucanase activity. Samples were taken at 0, 48 and 96 h of germination. Again steeping temperature was seen to have a large effect on the activity of β-glucanase. Addition of lactic acid to the steeping water gave a further reduction in activity of β-glucanase at 48 °C, giving almost no activity over time of germination. At 15 °C steeping, the development of β-glucanase activity was slowed down, though after 96 h an increase was seen.

Lactic acid bacteria did not give any significant effect on the β-glucanase activity, which was probably due to unfavourable conditions for its growth. The same trends were seen for both barley cultivars, naked or covered, and only the levels of β-glucanase activity differed.

The effect of β-glucanase activity on β-glucan during malting

Several studies have shown that that with a higher activity of β-glucanase a lower β-glucan content is obtained (Henry, 1989; Ellis *et al*., 1997). Degradation of β-

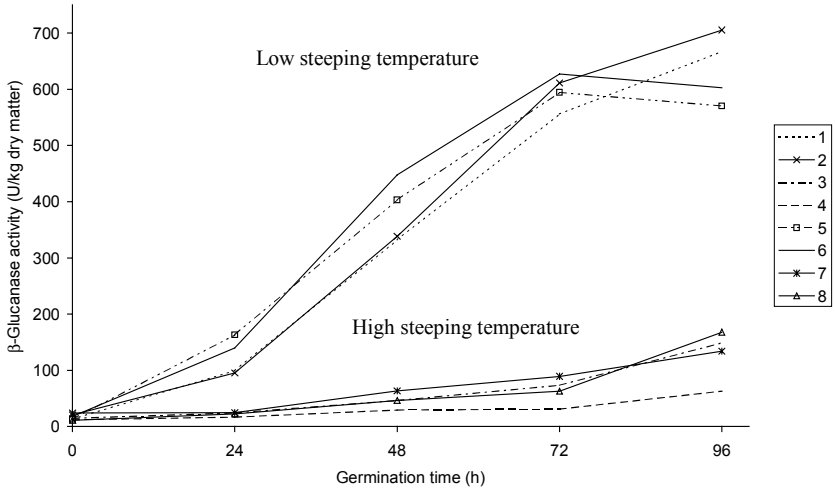


Figure 11. The activity of β-glucanase over the time of germination for the eight experiments with SW 8775 from malting trial 1.

glucan occurs during the germination phase of malting (Bamforth, 1982). This is desirable for malt used for beer or whiskey production, although as previously stated, our goal was to preserve β-glucan during malting. From the results in malting trial 1 it was seen that the samples with lower β-glucanase activity, i.e. steeped at 48 °C, had a higher content of β-glucan after germination compared to the samples with higher activity (15 °C steeping) (**Paper IV**), which was confirmed in malting trial 2 (**Paper IV**). In this malting trial, addition of lactic acid in combination with steeping at 48 °C gave an almost unchanged content of β-glucan (Fig. 12). Total content of β-glucan was higher in the covered barley Cindy than in the naked barley SW 8775, while the activity of β-glucanase was higher in SW 8775.

Unextractable β-glucan

The unextractable β-glucan in our experiments was that not extractable in water at 38 °C for 2 h (Åman & Graham, 1987). The change in content of unextractable β-glucan followed that of total β-glucan, but with an even stronger trend. This has also been observed previously by Bamforth & Martin (1981) and is probably due to initial breakdown of unextractable β-glucan yielding extractable β-glucan fragments detected in total β-glucan analysis. Samples steeped at 48 °C had higher levels than samples steeped at 15 °C. These trends were observed for both Cindy and SW 8775 and in both malting trial 1 and 2 (**Papers III & IV**). Addition of lactic acid to the steeping water at 48 °C, in malting experiment 2, left the unextractable β-glucans unchanged over the time of germination (Fig. 12).

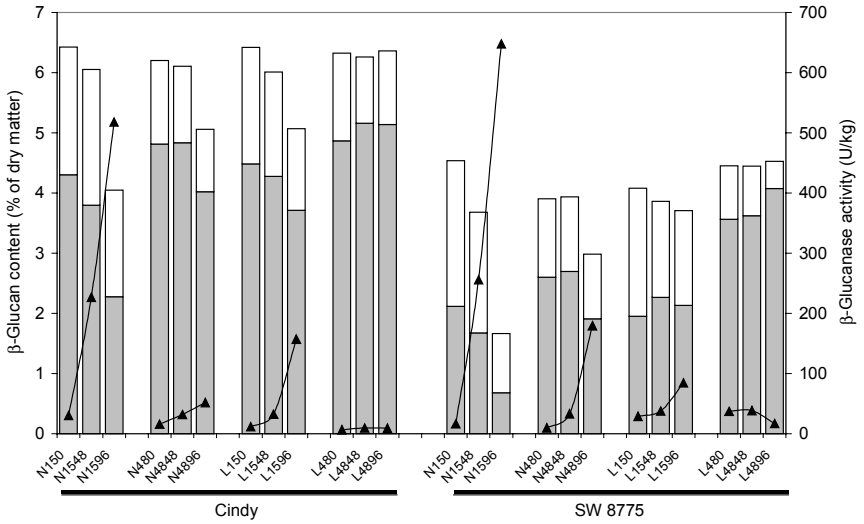


Figure 12. The content of total (grey and white bars) and unextractable β-glucan (grey bars) and activity of β-glucanase (▲) for samples steeped at 15 or 48 °C with no addition (N) or with addition of lactic acid (L) over the time of germination for Cindy and SW 8775 in malting trial 2.

Extractable β-glucan

As stated previously, the amount of extractable β-glucan can be different for the same material depending on conditions used at the extraction. However, one factor that can be interesting to look at when malt is used for food as an end-product is a quasi-physiological state. We therefore looked at the effect of β-glucanase activity on the content of β-glucan extractable at 38 °C (Åman & Graham, 1987). The higher β-glucanase activity in samples steeped at 15 °C reduced the extractable β-glucan over the time of germination compared to the lower activity at 48 °C steeping (Fig. 12)(**Papers III & IV**). However, this reduction was not as strong as seen for the unextractable β-glucan. It was concluded that this might be due to unextractable β-glucan becoming extractable during germination while extractable β-glucan was continuously being degraded to low-molecular-weight fractions and lost in analysis. Less extractable β-glucan was lost during the germination when lactic acid was added to the steeping water at 15 °C, while addition of lactic acid in the steeping water at 48 °C made the extractable β-glucan unchanged during malting (**Paper IV**).

We also looked at a fraction of β-glucan extractable at 100 °C. This was to minimise the effect of active β-glucanase during extraction, and thus this would reveal changes occurring during the malting process (**Paper III**). Contents of neutral sugar residues were determined according to the method of Theander *et al*. (1995) with some modifications according to Andersson *et al*. (1999). A stronger decrease of β-glucans extractable at 100 °C was obtained for samples steeped at the lower temperature (higher β-glucanase activity) than for samples steeped at the

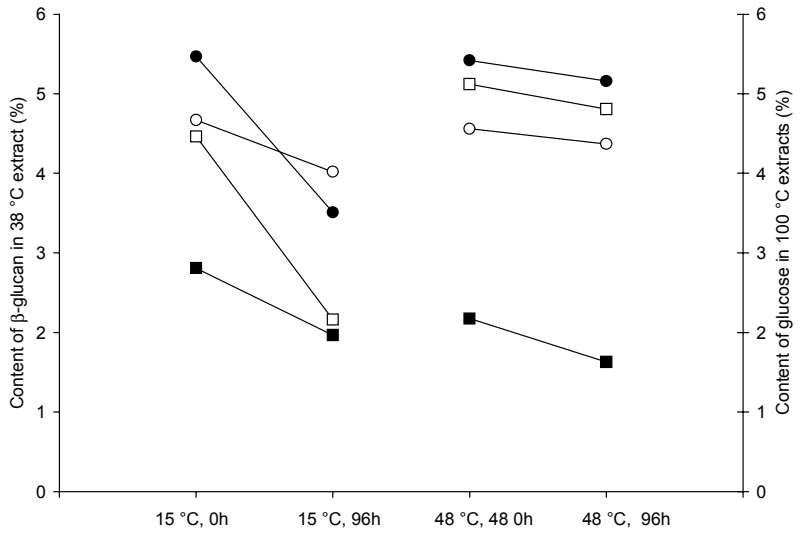


Figure 13. Content of 38 °C extractable β-glucan (■), 38 °C unextractable β-glucan (□), content of 100 °C extractable glucose (●) and 100 °C unextractable glucose (○) in barley (Cindy) steeped at 15 or 48 °C and germinated at 0 or 96 h in malting trial 1.

higher temperature (low β-glucanase activity) over the time of germination. A similar but weaker trend was seen for the β-glucan unextractable at 100 °C (Fig. 13). This was an interesting result since for the extraction at 38 °C, the unextractable β-glucan showed the highest influence of steeping temperature, while influence on the extractable fraction was low. Thus this indicated that the β-glucans extractable at 100 °C but not at 38 °C are those readily available for the endogenous β-glucanase.

Molecular weight distribution and structural features

For some of the samples in malting trial 1 the average molecular weight of the β-glucan extractable at 100 °C was determined using a HPSEC-RI-MALLS-Visc-system (**Paper III**). Data for molecular weight determinations were analysed using ASTRA software (Version 4.70.07, Wyatt Technology Corp., CA, Santa Barbara, USA) based on a dn/dc of 0.147 (Vårum, Smidsrød, & Brant, 1992). The results showed a significantly larger decrease in weight average molecular weight ($\overline{M}_w$) and number average molecular weight ($\overline{M}_n$) for samples with high β-glucanase activity (i.e. low steeping temperature) over the time of germination (Tab.2).

Another fraction of β-glucan was isolated from selected samples from malting trial 2 (**Paper IV**). Samples studied were steeped at 15 or 48 °C, with addition of lactic acid in the steeping water and germinated at 15 °C for 0 or 96 h (**Paper V**). These samples were chosen since they had the largest variation in β-glucanase activity.

Table 2. *Weight average molecular weight ($\overline{M}_w$) and number average molecular weight ($\overline{M}_n$) of β-glucan extracted at 100 °C from samples with different steeping temperatures and germination times. Germination temperature was 15 °C and moisture content was 42 % for all samples*

Cultivar	Steeping Temp. (°C)	Germination time (h)	$\overline{M}_w * 10^{-3}$ (g/mol)	$\overline{M}_n * 10^{-3}$ (g/mol)
Cindy	15	0	190.3	113.8
Cindy	15	96	143.1	81.8
Cindy	48	0	179.4	111.3
Cindy	48	96	176.9	103.2
SW 8775	15	0	169.8	94.7
SW 8775	15	96	121.7	72.1
SW 8775	48	0	169.3	96.0
SW 8775	48	96	149.4	82.3

This was to investigate whether this fraction had any structural differences since the content of β-glucan indicated that these β-glucans are more readily available for the enzyme (Fig. 13). The fractions were isolated by incubating samples at 38 °C for 2 h in water and then from the insoluble residue extract at 100 °C for 1 h. The extracted β-glucans were purified and thereafter freeze-dried. As in the previous malting trial (**Paper III**), the $\overline{M}_n$ and $\overline{M}_w$ were higher for samples with a lower β-glucanase activity (steeped at 48 °C with or without lactic acid). A larger $\overline{M}_w$ and $\overline{M}_n$ of β-glucan in the purified β-glucan was, as expected, found to give solutions with higher viscosity.

The ratio of oligosaccharides after lichenase digestion was determined in the germinated barley samples previously selected from malting trial 2 using the HPAEC-PAD system. The weight ratio of cellotriosyl to cellotetraosyl units in barley samples was higher for samples germinated for 96 h (2.04) compared to samples germinated for 0 h (1.96). However, the weight percentage of DP 5 and 6 was lower for samples germinated for 96 h (DP 5=7.25 and DP 6=4.70), than for samples germinated for 0 h (DP 5=7.62 and DP 6=4.93). Thus β-glucans with a high content of 3, 4 and 5 subsequent (1→4)-linkages were degraded to a higher extent than the rest of the β-glucans during germination (**Paper V**).

The same germinated barley samples from malting trial 2 were also analysed by the HPSEC-FD-system (**Paper V**). Two different extractions were made from the samples, one as described above (extract B) and another with only a 100 °C extraction for 1 h (extract A). No purification was necessary since the detection in this method is specific for β-glucan. This was to study the effect of the extraction at 38 °C for samples with varying β-glucanase activity. The extraction yield for extraction method A was larger than for B, though $\overline{M}_{cf}$ was generally similar. The difference (about 40-50%) was at least partly attributable to the fraction extractable at 38 °C, which had been extracted and discarded before the 100 °C extraction in

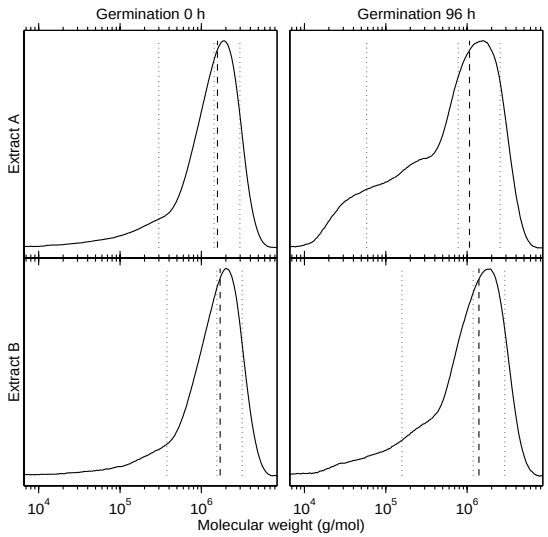


Figure 14. Molecular weight distribution of β-glucan from germinated barley after 100 °C extraction (A) or 38 °C in water and the insoluble residue extracted at 100 °C (B). Steeping was at 15 °C with lactic acid. Dotted lines are the percentiles 10, 50 and 90 %, while the dashed line is the Calcofluor average molecular weight ($\overline{M}_{cf}$) for the sample.

procedure B. However, slightly lower $\overline{M}_{cf}$ values were obtained for samples with high β-glucanase activity (germinated for 96 h) in extract A than those in extract B. This suggests that during the 38 °C extraction for extract B, β-glucans of lower molecular weights are degraded and lost, giving a higher molecular weight average. Comparison of the molecular weight distribution of β-glucan for these samples confirmed that fewer β-glucans were found in the lower molecular weight range (Fig. 14). Thus endogenous β-glucanase seems to have preference for hydrolysing β-glucan of lower molecular weight.

The molecular weight distribution of β-glucan in extracts A and B for samples germinated for 0 h (low β-glucanase activity) were also compared and found to be almost identical (Fig. 14) (**Paper V**). Thus, for these samples an extraction at 38 °C as in the extraction for extract B was mostly influenced by the solubility and not by the activity of endogenous β-glucanase. Thus it seems as though β-glucans with the same molecular weight range are extracted at 38 °C as at 100 °C since if they were not, the distributions would have been different. This result agrees with some previous findings, for example Woodward, Phillips & Fincher (1988) who found similar molecular weights of β-glucan from an extraction at 65 °C compared to an extraction at 40 °C for the same barley sample. However, others have found that an increasing temperature of extraction leads to an increase in molecular weight of the extracted barley (Fleming & Kawakami, 1977; Knuckles & Chiu, 1999).

It was found that the largest β-glucans in extract A were those mostly influenced by the factors affecting β-glucanase, while for extract B the β-glucans of middle-

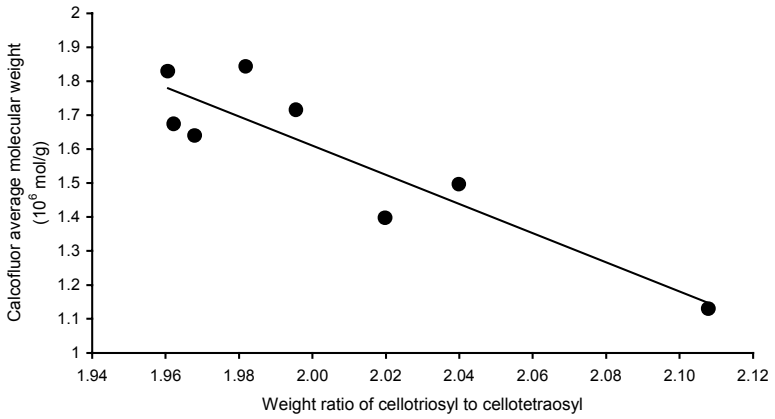


Figure 15. The relationship between weight ratio of cellotriosyl and cellotetraosyl units of β-glucan and $\overline{M}_{cf}$ from extract B of barley from malting trial 2.

range molecular weights were affected instead. A negative correlation was found between $\overline{M}_{cf}$ of β-glucan in extract B and the weight ratio of cellotriosyl to cellotetraosyl of barley samples (R=0.902) (Fig. 15), which was not found for extract A. Thus the extraction at 38 °C was influenced by this ratio, while the extraction at 100 °C was not (**Paper V**).

Physiological aspects of barley

Dietary fibre

Dietary fibre has for centuries been recognised as having several health benefits. However, today a lot of attention is on its cholesterol lowering (Newman *et al*., 1989; Davidson *et al*., 1991) and hypoglycemic effects (Jenkins *et al*., 2002; Cavallero *et al*., 2002). These effects arise from the water binding and gelling properties of dietary fibre, which in turn may influence gastric emptying, faecal flow and absorption of nutrients (Kritchevsky, 2001) and thus act positively on our health and therefore may also have an indirect protective role in other diseases.

The rate of glucose delivery from the gut to the portal vein, i.e. glucose absorption, is slowed down by dietary fibre. This is called the hypoglycemic effect of dietary fibre and is related to both the soluble and insoluble fraction of dietary fibre. It may for example arise from the delayed gastric emptying (Wood *et al*., 1989) and the slower diffusion of glucose from the intestine due to an increased thickness of the water layer (Johnson & Gee, 1981). The hypocholesterolemic effects of barley are due to the soluble fibre, which is mainly the β-glucan. It is suggested that soluble fibre creates a viscosity in the intestine that leads to an entrapment of bile acids followed by excretion, thereby decreasing their reabsorption. This results in less bile acid returning to the liver in a process known as enterohepatic circulation (Story *et al*., 1990). This means that new bile acids

have to be synthesised, which leads to a reduction in serum cholesterol since bile acid is synthesised from cholesterol in the blood (Eastwood, 1992).

High dietary fibre or whole-grain intake has been suggested to be connected to a reduction in incidence of, for example, coronary heart disease (CHD) and certain types of cancer. A reduced risk of CHD by dietary fibre or whole grain intake is associated with the soluble fibre, which produces favourable changes in serum lipoproteins, lower serum insulin levels, lower blood pressures and a lower risk for developing diabetes (Anderson, 2002). Reduction of the risk for colon cancer by dietary fibre or whole grain is based on its bulking effect in the colon, ability to bind to certain toxic metabolites and the generation of short-chain fatty acids (McIntosh & Jacobs, 2002). However, that dietary fibre is solely a protective factor against these multifunctional diseases is not easily established. For example, epidemiological studies have shown that a high fibre diet may be an indicator for a healthy lifestyle (Anderson, 2002).

Phytate

Phytic acid is the hexaphosphoric acid ester of *myo*-inositol, which forms a wide variety of insoluble complexes, in plants usually mineral complexes called *myo*-inositol hexaphosphate ($InsP_6$ or phytate). Phytic acid is strongly negatively charged and has therefore a great potential for complexing positively charged multivalent cations, most often calcium, potassium and magnesium salts. These are complexes either insoluble in the pH conditions in the gastrointestinal tract of humans or difficult to hydrolyse and thus difficult to absorb. This can be of nutritional importance in a diet high in phytate, since essential minerals such as iron and zinc become unavailable for absorption in the human intestine (Sandberg *et al.,* 1999). All cereals contain large quantities of phytate, mainly concentrated in the outer parts of the grain (Bergman, Autio & Sandberg, 2000). Phytate represents the major storage form of phosphorus in cereals. Cereals also contain phytases, endogenous enzymes that can hydrolyse phytate to free inorganic phosphate and *myo*-inositol via lower inositol phosphates (Fig. 16).

During germination and malting, phytase activity in barley has been reported to increase extensively (Bartnik & Szafranska, 1987). An extensive phytate hydrolysis has been shown to improve the absorption of iron and zinc in humans (Nävert, Sandström & Cederblad, 1985, Brune *et al*., 1992). The hydrolysis of phytate has

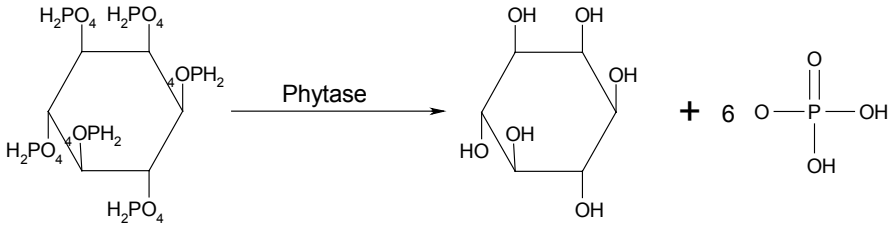


Figure 16. Structure of phytate and the hydrolysis to *myo*-inositol and phosphate groups

been reported to be optimal at 48 °C when soaking whole kernels during hydrothermal processing (Bergman, Autio & Sandberg, 2000). Therefore this temperature was used for steeping in malting trial 1 and 2. In malting trial 1 the content of phytate was decreased, but to a very small extent (**Paper III**). In malting trial 2 high steeping temperature (48 °C) in combination with addition of lactic acid resulted in a large phytate degradation after germination (**Paper IV**). These were also the conditions that were the most favourable for achieving a low β-glucanase activity.

Tocopherol and tocotrienol

The contents of tocopherol and tocotrienol during malting were analysed in samples from malting trial 2. These are compounds that possess vitamin E activity and are naturally occurring in plants. There they act as antioxidants to protect lipids in tissues (White & Xing, 1997), thus a larger content of tocopherol and tocotrienol might give an increased storage stability of a cereal product. All germinated barley samples contained all eight forms of tocopherol and tocotrienol, in accordance with previous results for barley (Peterson & Quershi, 1993, 1994). The germination itself did not alter the contents of tocopherol and tocotrienol significantly in the present study, but it was found that an addition of lactic acid to the steeping water reduced the amounts for both steeping temperatures over the time of germination (**Paper IV**).

A dynamic in vitro gastrointestinal model

To study the effects of barley dietary fibre in the gastrointestinal tract of animals, such as pigs (Johansen, Wood & Bach Knudsen, 1993), chickens (Sundberg, Pettersson & Åman, 1995) and rats (Wood, Weisz & Mahn, 1991) have been used as an *in vivo* model. One drawback with using animals as a model, especially pigs and rats, is their different bacterial flora in comparison to humans. Several human studies has also been performed (Newman *et al*., 1989; Davidson *et al*., 1991), though these are tedious and expensive. An *in vitro* gastrointestinal model was therefore set up by Minekus *et al*. (1995) and further developed by Marteau *et al*. (1997) and Minekus (1998). This computer-controlled model simulates the gastrointestinal transit, pH, composition and rate of secretions and absorption of digested products. It consists of four sections of flexible silicone tubing representing the stomach, duodenum, jejunum and ileum that are squeezed periodically by pump action on the surrounding water, to imitate peristalsis (Fig. 17). The secretion of digestive juices and the pH-adjustments in each section is simulated according to physiological data. This model was used to study ingested β-glucan and arabinoxylan in malted barley under dynamic and complex conditions.

Naked barley SW 1290 and two different malts from this sample were used (**Paper VI**). The malts were made to have a low β-glucanase activity (non-degraded β-glucan), while phytase activity was high (extensive degradation of phytate), the parameters used being based on previous studies (**Papers III & IV**). Malt A was made by steeping at 15 °C with 0.8 % lactic acid until a water content

of 39.2 % was obtained and malt B was made by steeping at 48 °C with 0.8 % lactic acid until a water content of 42.7 % was obtained. Both malts were germinated for 72 h at 15 °C followed by kilning at 50-60 °C for 21 h for malt A and kilning at 50-82 °C for 21 h for malt B. After malting both samples contained β-glucan of a high molecular weight and thus only minimal degradation of β-glucan had occurred during malting. However, the reduction in phytate was not sufficient to give an increased absorption of minerals, since it has previously been shown that an almost complete reduction in phytate is necessary to give an improved mineral absorption (Nävert, Sandström & Cederblad, 1985; Brune *et al*., 1992). Therefore the malted samples were incubated in water at 48 °C, which is a temperature that has previously been shown to minimize β-glucanase activity (**Paper III**). After incubation, the $\overline{M}_{cf}$ of β-glucan was determined on the HPSEC-FD-system in these samples. Only a slight change in $\overline{M}_{cf}$ of β-glucan from malt A occurred (Fig. 18), while for malt B a large part of the β-glucans had moved to a lower molecular weight range (Fig. 19). This decrease in $\overline{M}_{cf}$ during the incubation of malt B might be due to the higher bacterial total count (1.3×10^6) compared to malt A (1.0×10^3), since many bacteria are known to possess β-glucanase activity (**Paper VI**).

Porridges were made from the incubated malts and the raw material and introduced into the gastrointestinal model. First a small sample (1 ml) was extracted from the ileal delivery after 60 min ($ID_{0\text{-}60}$). Thereafter a sample was collected after 120 min ($ID_{0\text{-}120}$) (i.e. the total volume of delivered intestinal contents during this period), and the following two samples after 240 min ($ID_{120\text{-}240}$) and 360 min ($ID_{240\text{-}360}$) (Fig. 17). Extraction yield and $\overline{M}_{cf}$ of β-glucan were determined in porridges and samples collected from the gastrointestinal model using the HPSEC-FD-system as described previously (**Paper VI**).

The extraction yields were generally increased when samples of porridge were introduced into the gastrointestinal model (Fig. 18). This trend was found for all three samples, even though raw material and malt A had significantly higher extraction yields than malt B. That extraction yield increased might be due to an increase in concentration of β-glucan due to absorption of nutrients or that β-glucan becomes more easily extractable the longer the time spent in the model. For the $ID_{240\text{-}360}$ samples, the extraction yield of β-glucan decreased again, which might be due to a more extensive breakdown of β-glucan after a longer time spent in the model. This makes large parts of the β-glucan undetectable, since only β-glucans of molecular weights larger than 10 000 D are detected by this method (Jørgensen and Aastrup, 1988).

The 60 min sample taken from the collection outlet of the ileum gave only a slight change in $\overline{M}_{cf}$ of β-glucan for raw material and malt B and a slightly larger decrease for malt A (Fig. 19). All samples of $ID_{0\text{-}120}$ contained more β-glucan of lower molecular weights than the samples at 60 min, with a $\overline{M}_{cf}$ that were almost half of that found in $ID_{0\text{-}60}$ (Fig. 18). This would indicate that in the beginning, β-glucans of higher molecular weight pass through the model, while smaller β-glucans come later on. This may be for out of two reasons, namely that larger molecules for some reason pass more easily through the model and/or that the

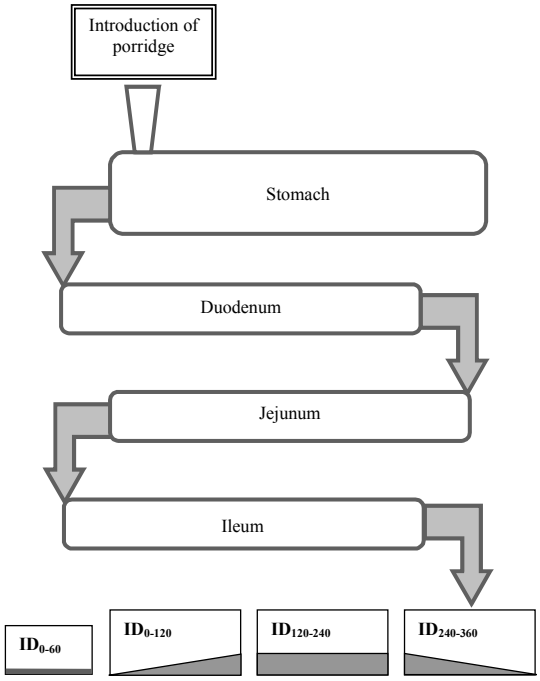


Figure 17. Illustration of samples collected from the gastrointestinal model.

activity of β-glucanase gradually increases, giving more degradation after some time.

The activity of β-glucanase was tested in the porridge introduced into the model and the accompanying $ID_{240\text{-}360}$ sample collected from the model. This was tested using the HPSEC-FD-system as described previously. In the porridges from raw material and malt B little activity was found, although in the porridge from malt A it was even less. In the $ID_{240\text{-}360}$ samples from raw material and malt B, a similar and larger increase was found, while in the $ID_{240\text{-}360}$ sample from malt A this increase in β-glucanase activity was smaller. Thus the activity of β-glucanase increased during the passage, which would explain the decrease in β-glucan molecular weight for raw material and malt B. However, it does not explain the almost as extensive breakdown in malt A. Thus this indicates that a fractionation is more likely (**Paper VI**).

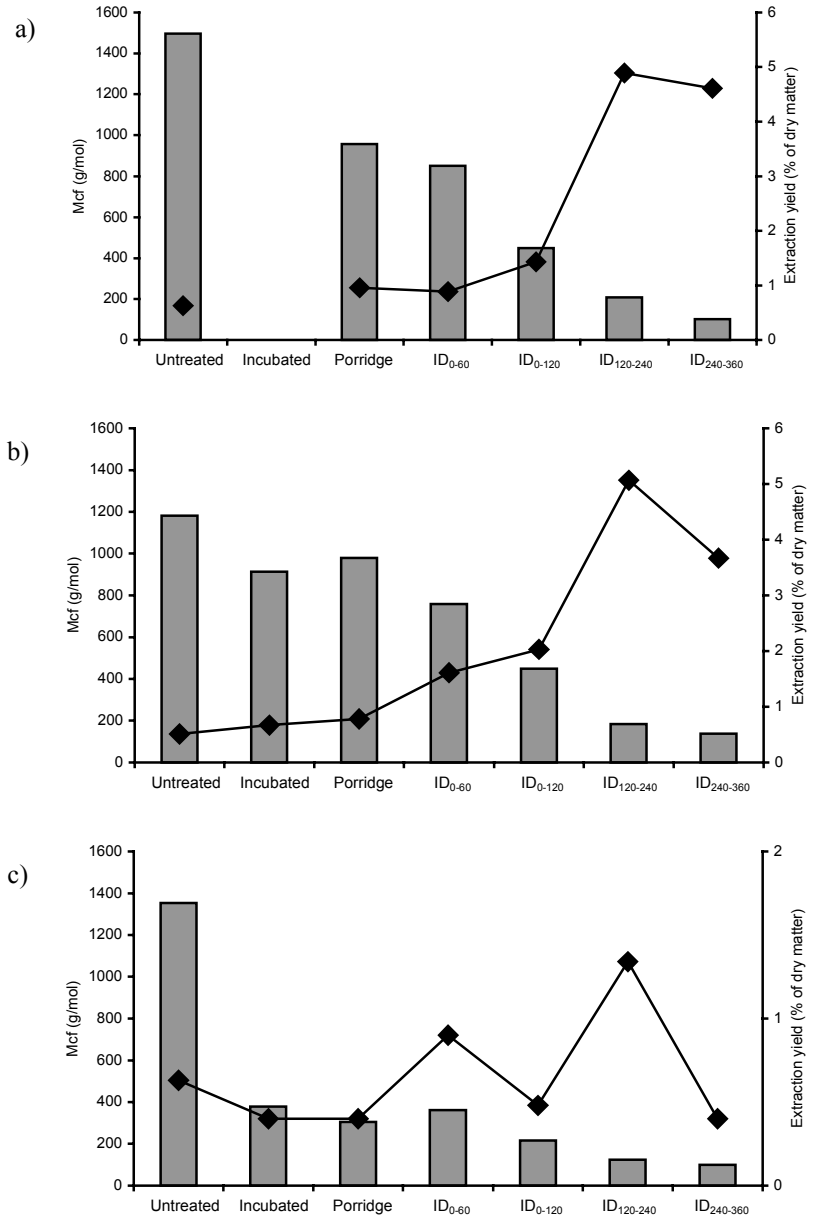


Figure 18. The Calcofluor average molecular weight and extraction yield of untreated, incubated, porridge and ileal deliveries from raw material (a), malt A (b) and malt B (c).

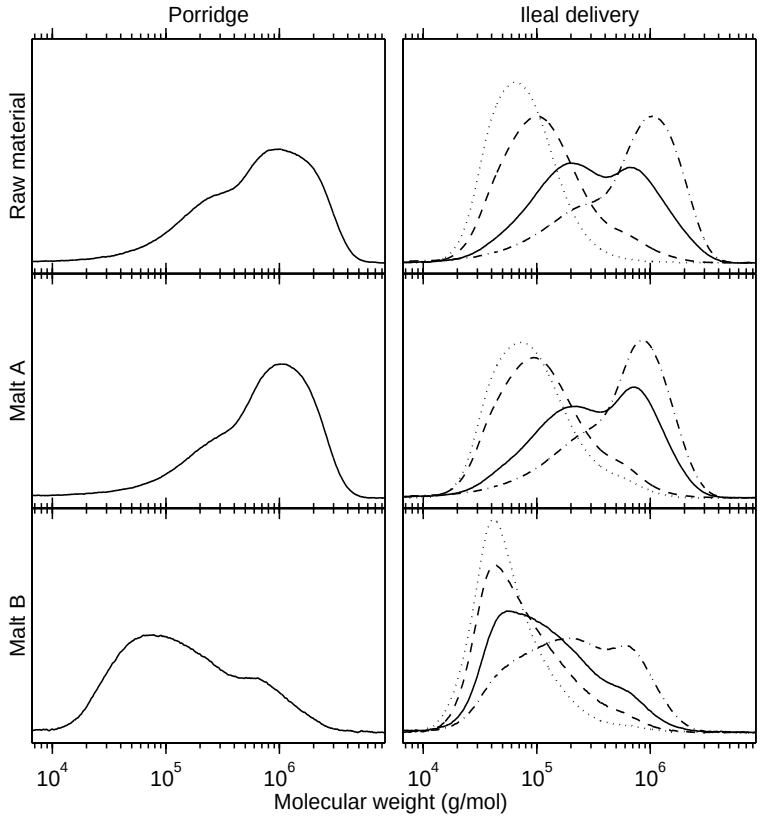


Figure 19. Molecular weight distribution of β-glucan from porridge and replicates of ileal deliveries after 60 min (·−·) and during the time 0-120 min (—), 120-240 min (---) and 240-360 min (¨) for raw material, malt A and malt B. average of two replicates, except for the ileal delivery of raw material at 60 min.

Arabinoxylan was also extracted from the raw material and malt A and B. These were precipitated at 60 and 90 % EtOH, to obtain one fraction with only large-sized molecules and another with both small and large-sized molecules. The differences in content of arabinose and xylose between the fractions were small in porridges, thus most of the arabinoxylans extracted were of a large size. For raw material and malt B, the large-sized arabinoxylan decreased with time spent in the model, while for malt A there was no significant change. The ratio of arabinose to xylose remained constant for all, thus no structural differences were found.

Main conclusions

- The results showed that ferulic acid dimers were present in arabinoxylan extracts of both untreated and ethanol treated cereals indicating that the dimers were not an extraction artefact. Thus, water-extractable arabinoxylan exist in a coupled form with ferulic acid in the endosperm.

- Fractionation of a sample with a broad molecular weight range was a reliable way of calibrating HPSEC-FD systems, since the fractions gave peaks with narrow molecular weight distribution over a large range.

- The β-glucanase activity can be reduced during germination by steeping at a high temperature.

- A reduced activity of β-glucanase gave a higher content of total β-glucan in the germinated samples, mainly due to a higher content of unextractable β-glucan when extracting at 38 °C. In contrast, extracting β-glucan at 100 °C gave a high influence of steeping temperature, while a low influence on the unextractable β-glucan at 100 °C was obtained. Thus the results indicated that the β-glucan extractable at 100 °C but not at 38 °C is readily available for the endogenous β-glucanase.

- Larger average molecular weights were obtained for samples with a reduced β-glucanase activity during germination.

- β-Glucanase has a preference to hydrolyse β-glucan of lower molecular weights.

- By steeping barley kernels at 48°C and with 0.8% lactic acid prior to germination, a well preserved content of β-glucan in malted barley was obtained at the same time as a substantial phytate degradation occurred.

- Average molecular weight of β-glucan from malted barley decreased, while extraction yield of β-glucan was found to increase with time spent in an *in vitro* gastrointestinal model.

Concluding remarks

Barley is a valuable cereal with an old tradition in malt, food and feed. With new food applications this cereal can be re-discovered. Naked barley has the greatest potential since no pearling is needed and can it therefore directly be used without any loss of its good nutritional balance. Addition of a malting step in a food process can give a changed texture and taste to a product. It is also a process with high potential for improvement of nutrient availability.

Primarily β-glucan is associated with lower plasma cholesterol and reduced glycemic index. However, arabinoxylan may also be involved since both dietary fibres have been shown to increase viscosity in a solution, which is believed to play a part in the mechanism. To exert these effects the molecular weight should be large enough, but the limit need to be established. This would be possible by using cereal products of different molecular weights in a human study.

Addition of lactic acid bacteria to the steeping water may improve the microbiological quality during malting. The conditions during steeping should then be optimised for the growth of the bacteria. With the HPSEC-FD-system the molecular weight and amount of extracted β-glucan can be determined on the same day on a large number of samples. This can for example be used to find a range of cereal products with different molecular weights of β-glucan. An *in vitro* gastrointestinal model gives a prediction of how soluble fibre change during digestion. However, the results should be confirmed in a human study.

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