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Cholesterol-lowering properties of oats: Effects of processing and the role of oat components

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Effects of processing and the role of oat components

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Abstract

The cholesterol-lowering effect of oats has been studied for almost fifty years. The effect has hitherto essentially been associated with its content of β-glucans, the primary soluble dietary fibre in oats. Few studies have been published regarding the extent to which other oat components may contribute to the effect and how processing of oats may influence the cholesterol-lowering properties. The objective with the current thesis was to increase the knowledge in this area, and was intended to provide a basis for the development of new functional foods from oats, with optimised cholesterol-lowering properties. For this purpose, different oat products or fractions of oats were produced, characterised and evaluated in mice.

The wild-type mouse strain C57BL/6 fed an atherogenic diet was shown to be an appropriate tool for evaluation of effects on plasma cholesterol, although substrain differences in response to oats were found. When oat bran was processed to different average molecular weight of β-glucans and then evaluated in C57BL/6NCrl mice, all products were found to lower plasma cholesterol to the same degree as untreated oat bran. This was also seen for a second mouse model (LDL-receptor deficient mice), although in this case there was a small but statistically significant decline in effect with reduced average molecular weight of β-glucans. It could, however, not be excluded that other components, apart from the β-glucans, might have contributed to the effects on plasma cholesterol in these studies.

Indeed, the cholesterol-lowering effect of oat bran, was found to be attributed to two complementary fractions: an oat bran oil and a β-glucan containing fraction, which supported previously published studies that components other than β-glucan may be of importance. The effect of the oat bran oil was shown not to be caused by the content of unsaturated fatty acids, but by other components.

An extraction method was developed in order to purify β-glucans from oat bran for studies in mice. The highest yield was obtained after a combined heat treatment and a starch digestion step followed by treatment with pancreatin. Using this method, about 80% of the β-glucans could be extracted. When purified β-glucans from oats were studied, the difference in purity, viscous properties and average molecular weight, seemed not to be crucial parameters for the cholesterol-lowering effect.

Further studies are needed to investigate whether purified β-glucan products, with average molecular weights lower than 285 kDa, are able to lower plasma cholesterol and which components in the oat bran oil fraction that cause the effect on plasma cholesterol. The results should also be confirmed in human intervention studies.

List of papers

Paper I

Effects of oats on plasma cholesterol and lipoproteins in C57BL/6 are substrain specific.

Andersson KE, **Immerstrand T**, Swärd K, Bergenståhl B, Lindholm MW, Öste R, and Hellstrand P.

British Journal of Nutrition 2010; 103: 513-521.

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Paper II

Effects of oat bran, processed to different molecular weights of beta-glucan, on plasma lipids and caecal formation of SCFA in mice.

Immerstrand T, Andersson KE, Wange C, Rascon A, Hellstrand P, Nyman M, Cui S, Bergenståhl B, Trägårdh C, and Öste R.

British Journal of Nutrition (In press); doi:10.1017/S0007114510000553.

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Paper III

Extraction of β-glucan from oat bran in laboratory scale.

Immerstrand T, Bergenståhl B, Trägårdh C, Nyman M, Cui S and Öste R.

Cereal Chemistry 2009; 86: 601-608.

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Paper IV

Cholesterol-lowering effects of oat components.

Immerstrand T, Andersson KE, Ulmius M, Nyman M, Bergenståhl B, Dimberg L, Hellstrand P, Trägårdh C and Öste R. (Manuscript, 2010)

The author's contributions to the papers

Paper I   The author T. Immerstrand was responsible for the design of diets, produced the diets and performed the β-glucan analysis of the oat bran products.
T. Immerstrand also took part in evaluating the results and writing the manuscript.

Paper II   The author T. Immerstrand, was involved in the design of the animal experiments, partly prepared and characterized oat bran products, took part in the production of the diets, evaluated the results together with K. Andersson and was responsible for writing the manuscript.

Paper III   The author T. Immerstrand, was involved in the design of the experiments, performed experimental work, evaluated results, and was responsible for writing the manuscript.

Paper IV   The author T. Immerstrand, took part in designing the animal studies, coordinated experimental work, isolated and characterized oat products, designed and produced the diets and took part in performing the animal studies. T. Immerstrand also evaluated the results together with K. Andersson and was responsible for writing the manuscript.

Paper I and II have been included in the thesis by K. Andersson (2009), Lund University.

Abbreviations

AACC	American Association of Cereal Chemists
cv.	Cultivated variety
c n v	Concentration-normalised viscosity
DM	Dry matter
EFSA	European Food Safety Authority
FDA	U.S. Food and Drug Administration
GI tract	Gastrointestinal tract
LDL	Low density lipoprotein
LDLr$^{-/-}$ mice	LDL-receptor deficient mice
MW	Molecular weight
MW$_p$	Peak molecular weight
SCFA	Short chain fatty acids
SNR	Swedish Nutrition Recommendations
VLDL	Very low density lipoprotein

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1 Introduction

An elevated level of blood cholesterol, total and low density lipoprotein (LDL) cholesterol, is considered as a major risk factor for atherosclerosis (Glass and Witzum 2001; Chen et al. 2008). In order to circumvent cardiovascular diseases patients with elevated cholesterol levels are treated with drugs (e.g. statins). Another desirable therapeutic alternative is that by dietary regimes (Jenkins et al. 2005). Examples of dietary advices are low contents of saturated fatty acids and a high intake of water-soluble fibres in the diet (Theuwissen and Mensink, 2008). Indeed, a portfolio diet containing plant sterols, soy protein, almonds, and viscous fibre (from oats, barley, psyllium) was reported to be as effective as statins in reducing LDL cholesterol levels in humans (Jenkins *et al.* 2005). Commercial products enriched in physiologically functional ingredients are now available and are sometimes referred to as "functional foods", i.e. foods that contribute to beneficial health effects and/or reduce the risk of disease, besides providing energy and essential nutrients (Sirtori *et al.* 2009). Oats are an interesting food candidate in this respect, as it can lower the blood cholesterol level, as well as blood glucose and insulin levels (Wood, 2007), which is of great interest in prevention and control of atherosclerosis and diabetes.

Oats contain the soluble fibre β-glucan. A purified oat β-glucan product (80% β-glucan content) has been shown to have cholesterol-lowering effect in humans (Braaten *et al.* 1994) and therefore the β-glucan is the primary component hitherto associated with such effects of oats. Apart from β-glucan, oats also contain significant amounts of other types of dietary fibre (e.g. cellulose, lignin and arabinoxylans). Dietary fibres generally are known for reducing the energy density in foods and may thereby reduce the food intake and an increased intake of dietary fibre may have several health-promoting effects (Westerlund *et al.* 1993; Lairon, 2001; Anderson *et al.* 2009). Moreover, oats contain antioxidants that protect foods against rancidity. One group of antioxidants is the oat-specific avenanthramides; polyphenolics potentially beneficial to health (Skoglund *et al.* 2008).

Among the cereals, oats not only have the highest protein content but also the best protein quality, as the primary storage protein is globulin, containing higher levels of essential amino acids than prolamine (the predominant storage protein in other cereals; Webster, 2002). Oats also contain the two essential fatty acids, linoleic (18:2, n6) and

linolenic acid (18:3, n3), although the content of the latter is relatively low (<5% of total fatty acids; Bryngelsson *et al.* 2002; Peterson, 2002). The above-mentioned nutritional properties of oats have led to an increased interest in the development of oat-based foods.

Many research studies on the cholesterol-lowering effect of oats have been performed in both human and animal models, and extend almost fifty years back in time (De Groot *et al.* 1963). In order to develop foods from oats and oat components with improved cholesterol-lowering properties, more detailed knowledge of the nature and properties of the active cholesterol-lowering mechanism(s) of oats, and the way in which they may be affected by food processing is needed. This was the subject of the studies presented in this thesis. Various fractions or components of oats have been isolated and characterised, and the related cholesterol-lowering effects and other nutritional effects, evaluated in mice.

2 Background

2.1 Oats & health claims

After reviewing 37 human studies, the US Food and Drug Administration (FDA) approved a health claim for oat-based foods in 1997, stating that a diet high in soluble fiber from whole oats (oat bran, oatmeal or rolled oats, and whole-oat flour) and low in saturated fats and cholesterol may reduce the risk for heart disease (FDA, 1997). An intake of 3 g /day was regarded as the minimum required to bring about a decrease in cholesterol level. To be eligible for the claim, a minimum of 0.75 g β-glucan per serving is required. It is important to consider that oat bran or oatmeal products were evaluated in the 37 reviewed studies, and that only one of these studies included a purified β-glucan product derived from oats (Braaten *et al.* 1994).

Recently, the European Food Safety Authority (EFSA) approved the following health claim for foods containing β-glucan derived from barley, barley bran, oats or oat bran, or mixtures of non-processed or minimally processed β-glucans**:** "Regular consumption of β-glucans contributes to maintenance of normal blood cholesterol concentrations"(EFSA, 2009). The EFSA, like the FDA, also concluded that an intake of 3 g β-glucans per day was the minimum dose. The EFSA also stated that the intake could be distributed over one or more servings, without giving any lower limit for the β-glucan content of the food product. From now on, not only foods containing β-glucans from oat bran, rolled oats and whole-oat flour may make this claim, but also foods fortified with purified "minimally-processed" β-glucans. However, the EFSA has not yet defined the meaning of the term "minimally processed".

2.2 Oats (Avena sativa)

2.2.1 Composition

The composition of the oat grain is illustrated in Fig. 1a. The "hull" (also called the husk) of the oat grain comprises 25-30% of the weight of the grain, and is normally separated from the "groat" before use (Butt *et al.* 2008). However, in for example China, "hull-less" oats are commonly grown. During harvest the hull separates easily from the seed and no de-hulling process is required (Webster, 2002). The oat groat, i.e. the de-hulled oat grain, is composed of three parts: the bran, the endosperm and the germ. The nutritional composition of the oat groat is given in Table 1 and is based on data for oats cultivated in North America.
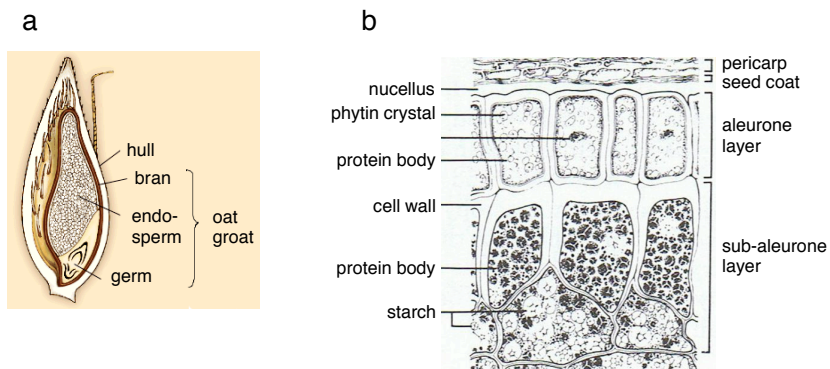
Figure 1. (a) The composition of the oat grain (courtesy of the Encyclopaedia Britannica, Inc., copyright 1996; used with permission). (b) Oat bran (figure provided by RG Fulcher, University of Manitoba, reprinted from Fulcher, 1986).

Table 1. Typical composition of the oat groats[a]

Component	Range (%)
Protein	11-20
Fat	5-9
Starch	44-61
Total dietary fibre	7-11
β-glucan	2.2-6.6
Free sugars	0.9-1.3
Moisture	9-14

[a] Data are based on values for oat groat from
North America (Webster, 2002)

The bran is the outermost layer of the oat groat (not including the hull) and consists of several cell layers: the pericarp, seed coat, nucellus, aleurone layer and sub-aleurone layer, from the outer to the inner layers of the groat (Fig. 1b). The pericarp, seed coat and nucellus contain empty cells and cell walls (Webster, 2002). The largest part of the oat groat is the endosperm, which may constitute 55-80% of the oat grain (Mälkki, 2001). In contrast to the bran, the cells in the endosperm are generally larger and the cell walls thinner (Webster, 2002).

The β-glucans are found in the cell walls of the oat groat. The distribution of β-glucans in the oat groat is more concentrated in the bran fraction than in the endosperm fraction. In contrast, the β-glucans in barley and rye are more evenly distributed (Cui and Wang, 2009). Closest to the endosperm is the sub-aleurone layer, where the cell walls are thicker and contain higher amounts of β-glucans than those in the aleurone layer.

The third fraction, the oat germ, contains 30-35% of the total oat protein content and 10% of the total lipid content (Webster, 2002). The oat bran used in the current work also contained parts of the germ, as the germ was not specifically removed from the oat groat during production (Börjesson, 2010).

2.2.2 Processing

Besides the mechanical treatment needed to remove the hull from the oats, the oat groats are subjected to a heat-steaming procedure (0.5-2 hours at 95-100°C and about 15-17% moisture content) to prevent the oxidation of fat during storage. The heat treatment inactivates enzymes such as lipase and lipoxygenase (Mälkki, 2001; Börjesson, 2010).

Oat products are widely used as ingredients in foods, e.g. baked products, beverage products, pancake mixes and infant foods (Webster, 2002). One of these oat products is oat bran, which was defined by the American Association of Cereal Chemists (AACC) in 1989 as: "The food which is produced by grinding clean oat groats or rolled oats and separating the resulting oat flour by sieving, bolting and/or other suitable means into fractions such that the oat bran fraction is not more than 50% of the original starting material and has a total β-glucan content of at least 5.5% (based on dry matter, DM) and a total dietary fiber content of at least 16.0% (based on DM), and such that at least one-third of the total dietary fiber is soluble fiber" (AACC, 1989). However, Luhaloo *et al.* (1998) compared different commercial oat brans from five different countries (Australia, Denmark, Finland, Norway and Sweden), and found that the β-glucan content varied between 4.7 and 8.3%. Apart from the β-glucan content there were wide variations in the nutritional composition of fat, carbohydrates and protein.

Other oat products are oat bran concentrates, rolled oats and oatmeal. Oat bran concentrates are enriched in β-glucans (e.g. OatWell® containing 22% β-glucan), and are sold in health food shops and used as an ingredient in the food industry. Rolled oats (also known as oat flakes) are produced by rolling heat-treated groat between cast-iron rolls rotating at equal speeds, while whole-oat flour is produced from oat groats by milling (Mälkki, 2001).

2.3 Oat β-glucans

2.3.1 Structure

Cereal β-glucans belong to the group of linear homopolysaccharides, as they are composed of only one type of monomer (β-D-glucopyranose). These units are linked by two kinds of β-linkages: about 70% β-(1→4) and 30% β-(1→3) (Wood, 2002). Cellulose, in contrast, contains only β-D-glucopyranose units linked by β-(1→4) bonds.

Over 90% of the monomers of cereal β-glucans exist as two units: either as cellotriose or as cellotetraose units, separated by a single β-(1→3) linkage (Fig. 2a). The distribution of the two units is believed to be random. The molar ratio between the two units is often used as a "fingerprint" to describe the structure of β-glucans, and can be determined after complete hydrolysis by lichenase (EC 3.2.1.73). Lichenase is a type of β-glucanase which specifically cleaves the β-(1→4) linkage after a β-(1→3) linkage (Fig. 2b). The ratio of trisaccharides/tetrasaccharides after hydrolysis reflects the ratio of cellotriose units/cellotetraose units in the β-glucan chain. The remaining 10% of the monomers in the chain consists of larger cellulose-like segments (5 ≤ degree of polymerisation ≤14) (Cui and Wang, 2009).

The ratio of cellotriose units/cellotetraose units varies between cereals. The highest ratio has been found in wheat (3.0-4.5), while apparently lower ratios have been reported for barley, rye and oats (1.8-3.5, 1.9-3.0 and 1.5-2.3, respectively), according to a review by Lazaridou and Biliaderis (2007). Moreover, the ratio of cellotriose units/cellotetraose units has been shown to be higher for β-glucans in the pericarp of oat bran (containing little endosperm) than in flour from the whole oat groat (2.6 vs. 2.2) (Wood *et al.* 1994).
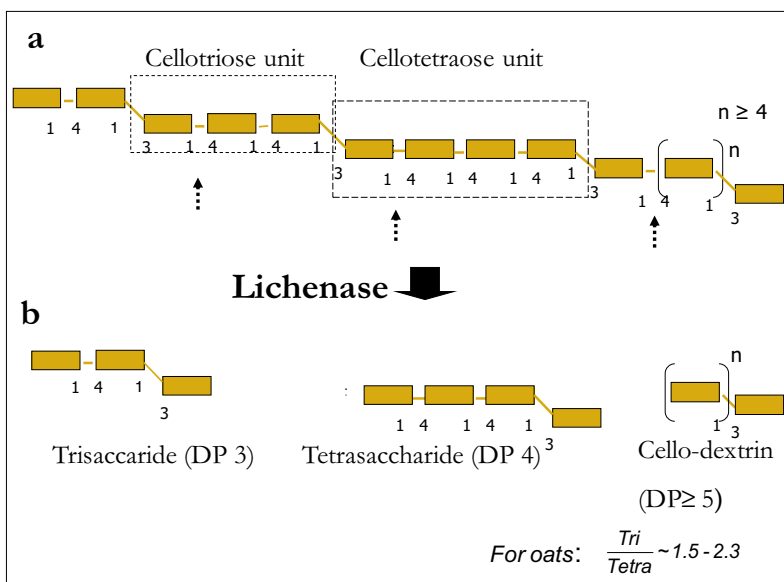
Figure 2. Cereal β-glucans: a) Their schematic structure and b) The units formed after hydrolysis by lichenase. Each box represents a β-D-glucopyranosyl unit. Dotted arrows indicate the links broken by lichenase. Modified after Lazaridou and Biliaderis (2007). DP= degree of polymerisation.

2.3.2 Molecular weight

The theoretically lowest possible molecular weight (MW) of an oat β-glucan is about 0.5 kDa (i.e. 3 units, as illustrated in Fig. 2). Values of the average MW of oat β-glucans in the literature range between 20 kDa and 3100 kDa (Lazaridou *et al.* 2003), and reasons for the large scatter may be due to a number of factors such as oat sample preparation, the presence of non-inactivated endogenous enzymes, the analytical method used (including the choice of dissolution method, the use of inappropriate standards such as pullulan, or the detector used) (Lazaridou *et al.* 2003; Lazaridou and Biliaderis, 2007).

2.3.3 Solubility and viscous properties

Cellulose is composed of β-(1→4) linked units and is a water-insoluble fibre (Wood, 2007). The β-(1→3) bonds of the β-glucan chain make the chain more flexible and soluble in water (Lazaridou and Biliaderis, 2007). It has been reported that the

solubility of freeze-dried cereal β-glucans from oats is higher than that of β-glucans from barley, which in turn is higher than that of β-glucans from wheat. This reflects the order of the molar ratios between the two cellulose units (Fig. 3), i.e. a higher ratio of the two units (cellotriose/cellotetraose) seems to reduce the solubility (Lazaridou *et al.* 2003).

Cereal β-glucans generate high viscosities at low concentrations (Wood, 2007). Solutions of cereal β-glucans may also form gels under certain conditions (5-45°C; 4-12% w/v β-glucan) (Lazaridou and Biliaderis, 2007). The gelling ability of oat β-glucans has been shown to depend on their MW: i.e. the lower the MW, the shorter the gelation time. Lazaridou *et al.* (2004) reported that the gelation time of 100 kDa β-glucans was 42 hours, while that of 200 kDa β-glucans was 167 hours (8 weight% β-glucan; strain 0.1; 25°C). It remains yet to be resolved whether the correlation between gelation time and the MW of β-glucans is also valid under the conditions found in the intestine.

2.3.4 Effects of processing

The average MW of the β-glucans in oat bran, rolled oats and oatmeal products has been reported to be in the range of 1700-3100 kDa, based on results from high-performance size-exclusion chromatography (HPSEC) and post-column addition of Calcofluor, employing calibration against pure β-glucans (Wood *et al.* 1991; Åman *et al.* 2004; Shewry *et al.* 2008). Wood *et al.* (1991) found that the average MW of β-glucans was about the same for raw oats, heat-treated oats, rolled oats and oat bran. It thus appears that dry processes, such as milling, sieving and rolling, do not depolymerise β-glucans. Åman *et al.* (2004) compared the average MW of β-glucan in different types of commercial oat-based foods and found that the MW was maintained following heat treatment (e.g. boiling of porridge or frying of pancakes). Crispbread and pancake batter were, however, found to contain partially degraded β-glucans (950 kDa and 19 kDa, respectively).

There are several possible reasons for the depolymerisation of β-glucans, including enzymatic hydrolysis, acid hydrolysis, shear forces and OH-radical induced degradation. Endogenous β-glucanases may be responsible for enzymatic hydrolysis if they have not been inactivated (e.g. during baking). Side activities of β-glucanases from added enzymes (e.g. amylase) may also cause hydrolysis (Kerckhoffs *et al.* 2003;

Rimsten *et al.* 2003; Åman *et al.* 2004). β-glucans may also be depolymerised by acid hydrolysis. Noteworthy is, however, that agitation for 12 hours at 37°C in 0.1 M HCl (which roughly mimic the conditions in the human stomach) seems not to digest oat β-glucan (Johansson *et al.* 2006). Shear forces (direct or indirect) during agitation, homogenisation or sonication may cause β-glucan depolymerisation (Wood, 1993; Yokoyama *et al.* 2002). The shear force may cause direct breakage of the molecules (the direct effect), or improve the cleavage capacity of β-glucanases (i.e. an indirect effect). Finally, OH-radical-induced depolymerisation is possible in aqueous solutions in the presence of ascorbic acid and ferrous ions (Kivelä *et al.* 2009).

Freeze-storage of oat products for long times has been shown to reduce the extractability (solubility) of β-glucans after *in vitro* treatment. Oat bran muffins stored for 8 weeks at -20°C exhibited up to 45% lower solubility of β-glucans than those stored frozen for 4 weeks (Beer *et al.* 1997a). Moreover, repeated freezing and thawing cycles of oat bran muffins have been found to decrease the solubility of β-glucans *in vitro* and has been suggested to be due to aggregation of β-glucan chains during freeze-concentration (Lan-Pidhainy *et al.* 2007; Lazaridou and Biliaderis, 2007).

2.4 Oats & plasma cholesterol

2.4.1 Previous studies in humans

Examples of oat products that have been shown to possess cholesterol-lowering properties (total cholesterol and/or LDL cholesterol) in humans are: oatmeal, oat flakes, oat bran, oat bran concentrates, oat milk, and products enriched in β-glucan (Ripsin *et al.* 1992; Brown *et al.* 1999; Önning *et al.* 1999).

Some of the reported studies of oat bran in man have, however, failed to show any effects on total plasma cholesterol (Bremer *et al.* 1991; Leadbetter *et al.* 1991; Lepre and Crane, 1992; Stewart *et al.* 1992). The reasons for the lack of effect of oat bran in these studies are not known. Genetic differences between subjects may influence the outcome of clinical studies, such that only some people "respond" to oats. Another factor of importance may be the subjects' baseline cholesterol levels, as the effects of oat products on total cholesterol have been found to be greater in subjects with elevated baseline levels, as concluded by Ripsin *et al.* (1992) in their meta-analysis of

20 studies on the effects of oat products. In a study on oat milk, the reduction of LDL cholesterol was shown to be greater in subjects with elevated baseline LDL levels (Önning *et al.* 1999). Furthermore, a too low daily dose of β-glucan may also influence the outcome of the results, as discussed by Leadbetter *et al.* (1991). In their study, 3.6 g β-glucan per day did not lower plasma cholesterol levels, despite the fact that this amount exceeds the recommended lower limit of 3 g per day (FDA, 1997 and EFSA, 2009). The duration period in the study and the diet composition (e.g. amount of saturated fatty acids and cholesterol) might also have influenced the outcome of the results.

Human studies dealing with the effects of products enriched in β-glucans from oats on blood cholesterol are summarised in Table 2. The MW of β-glucans in the products was not always reported in these studies, but it is interesting to note that β-glucans with MWs of both 80 kDa and 932 kDa have been proven to reduce cholesterol levels in humans. Only three studies on the effects of a product containing >50% oat β-glucan on blood cholesterol (i.e. total and LDL cholesterol) appear to have been published (Table 2), where two showed effects. No effect was observed in the study by Beer *et al.* (1995), and the authors speculated that this might have been due to too low baseline levels of plasma cholesterol, or the low solubility of the high-MW β-glucan product used (62% β-glucan content, average MW 1000 kDa). Similarly, Törrönen *et al.* (1992) discussed whether the lack of effect of their oat bran concentrate was due to the low MW (370 kDa) and the low solubility of β-glucans in the bread product tested. In the study by Kerckhoffs *et al.* (2003) effects were obtained when the oat bran concentrate was mixed with orange juice, but not when it was incorporated into bread and biscuits. This suggests that the choice of food matrix or the treatment conditions may play an important role in the outcome when investigating the effects of oat products in humans.

Table 2. Studies of the effects of products enriched in β-glucan from oats in humans.

Number of subjects (men/female)	Törrönen (1992)	Braaten (1994)	Beer (1995)	Kerckhoffs (2003)	Kerckhoffs (2003)	Naumann (2006)	Queenan (2007)
Study design	Parallell	Cross-over	Cross-over	Parallell	Cross-over	Parallell	Parallell
Number of subjects (male/female)	28 (28/0)	19 (9/10)	14 (14/0)	48 (21/27)	25 (10/15)	47 (18/29)	75 (25/50)
Baseline TC (mmol/l)§	6.4	6.8 ± 0.1	~4	6.0	~6	~5.8	6.2 ± 0.1
Duration (weeks)	6	4	2	4	2	5	6
Control	Wheat	Maltodextrin	Placebo whip	Wheat fibre	Wheat fibre	Rice starch	Glucose
Purity of β-glucan (%)	15	80	62	21	21	22	54
Average MW of β-glucan (kDa)	370	932#	1000	*	*	80	ni
Test food	Bread	Beverage	Instant whip	Bread & cookies	Beverage	Beverage	Beverage
Dose β-glucan/day (g)	11.2	6	9	5.9	5.0	5	6
Cholesterol-lowering effect? (P<0.05)	No	Yes	No	No	Yes	Yes	Yes

§ European guidelines recommend baseline levels < 5.0 mmol/l (Mackay and Mensah, 2004)

Wood (2010); Beer et al. (1997b)

* The average MW was not reported, only the MW distribution

ni=no information

2.4.2 Proposed mechanisms of soluble oat β-glucan

The main soluble fibre in oats is β-glucan (Braaten *et al.* 1994). A number of mechanisms have been proposed by which soluble dietary fibre may lower cholesterol (Kerckhoffs *et al.* 2002; Theuwissen and Mensink, 2008). These can be summarised as follows.

i. The water-soluble fibre increases the viscosity of the intestinal contents, which in turn creates a thick unstirred layer next to the intestinal wall. This unstirred layer acts as a physical barrier for the absorption of e.g. glucose, fat and cholesterol, and bile acids.

ii. Binding (directly or indirectly) of bile acids by water-soluble fibre leads to reduced re-absorption of bile acids from the small intestine by the liver. As a consequence, the liver will be forced to produce more bile acids from cholesterol in the blood to compensate for the faecal excretion of bile acids. Bowles and co-workers (1996) suggested that bile acid molecules do not bind to specifics sites on barley β-glucan polymer, and the authors speculated whether β-glucans might bind micelles (of bile acids and fatty acids) and not the bile acids alone. It has also been discussed whether β-glucans may prevent reabsorption of bile acids by an increased viscosity of intestinal contents, i.e. through mechanism i) above (Sayar *et al.* 2005). *In vitro* studies have proposed that the MW of β-glucans may not influence the binding of bile acids (Sayar *et al.* 2005; Bae *et al.* 2009a).

iii. A reduction in the absorption of glucose, through the first mechanism i) described above, leads to lowered insulin levels. Reduced insulin levels will in turn lead to a reduction in hepatic cholesterol biosynthesis.

iv. Inhibition of the biosynthesis of cholesterol in the liver by propionic acid, a short chain fatty acid (SCFA) produced by bacterial fermentation of the water-soluble dietary fibre in the large intestine. Animal studies suggest that the synthesis of cholesterol is reduced by the inhibition of two enzymes: 3-hydroxy-3-methylglutaryl-CoA synthase and 3-hydroxy-3-methylglutaryl-CoA reductase (Wong *et al.* 2006). Indeed, the commonly used cholesterol-lowering drugs, statins, work by inhibiting the latter of these enzymes (Charlton-Menys

and Durrington, 2008). Furthermore, it has been suggested that propionate increase the activity of cholesterol-7-α-hydrolase (i.e. the rate limiting enzyme for bile acid synthesis) in rat hepatocytes, which may result in an increase in the synthesis and excretion of bile acids. Thus, this may also explain the lowering of cholesterol levels (Imaizumi *et al.* 1992).

A study in hamsters underlines that also other oat components than β-glucans may lower plasma cholesterol, as one of the oat fractions that did not contain any β-glucans, had an effect on cholesterol (Yokoyama *et al.* 1998). Protein, fat, ash and total dietary fibre explained 4.8% of its composition and the remaining part was assumed to be mainly digestible carbohydrates. No analysis of potentially bioactive compounds was made. There are also a few other indications in the literature that other oat components may contribute to the cholesterol-lowering effect of oat products, and these are discussed below.

2.4.3 *Effects of other oat components*

Insoluble dietary fibre. Oats contain significant amounts of insoluble fibre, mainly cellulose and arabinoxylans. Oat bran has been shown to contain about 13-15% of the arabinoxylans in soluble form, determined by using conventional methodologies which included enzymatic treatments (Westerlund *et al*. 1993). Similarly, Frølich and Nyman (1988) found that the proportion of soluble arabinoxylans in different oat bran fractions ranged between 9 and 14% while that in oat groat was about 7%, when a similar enzymatic fibre methodology was used. A part of the β-glucans in oat products may also be insoluble. For example, Luhaloo *et al.* (1998) found that the proportion of insoluble β-glucans in commercial oat bran from five European countries varied from 57-70%. However, this was determined after 2 hours extraction with water at 38°C, without any added enzymes. The cholesterol-lowering effect of dietary fibre has generally been attributed to the viscous water-soluble fibre, and not to the water-insoluble part (Chen *et al.* 2008). The number of reported studies showing cholesterol-lowering effects of insoluble fibre is limited. An example of such a study is that by Goel and co-workers (1997), where a rhubarb stalk fibre, with a total dietary fibre content of 74% (66% insoluble and 8% soluble) was shown to lower plasma cholesterol in humans.

Welch *et al.* (1988) observed no cholesterol-lowering effect of an insoluble fraction isolated from oat bran in chickens. This fraction consisted of 67% starch, 8% protein, 4% ash, 0.1% β-glucan and the remaining 20% was assumed to be insoluble dietary fibre based on DM. On the other hand, a purified β-glucan fraction (68% β-glucan, 19% protein, 1.6% starch, 1.7 % ash and 0.1% fatty acids, based on DM) from oat bran was found to reduce cholesterol levels, which implies the importance of soluble oat fibres in the lowering of cholesterol levels.

Lipids & protein. Oats contain more lipids than any other cereal. The distribution of the main groups of lipids in the oat groat is given in Table 3. Oat oil has the following fatty acid composition: palmitic acid 13-28%, stearic acid 1-4%, oleic acid 19-53%, linoleic acid 24-53% and linolenic acid 1-5 % (Peterson, 2002). Indeed, linoleic acid (i.e. 18:2, n6) has been shown to reduce both total and LDL cholesterol in humans (Lunn and Theobald, 2006).

Table 3. Compositon of oat groat lipid,
extracted with chloroform-methanol (2:1)[a]

Lipid group	%
Triacylglycerols	51
Phospholipids	20
Glycolipids	8
Partial acylglycerides	7
Free fatty acids	7
Free sterols	3
Sterol esters	3

[a] Data are reproducced from Peterson (2002).

In the study by Welch *et al.* (1988) on chickens it was concluded that a protein fraction, (85% protein, 1% β-glucan and 7% starch, based on DM) was able to lower the plasma cholesterol level, whereas an oil fraction (73% fatty acids, 3% protein, 0.4% ash based on DM) isolated by extraction with petroleum spirit at 40-60°C did not. The effect of the protein-rich fraction was, however, small in comparison to that of the β-glucan rich fraction (as mentioned above). The authors discussed whether the globulin fraction of oats was responsible for the effect. Globulin is the main protein fraction of oats, accounting for 70-80% of the total protein (Webster, 2002).

Other previously reported studies have shown cholesterol-lowering effects of oat lipids. Already in 1963, De Groot *et al.* observed in a preliminary study that when hypercholesterolemic rats were fed a diet containing 25% rolled whole-grain oats, their blood cholesterol level was reduced, by 82%, compared with the control diet. Extracted oat lipids (1.8% in the diet, isolated by extraction with ether/ethanol 1:1) led to a reduction in total cholesterol of 54%, while rats fed defatted oats (23.2% in the diet) showed a reduction of 61%. They thus concluded that both the lipid fractions and the defatted fraction of oats contributed to the cholesterol-lowering effects of whole oats. Another study in rats showed that the effect of oat bran was partly due to a pentane-soluble fraction (Illman *et al.* 1991).

Oats also contain sterols, mainly β-sitosterol, but also campesterol, stigmasterol and Δ^5- and Δ^7-avenasterols (Määtä *et al.* 1999; Shewry *et al.* 2008). Määtä *et al.* (1999) reported that the sterol content in seven different oat cultivars varied between 350 and 491 µg/g in de-hulled oats. In the same study, it was also shown that the sterol content differed significantly between the cultivars but the growth location did not seem to influence the sterol content. An intake of 2 g sterols per day has been shown to reduce cholesterol levels in humans (Law, 2000). In this respect, the content of sterols in whole-oat products seems low. Two mechanisms have been proposed for the cholesterol-lowering effect of plant sterols: i) the competition of sterols with cholesterol in the intestine thereby preventing cholesterol from being absorbed, and ii) a reduction of cholesterol synthesis (Chen *et al.* 2008).

Avenanthramides, flavonoids and phenolic acids. Oats contain a group of polyphenolics not found in other cereals, so-called avenanthramides (AVAs) (Bratt *et al.* 2003). There is considerable variation in reported values of total contents of AVAs. Jastrebova *et al.* (2006) reported a variation from 3 to 289 mg/kg, based on a review of data from several studies. The content of AVAs has been shown to vary with environmental and growing factors such as fertilization, location and year (Shewry *et al.* 2008). Moreover, the bran fraction of oats contains higher amounts of AVA than de-hulled oats (Chen *et al.* 2007).

Chen *et al.* (2007) evaluated the bioavailability of AVAs in humans, by measuring their concentration in plasma for a 10-hour period after the consumption of a beverage containing an AVA concentrate derived from oats. The highest maximum concentration was observed for N-(4´-hydroxy-(E)-cinnamoyl-5-hydroxy-anthranilic

acid (AVA 2p). Interestingly, polyphenolic-containing extracts from red grapes have been found to reduce LDL cholesterol in humans (Castilla *et al.* 2006) and in hamsters (Auger *et al.* 2002). Mechanisms behind the cholesterol-lowering effects of grape polyphenols have been suggested to be: i) up-regulation of the rate-limiting enzyme for bile acid production, cholesterol 7α-hydroxylase (CYP7A1), and ii) activation of the LDL receptor (Chen *et al.* 2008). The effects of AVAs on the cholesterol levels in humans and animals have not been reported.

Besides AVAs, oats contain other types of phenolic compounds: i.e. flavonoids (e.g. apigenin, kaempferol and tricin) and phenolic acids (e.g. caffeic, p-coumaric, ferulic, sinapic and vanillic acids).

Vitamin E (tocotrienols and tocopherols). There are 8 natural forms of vitamin E (tocols), four compounds of tocopherols (α, β, γ, δ) and four compounds of tocotrienols (α, β, γ, δ) (Bramley *et al.* 2000). Bryngelsson *et al.* (2002) reported that the total content of tocols in Swedish oat groats was on average 18 mg/kg, with a variation between the seven different oat cultivars from 13.8-25.3 mg/kg. In that study, α-tocotrienol was the primary tocol in oat groats (12.8 mg/g oat groat), and constituted 71% of the total tocols. The primary tocol in the hulls of the Swedish oat cultivars was reported to be α-tocopherol. Shewry *et al.* (2008) reported that α-tocotrienol accounted for 57-69% of total tocols in Hungarian oat grains (including bot oat groat and hull), while α-tocopherol contributed to 23-32%. Forms of γ- and δ- tocols have been reported to be found only in low concentrations amounts in oats and sometimes not detectable by analysis (Peterson, 1995; Bryngelsson *et al.* 2002). Thus, it seems that α-tocotrienol is the main form of vitamin E in oats. It is interesting to point out that a tocotrienol-rich fraction extracted from palm oil has been found to reduce cholesterol levels in humans (Qureshi *et al.* 1991). Moreover, tocotrienols have been shown to inhibit the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase, an enzyme which promotes cholesterol synthesis, whereas α-tocopherol instead increases the activity the enzyme (Qureshi *et al.* 1989, 1996; Parker *et al.* 1993). Tocotrienols therefore seem to have better cholesterol-lowering properties compared with α-tocopherol.

In a study in chicks, a diet containing 68% ethanol-extracted oat bran resulted in similar effects on cholesterol levels as a diet containing 68% non-extracted oat bran, although the total content of tocotrienols was significantly lower, and the content of dietary fibre somewhat higher (14%) in the ethanol-extracted bran. The authors

concluded that the effect of tocotrienols may be small in comparison to that of β-glucans (Petterson and Åman, 1992).

To summarise, oats contain other components apart from β-glucans that may contribute to a lowering of the blood cholesterol level, but to our knowledge, whether they do so or not remains to be settled.

3 Objectives

The objective of the work was to increase the understanding about which components and properties of oats that are relevant for the lowering of blood cholesterol levels, and to describe effects of industrial processing of oats.

The specific objectives were:

i. to develop a strategy for the systematic evaluation of oats in mice, i.e. the choice of mouse model and the design of experimental diets (Paper I)

ii. to elucidate the role played by the physico-chemical properties of β-glucans (i.e. solubility, MW and viscosity) in their cholesterol-lowering effects in mice (Papers II and IV)

iii. to develop an extraction method for the isolation of β-glucans from oat bran, resulting in a high yield and a high MW of β-glucans (Paper III). The method developed was intended to be used for the isolation of β-glucan from oat bran for a physiological study in mice (Paper IV).

iv. to describe the extent to which different oat components contribute to the cholesterol-lowering effect of oat bran (Paper IV)

v. elucidate which role the degree of β-glucan purification play in the cholesterol-lowering effect of β-glucan products (Paper IV)

In order to achieve this, a system was required to allow the evaluation of small amounts of isolated fractions and components of oats within a short period of time. A mouse model was chosen since it requires small amounts of food and consequently small amounts of test products. Methods for the isolation and characterisation of various oat fractions were further developed from established methods. The work also included design and production of experimental diets.

The results of this work are intended to form the basis for the development of new, health-promoting foods with effective cholesterol-lowering properties.

4 Material and Methods

A brief summary of the materials and the methods used is given below. Detailed descriptions of the specific experiments and assays can be found in the papers.

4.1 Oat products

A commercial oat bran produced in Sweden (Lantmännen AB, Järna, Sweden) was used as the test product in the animal experiments (Papers I, II and IV), and as the starting material for the extraction of β-glucans (Paper III). The oat bran was also used as a starting material when producing oat products for nutritionally evaluation in mice, including five processed oat bran (POB) preparations with different peak MW of β-glucans (Paper II), and three fractions of oat bran (i.e. an ethanol-extracted oat bran, an oat bran oil and a β-glucan enriched product; Paper IV).

Oat flakes was produced by the same company as the oat bran (Lantmännen AB, Järna, Sweden). The oat bran and oat flakes used were based on a Swedish cultivated variety (cv.) of oats named "Sang", except for one batch of oat bran, which consisted of a mixture of oat varieties (43% Sang, 10% Kerstin and 47% mixed oats, mainly Belinda). The β-glucan content (based on DM) in the different batches of oat bran ranged between 6.3-8.2 % while that of oat flakes was 3.5% (± 0.07 %) (± SEM, n=12) (Papers I-IV).

4.2 Analysis of nutritional composition

The nutritional composition (the content of fat, protein, total dietary fibre, ash, DM and indigestible carbohydrates) in oat bran and oat flake products (Paper I-IV) was determined by Eurofins Food (Lidköping, Sweden). In Paper II, III and IV the various POB preparations and the purified β-glucan products were analysed at our department.

4.3 β-glucans

4.3.1 Extraction yield

The various extraction methods of oat bran were evaluated after centrifugation, by determination of three parameters: the extractable β-glucan, the β-glucan in the supernatant, and the β-glucan in the residue (Papers III and IV).

The parameter extractable β-glucan (i.e. the maximum extraction yield) is referred to as the the amount of soluble β-glucan present in the supernatant as well as the soluble β-glucan present in the capillary water present in the residue (see Equation 1; Paper III). The principal behind this terminology is that the supernatant is assumed to be a one phase solution region and the residue a two phase region (solution phase plus solid phase). Thus, this parameter expresses the amount (*m*) of β-glucan distributed in the solution phase between the supernatant and the residue. The amount of soluble β-glucan in the capillary water of the residue can be described by the following equation:

$$m_{\text{soluble } \beta\text{-glucan, residue}} = \frac{m_{\beta\text{-glucan, supernatant}}}{m_{H_2O, \text{ supernatant}}} \cdot m_{H_2O, \text{ residue}} \qquad [1]$$

The parameter β-glucan in supernatant, on the other hand, represents the part of β-glucan that could be extracted in practice (i.e. the pratical extraction yield). The final parameter is that in residue, and refers to the part of the β-glucan in the starting material that cannot be extracted in practice.

4.3.2 Content

The total β-glucan content was determined using an enzymatic method, following the procedure described by McCleary and Codd (1991). An additional step was included in the assay (after addition of ethanol and sodium phosphate buffer) to enhance the solubilisation of β-glucans from solid oat samples, and consisted of a heat treatment for 15 minutes in a water bath at 80°C with agitation. When analysing the β-glucan content in the liquid samples, the method by McCleary and Codd was modified to suit the expected concentration of β-glucan in the liquid samples and/or to adjust for pH.

In this enzymatic method the β-glucans are completely hydrolysed to glucose in two steps by two β-glucanases. The reactions are as follows:

$$(1{\rightarrow}3)\ (1{\rightarrow}4)\text{-}\ \beta\text{-D-glucan} \xrightarrow{\textit{Lichenase}} \beta\text{-gluco-oligosaccharides}$$

$$\beta\text{-gluco-oligosaccharides} \xrightarrow{\beta-glu\cos idase} glucose$$
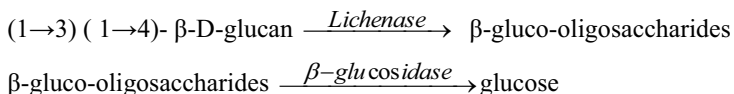
A blank was included with each set of samples to allow correction for the glucose content in the starting material. Oat flours with known β-glucan contents and standards containing glucose were also included. The amount of β-glucan in the sample can be derived from the amount of glucose that is formed during enzymatic hydrolysis, using a factor of 0.9 to account for the difference in the MW of free glucose (180 Da) and glucose in polysaccharides (162 Da).

For dissolved samples that were analysed for MW, the β-glucan contents were determined by flow-injection analysis (FIA), as described by Jørgensen (1988), but as in the method for MW determination, the method does not analyse β-glucans <9200 kDa (Gomez *et al.* 2000). FIA is based on fluorescence detection of complexes between β-glucans and calcofluor, where the increase in fluorescence intensity is proportional to the concentration of β-glucan in the sample (Kim and Inglett, 2006). Standards with known concentration of β-glucans were used to determine to concentration of β-glucan in the samples.

4.3.3 Molecular weight

The oat samples were dissolved in 0.1 M NaOH (2 hours; room temperature). Some samples were extracted with ethanol prior to solubilisation in NaOH to inactivate possible β-glucanases. Ethanol extraction (82 %, v/v) was carried out in a round flask equipped with a reflux system, agitation and a water bath at 100°C. This was the same procedure used as when isolating β-glucans from oat bran (Papers III and IV). After extraction with ethanol, the mixture was centrifuged, the supernatant was discarded and the remaining residue oven-dried (13 hours at 60°C).

The choice of 0.1 M NaOH was based on results from an initial preliminary study to evaluate different dissolution methods regarding their ability to solubilise β-glucans (data not shown). Another reason for why dissolution in 0.1 M NaOH was chosen is

that aggregation of β-glucans may be prevented; as 0.5 M NaOH has been reported to eliminate aggregates of wheat β-glucans (Li *et al.* 2006). Since the pH of 0.1 M NaOH (13.0) is higher than the pKa of the glucose unit (12.3) the β-D-glucopyranosyl units in the β-glucan chain are essentially deprotonised. The β-glucans thus become negatively charged and repel each other, reducing the possibility of aggregation. After dissolution in NaOH and subsequent centrifugation, the supernatant was neutralised to ensure that the exposure time in NaOH was the same for all samples.

The MW distribution and peak MW were analysed using high-performance size-exclusion chromatography (HPSEC) with post-column addition of calcofluor. Using this technique, the β-glucans are separated according to their size through columns, and then form a β-glucan-calcofluor complex, which can be detected with a fluorescence detector (Beer *et al.* 1997b; Brummer and Cui, 2005). This methodology has been used for MW determination in a number of studies (Åman *et al.* 2004; Suortti, 1993; Shewry *et al.* 2008; Tosh *et al.* 2008). However, it cannot detect β-glucans <9200 kDa (Gomez *et al.* 2000).

The analytical procedure was calibrated against different β-glucan standards by plotting the logarithm of the MW against the retention time. MWs corresponding to retention times within the calibration range were obtained by linear interpolation. Values outside this interval were obtained by linear extrapolation, and should thus be considered as somewhat uncertain. The average MW of β-glucans was expressed as the peak MW (MW_p) in the current work, and was obtained from the maxima of the fluorescence intensity.

4.3.4 Viscosity

The viscosity of the purified β-glucan products from the current work were analysed after solubilisation according to the product specification of a pure (97-98%) oat β-glucan (Megazyme International, Ireland). The method comprised a heat treatment of the sample (0.5 g β-glucan/100 mL) in a mixture of deionsed water and ethanol (13:1; v:v) with agitation for 10 minutes in a boiling water bath. When evaluating the viscous properties of the β-glucanase treated oat bran products, the samples were dissolved by using another dissolution procedure by agitation for 1 hour at room temperature, using a ratio 1: 10 between amounts of oat sample (DM) and deionised water (Paper II).

Rheological measurements were carried out on a stress-controlled rheometer (StressTech, Reologica, Lund, Sweden) using a concentric cylinder (25 mm diameter: CC25). The temperature used was 20°C. An exception was when the β-glucanase activity of two enzymes was estimated by meausring the reduction in viscosity of a solution containing a pure β-glucan (37°C or 40°C; Paper III). Different shear stresses were used to give a change in shear rate from 5 to 50 s^{-1}. At least two solutions with different concentrations of sucrose in deionised water, with known viscosities, were used to verify the method.

The concentration-normalised viscosity (c n v) of the solubilised fraction was calculated as follows:

$$\frac{\eta_{sp}}{c} = (\eta_r - 1) \cdot \frac{1}{c} = \frac{\eta_s - \eta_0}{\eta_0} \cdot \frac{1}{c} \quad [\,L/g\,] \qquad\qquad [2]$$

where η_{sp} is the specific viscosity, η_r is the relative viscosity (η_s/η_0), η_s is the viscosity of the solution containing the oat product, η_0 is the viscosity in the absence of the oat product, and c is the concentration of the β-glucan (g/L).

In comparison, the intrinsic viscosity [η] is equal to the limit of $\frac{\eta_{sp}}{c}$, as $c \to 0$. Another way to express the intrinsic viscosity is by the following equation:

$$[\eta] = \cdot v \cdot \upsilon \qquad\qquad [3]$$

where v expresses the molecular shape (2.5 for spherial particles) and υ is the specific volume (Van Holde, 1971).

4.4 Animal studies

All animal experiments were approved by the Ethics Committee for Animal Studies at Lund University. An overview of these experiments, including the dietary groups in each experiment, is given in Table 4. The animal experiments were made together with the Department of Experimental Medical Science at Lund University.

Table 4. Overview of the design of animal experiments.

Dietay group	Mouse model	Number of mice/ dietary group	Paper or section
Control Oat bran (270 or 400 g/kg diet)	C57BL/6*	10/14*	I
Control Oat bran (1800 kDa) Processed oat bran (1311 kDa)	C57BL/6NCrl	10	(II)#
Control Processed oat bran (1311 kDa) Processed oat bran (241 kDa) Processed oat bran (56 kDa) Processed oat bran (21 kDa)	C57BL/6NCrl	10	II
Control Oat bran (1800 kDa) Processed oat bran (1311 kDa) Processed oat bran (21 kDa)	LDLr$^{-/-}$	10	Section 5.4
Control Oat bran Ethanol-extracted bran OB oil	C57BL/6NCrl	10	IV
Control Oat bran (2348 kDa) Oat β-glucan (356 kDa) Oat β-glucan (256 kDa) Processed oat bran (< 10 kDa)	C57BL/6NCrl	10	II, IV
Control Oat flakes (270g/kg diet) Oat flakes (490g/kg diet)	C57BL/6NCrl	10	Section 5.3

The concentration of oat bran was 270g/kg diet, except in Paper I, where 400g/kg was also used.

[*] Four separate experiments were performed, with an oat bran concentration of either 270 or 400 g/kg diet, two substrains of C57BL/6 (C57BL/6NCrl or C57BL/6JBomTac) were used; 10 or 14 mice/dietary group.

[#] Described as a control experiment in material and methods of Paper II and in section 5.4

4.4.1 Mouse models

Two different mouse models were used: 1) a wild-type strain of C57BL/6 and 2) LDL-receptor deficient mice (LDLr$^{-/-}$ mice).

A wild-type substrain of C57BL/6 mice (C57BL/6NCrl) was used in all the studies described in this thesis, while the results from the genetically modified LDLr$^{-/-}$ mice are yet to be published. C57BL/6 mice have been used previously to study the cholesterol-lowering effects of various dietary components, e.g. psyllium husks and soy isoflavones (Kirk *et al.* 1998; Chan and Heng, 2008). The wild-type mice used in this thesis were fed an atherogenic diet, i.e. a diet with a high fat content, containing cholesterol and bile acids, as hypercholesterolaemia can be induced in these mice by such a diet (Paigen *et al.* 1987). The method development of using this mouse model for evaluation of the cholesterol-lowering effect is further discussed in paper I and has also been described by Andersson (2009).

Results from LDLr$^{-/-}$ mice were compared with those obtained in C57BL/6NCrl mice. The LDLr$^{-/-}$ mice have elevated levels of LDL in their plasma due to the lack of the LDL receptor, which is responsible for uptake of lipoproteins in the liver and thereby clearance of plasma lipoproteins (Espirito Santo *et al.* 2005). Due to their elevated plasma cholesterol and atherogenic lipoprotein profile (i.e. high level of LDL cholesterol), LDLr$^{-/-}$ mice are commonly used for studies of atherosclerosis development (Lichtman *et al.* 1999). LDLr$^{-/-}$ mice were fed a Western diet (described in next section), while C57BL/6 mice were fed an atherogenic diet.

4.4.2 Diets

The diets were designed and semi-produced at our department from a premix purchased from Research Diets Inc. (New Brunswick, NJ, USA) in order to be able to control the composition of the diet and the conditions used during preparation (see Paper I). All diets were administered as a powder, and not as pellets, in order to retain the physical state of the oat components as much as possible during diet production.

The oat and control diets were designed to contain approximately equal energy ratios of protein, fat and carbohydrates and the same content of total dietary fibre. The proportions of milk fat and maize oil were the same as in the "Western diet"(D12079B; Research Diets, 2010a). In a review article on a number of surveys

performed in different countries it was reported that the intake of total fat in several European countries ranged between 29 and 46 energy%, being 34% in Sweden (Elmadfa and Kornsteiner, 2009). In comparison, an energy intake of about 30% total fats is recommended in Swedish guidelines (SNR, 2005). The vitamin mix and minerals were the same as those in a formula designed by Research Diets (D12451; Research Diets, 2010a).

The diet was made atherogenic by adding cholate and cholesterol (see, e.g. Table 1 in Paper I), and was similar to a diet reported previously (Nishina *et al.* 1990). Cholate improves the intestinal absorption of fat and cholesterol and suppresses the production of bile acids from cholesterol (Ando *et al.* 2005). Furthermore, cholate has been included in diets when studying cholesterol-lowering effects of oats in rats (Ney *et al.* 1988; Shinnick *et al.* 1990; Mälkki *et al.* 1993). The level of cholate in this work was reduced from 0.5% to 0.1% to prevent the formation of gallstones (see Paper I).

Compared with the study by Nishina *et al.* (1990), the diets in the current work contained less sucrose and more of the carbohydrates maltodextrin (C*Dry DM 01910, Cerestar, Dextrose equivalent = 14) and a waxy maize starch (Amioca™, National Starch & Chemicals). The amount of sucrose added to the diet, 100 g/kg diet, i.e. 9 energy%, was based on data from a survey of the human intake of nutrients in Sweden, in which it was found that the average intake of sucrose in men and women was approximately 9 energy% (Swedish National Food Administration, 2010).

As maltodextrin is known to form a glassy state during freeze-drying (Roos, 1995) it was used as a carrier for the oat products in some of the diets (Papers II and IV). A glassy state may increase the re-solubilisation after drying (Roos, 1995) and may thereby increase the release of β-glucans during the passage through the gastro-intestinal tract *in vivo*. When preparing the β-glucan products (Paper IV), a ratio of maltodextrin to sucrose of 10:3 was used to minimise the crystallisation of sucrose during freeze-drying, since it has been shown that at least 50% maltodextrin is needed to prevent the crystallisation of sucrose (Christensen, 2000).

In initial experiments, 400g oat bran/kg diet was used (Paper I) and the level was then reduced to 270g/kg diet, as this concentration of bran in the diet was sufficient to statistically significantly lower plasma cholesterol in the mice compared with the control diet (19% vs. 24% reduction with 400g/kg). A lower level of oat bran in the

diet is also more advantageous, as the composition of protein, carbohydrates then becomes more similar compared with the control diet. The oat bran and oat flakes were dry-milled to a particle size <0.8 mm before being incorporated into the diets, as a small particle size has been shown to increase the extractability of β-glucan (Mälkki and Virtanen, 2001; Regand *et al.* 2009). After milling, the oat products were stored in a refrigerator (5°C) until the diets were prepared. The products were not frozen as the extractability of β-glucan from oat bran muffins has been found to be reduced by prolonged frozen storage (Beer *et al.* 1997a).

The design of an optimal control diet can be discussed. In this work, a type of microcrystalline cellulose was added to the control diet to compensate for the amount of oat fibre in the oat diets, assuming that this type of cellulose act as an indigestible fibre without any effect on cholesterol. Microcrystalline cellulose has been shown to be a good control (i.e. placebo) in previous studies, for psyllium in humans (Anderson *et al.* 2000) and for oat bran in animals (Mälkki *et al.* 1992). Both these fibre sources constitute of high amounts of soluble fibre. Significant cholesterol-lowering effects were seen in both these studies in relation to the cellulose diet. A further advantage of adding cellulose is that the energy density of control diet and test diets becomes similar.

Extra DL-methionine was added to the oat bran diets than in the control diet, in order to compensate for the difference in methionine content between oat bran protein (Fulcher, 1986) and casein protein in the control diet (Research Diets 2010b). The Western diet (given to the LDLr^{-/-} mice), was based on the same formula as our atherogenic diet, but 0.8% cholesterol and 0.1% cholate were replaced by an equivalent amount of maize starch.

4.5 Statistical evaluation

Minitab software (package 14.0) was used to evaluate data presented in Papers II-IV and the data presented in this thesis.

The data on plasma cholesterol obtained from the animal studies (at baseline and after 4 weeks) was first checked for outliers, which were identified as observations deviating from the third quartile by more than 150% of the interquartile range (by distribution plots, so-called Boxplots in Mintab® software). The mice to which these outliers belonged, were removed from the interpretation of all data. Next, the Anderson-Darling test was used to determine whether the data were normally distributed (Papers II and IV). Normally distributed data were generally evaluated by the general linear model (ANOVA) for multiple comparisons, followed by Tukey´s test for pairwise comparisons of the means. One set of data was also evaluated with Dunett's test, which is a test designed for multiple comparisons against a reference group. This test was not applied to the data given in the Papers, but in the Results & Discussion of this thesis (see section 5.5.1). For non-normally distributed data the non-parametric Kruskal-Wallis test was performed to compare the median values between the groups (Siegel and Castellan, 1988).

When evaluating the plasma cholesterol levels in mice fed oat bran, ethanol-extracted bran or oat bran oil, multiple regression was used in order to consider the composition terms:

Oat bran = ethanol-extracted oat bran + oat bran oil.

In the experiment in $LDLr^{-/-}$ mice, a regression tool was used in order to find out if the cholesterol-lowering effect was a function of the peak MW of β-glucans in the oat products.

5 Results & Discussion

Dry-milled oat bran (particle size <0.8 mm) is referred to as untreated oat bran in this section.

5.1 Physico-chemical properties of oat β-glucans

5.1.1 Molecular weight

One of the proposed mechanisms behind the cholesterol-lowering effect of soluble dietary fibre is their ability to increase the viscosity of the intestinal contents (Kerckhoffs *et al.* 2002). The viscous properties of soluble fibre are influenced by their MW, structure and solubility (Wood, 2007). In this section, the physico-chemical properties of the β-glucan-containing oat products are summarised (Papers II, III and IV). Preliminary results describing more precisely how these physico-chemical properties are correlated with each other and how they are affected by processing are also presented.

The peak MW of β-glucans in the different batches of commercial oat bran used in this work was found to range from 1800 to 3000 kDa. The peak MW of oat flakes, produced by the same Swedish company as the oat bran used, was on average 1995 ± 106 kDa (± standard deviation, n=2). These values are in the same range as those reported previously for oat bran and oat flakes produced in Sweden (2150 kDa and 2300 kDa, respectively; Åman *et al.* 2004). In order to describe the MW distribution of β-glucan in oat bran, I used our data for an oat bran which was used as the starting material when studying the change in MW of β-glucans following extraction. The peak MW of β-glucans for that oat bran was determined to 3.0 MDa (Paper III). Using the calibration curve obtained with β-glucan standards:

$$Log\ M_w = -0.544 \cdot R_t + 10.47 \quad (R^2 = 0.996),$$

where MW is expressed in kDa and the retention time (R_t) in minutes, it was possible to transform the chromatogram showing the intensity (response) as a function of the retention time (Fig. 2b in Paper III) into a plot of the intensity against the MW of β-glucans (Fig. 3).
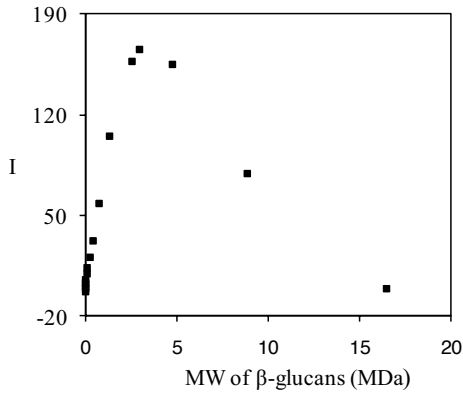
Figure 3. MW distribution of β-glucans in oat bran (cv. Sang 2006, Lantmännen AB, Järna, Sweden. (For original data, see Fig. 2b in Paper III). Peak MW of β-glucans = 3.0 MDa. I= intensity

However, it should be borne in mind that this estimate was based on extrapolation of the calibration curve obtained with β-glucan standards ranging up to 1.2 MDa. Interestingly, the MW distribution of β-glucans in that oat bran extended up to 16 MDa.

Åman *et al.* (2004) reported that heat treatment such as the cooking of oat porridge, or frying pancakes did not reduce the average MW of β-glucans. When a commercial β-glucan product (97-98% β-glucan content, based on DM) was solubilised in the presence of maltodextrin and sucrose (2 hours, 100°C) and then freeze-dried, the peak MW of β-glucans in the original and freeze-dried product did not differ significantly (Paper IV). The estimated MW distributions are shown in Fig. 4. Based on this finding, it can be concluded that neither heat-treatment nor freeze-drying itself, not necessarily has an effect on the MW distribution, and consequently the average MW of β-glucans. The probability of depolymerisation during heat treatment may be dependent on remaining β-glucanases in the oat product.
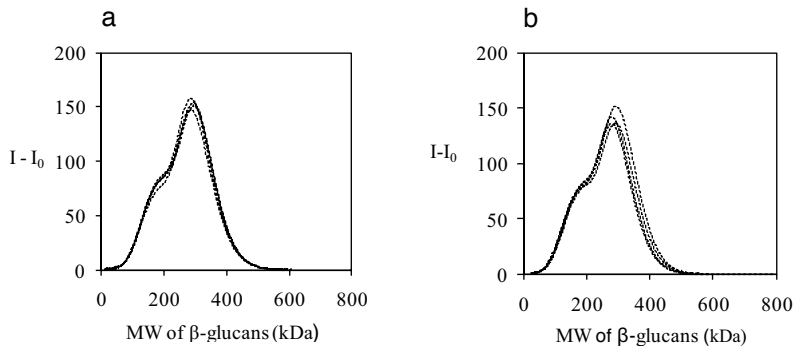
Figure 4. MW distribution of β-glucans of: a) Untreated oat β-glucan; MW_p= (289 ±2) kDa and b) Heat treated (2h, 100°C) and freeze-dried oat β-glucan; MW_p= (285 ± 4) kDa (± SEM, n=4). I=intensity.

5.1.2 Water solubility

In order to investigate how the MW of β-glucans affects their water solubility, different oat bran preparations, containing β-glucans of different peak MWs, were solubilised according to a standardised solubilisation procedure in deionised water at room temperature. The water solubility of the high-MW product (1311 kDa) was only half that of the low-MW product (56 kDa) ($P<0.05$), while the solubility of the 56 kDa β-glucans did not differ from that of the 21 kDa product or the product with a MW <10 kDa. Untreated oat bran which contained the highest MW of β-glucans, was shown to have the lowest amount of soluble β-glucans (23%). Luhaloo *et al.* (1998) found that 30-43% of the β-glucans in different European commercial oat brans were soluble, after extraction with water for 2 hours at 38°C. When two purified β-glucan products (57% pure) with different peak MWs (56 and 908 kDa, Paper III) were dissolved in deionised water at room temperature (same method as in Paper IV) the same trend was found, i.e. the water solubility of β-glucan apparently increased with decreasing MW (Fig. 5).
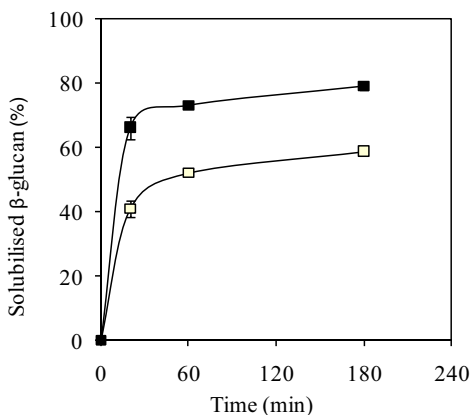
Figure 5. Amount of solubilised β-glucan from two β-glucan concentrates (57% pure) with different peak MWs (56 kDa; filled symbols and 908 kDa; open symbols) after 1 hour at room temperature. Data are presented as means ± SEM (n=2).

However, when the water solubility of the freeze-dried β-glucan products in Paper IV was evaluated, the solubility after 1 hour was generally higher than that found for the two β-glucan products in Paper III (Fig. 5), that were produced by precipitation with ethanol, and was on average ~ 90% or higher. One reason for this may be that precipitation with ethanol creates aggregates that are difficult to dissolve in water, whereas freeze-drying in the presence of maltodextrin and sucrose may prevent formation of aggregates during drying (as discussed in Paper IV). In line with these findings, Beer *et al.* (1996) reported that oat β-glucans precipitated in ethanol were difficult to dissolve in water, whereas the freeze-dried material dissolved quickly.

5.1.3 Viscous properties

The viscous properties of the various β-glucan products used in the current work were evaluated after a solubilisation procedure, with the aim of obtaining a high dissolution yield of β-glucans (77-87%) (Papers III and IV). To compare the results of the viscous properties of solubilised fractions of the β-glucan products in the different studies, their concentration-normalised viscosities were evaluated (Table 5). We chose to use the term c n v instead of intrinsic viscosity, as the concentration of β-glucan in some cases seemed not to be sufficiently low to equalise with intrinsic viscosity (see Material & Methods, section 4.3.4). In fact, our results (c n v) were sometimes higher than previously reported values for intrinsic viscosity for solutions of pure β-glucans

(0.03 - 0.96 L/g), according to a review article by Lazaridou and Biliaderis (2007). For example, a solution of a purified β-glucan product (57% pure; peak MW 908 kDa) had an intrinsic viscosity of 9.4 L/g (Table 6), i.e. about 10 times higher than the previously reported values in literature.

Table 5. Data on viscous properties of a solubilised fraction of β-glucan product.

β-glucan product		Viscosity[1]	Concentration[2]	c n v	Paper
MW$_p$ (kDa)	β-glucan content (%)	(mPas)	(g/L)	(L/g)	
908	57	39.5	4.1	9.39	III
356	11	9.2	2.4	3.45	IV
285	13	10.0	4.1	2.18	IV
56	57	3.1	4.1	0.51	III

MW$_p$ = peak molecular weight of β-glucans, c n v = concentration-normalised viscosity

[1] Data of viscosity is presented as the mean values at shear rates between 5 and 50 s^{-1}. Results showed Newtonian behaviour for all samples using this interval of shear rate.

[2] Concentration of β-glucan during measurement of the viscosity

Another reason for why we chose the term c n v was that some of the analysed products contained a low amount of β-glucans (5.5-6.6% based on DM, Paper II), in contrast to the literature data on intrinsic viscosity above which were based on highly purified β-glucans. Thus, as the samples were not containing only β-glucan, it can therefore not be ruled out that our results on viscosity were influenced by other oat components than β-glucans.

Previous studies have shown that the intrinsic viscosity of a solution of pure β-glucan is a potential function of the MW, according to Mark-Houwink-Sakarda relationship for random coils: $[\eta] = K \cdot MW^{\alpha}$. Examples of previously reported values for the exponent alpha were 0.62 (Wang *et al.* 2003), 0.75 (Vårum and Smidsrød, 1988) and 0.86 (Roubroeks *et al.* 2001). However, in this work the c n v of the solubilised fraction of β-glucan products (in Table 5) appeared to be a linear function of the average MW of the β-glucans (expressed in kDa) (Fig. 6) according to the following regression equation:

$$c\,n\,v = 0.01 \cdot MW - 0.39 \qquad (R^2 = 0.99) \qquad [L/g]$$
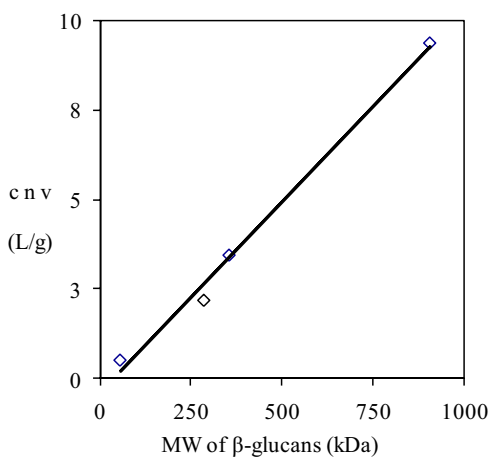
Figure 6. The concentration-normalised viscosity (c n v) of the solubilised fraction of β-glucan products as a function of the peak MW of β-glucans (Papers III and IV).

In another study, the c n v of the solubilised fractions of various enzyme-treated oat bran preparations was correlated to the peak MW of their β-glucans (Paper II, Figure 1). These values on c n v were determined after a dissolution procedure in deionised water (DM: H_2O; 1:10) for one hour at room temperature (with agitation). The c n v for the solubilised fraction of the enzyme-treated oat bran, containing β-glucans with a peak MW at 1311 kDa, was approx. 18 L/g. Reasons for why our results deviate from what has been reported before may, again, be due to that our β-glucan products contained not merely β-glucan and consequently the raise in viscosity could have been caused by other components (e.g. maltodextrin, sucrose, proteins, solubilised starch and soluble arabinoxylans). In fact, the concentration of β-glucan in the solubilised fraction of enzyme-treated oat bran with highest MW (1311 kDa) was 1.4 g/L while the concentration of total DM in solution was approximately 30 g/L.

5.2 Extraction of β-glucan from oat bran

An important objective of this work was to develop an extraction method for the isolation of β-glucans with a high extraction yield and high MW, in order to: 1) obtain a material that was representative of the native β-glucans in oat bran, and 2) obtain amounts that were sufficient for evaluation in mice (Paper III). The reason why it was deemed important to develop an extraction method was the limited published information about how different extraction techniques affect the extraction yield and the MW of β-glucans (Paper III).

The optimal maximum extraction yield of β-glucans from ethanol-extracted oat bran (~73%) was obtained by combining hot-water extraction with enzymatic digestion using a pre-heated thermo-stable α-amylase (3 hours at 100°C), followed by a treatment with pancreatin as a source of protease. The treatment with pancreatin was found not to play a crucial role in further improvement of the extraction yield of β-glucans from oat bran (Paper III). Three hours' extraction in water at 48°C and 100°C without the enzymes resulted in maximum extraction yields of 28% and 50%, respectively (Table II in Paper III).

When isolating the β-glucans from oat bran for the mice study (Paper IV), the maximum extraction yield of β-glucans for 4 batches was on average $84 \pm 1.4\%$ ($\pm$ SEM, n=4). In the previous study (Experiment V in Paper III), when essentially the same extraction conditions were used, 73% (n=1) was obtained. The first extraction step of oat bran, i.e. the hot water extraction combined with starch digestion using a pre-heated heat stable α-amylase, was shown to retain a high MW of β-glucans (1.6 MDa; Paper III).

Pancreatin and papain are two enzymes that previously have been used to purify β-glucans from oats (Delaney *et al.* 2002; Lazaridou *et al.* 2004). In the current work, it was found that papain digested oat protein efficiently (Paper III). However, when papain was used under the conditions described, the peak MW of the final, isolated β-glucan product was found to be 56 kDa. The use of pancreatin, in contrast, resulted in a higher peak MW (908 kDa). The finding that the papain premix used also contained enzymes with β-glucanase activity may explain these results. As it has been suggested that interactions between β-glucan and protein may exist (Autio *et al.* 1992), it could,

however, not be excluded that the hydrolysis of oat proteins caused inhibition of these interactions, which might have reduced the size of the β-glucan molecules.

When isolating a β-glucan product from oat bran for physiological studies in mice (Paper IV), the developed extraction method in Paper III was used (i.e. hot-water extraction with enzymatic digestion using a thermo-stable α-amylase followed by a treatment with pancreatin). The peak MW of β-glucans in the isolated material was considerably lower than what we found for the oat bran used as starting material (356 kDa vs. 2348 kDa, respectively). Reasons for the reduction in MW are not known but one might speculate in that it might have been due to some β-glucanase activity also found in the pancreatin premix.

5.3 Effects of oat bran and oat flakes in mice

It has previously been reported that 30% oat bran in the diet (i.e. 2-4.5 % β-glucan) lowers plasma cholesterol levels in rats (DeSchrijver *et al.* 1992) and chicks (Welch *et al.* 1988). As demonstrated in Paper I, the cholesterol-lowering properties of oats can be evaluated using a wild-type substrain of C57BL/6 mice (C57BL/6NCrl) together with an atherogenic diet (0.8% cholesterol and 0.1% cholic acid) reflecting the Western diet (41 energy% fat). The addition of 27% oat bran to the diet reduced the plasma cholesterol level by 19% compared to a cellulose-containing diet (*P*<0.05), while 40% oat bran caused a reduction of 24% (*P*<0.05).

In order to confirm that this mouse model can also be used to evaluate foods based on "whole" oat groat products, an additional experiment was carried out with oat flakes instead of oat bran, using the same type of atherogenic diet, i.e. 0.8% cholesterol and 0.1% cholic acid as in Paper I. The addition of 27% oat flakes to the diet reduced the plasma cholesterol by 17% compared with the control diet (Fig. 7), i.e. to about the same extent as 27% oat bran in the diet did (Paper I).
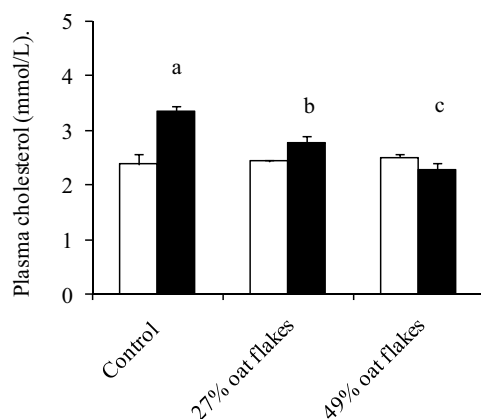
Figure 7. Effect of oat flakes on plasma cholesterol in mice (n=8-10 per group). The total dietary fibre content in all diets was 5.6%. Baseline values (□) were not significantly different between the experimental groups. Groups with different letters had significantly different plasma cholesterol levels (P<0.05) after 4 weeks (■). Data are presented as mean values ± SEM.

The β-glucan content in the diet with 27% oat flakes was only half of that obtained with the 27% oat bran diet (1 vs. 2%), but the cholesterol-lowering effect was similar. In addition, inclusion of 49% oat flakes in the diet, with about the same β-glucan content as the 27% oat bran diet, nearly doubled the reduction of plasma cholesterol (32%; 2% β-glucan) in the mice compared with 27% oat flakes (17%; 1% β-glucan). Thus, oat flakes diets (1-2% β-glucan) apparently showed a linear response with respect to cholesterol-lowering effect and β-glucan content whereas oat bran diets (2-3% β-glucan) did not. Accordingly, the cholesterol-lowering effect of oat bran and oat flakes in the mice seemed to decline above 2% β-glucan content.

The major differences in the nutritional composition of oat bran and oat flakes were found in the contents of protein, total fibre and β-glucan, and consequently the amount of digestible carbohydrates (Table 6). Furthermore, the peak MW of β-glucans was similar in the oat bran and the oat flakes (2-3 MDa).

Table 6. Nutritional composition of oat bran and oat flakes (g/100g, DM).

Nutrient	Oat bran[1]	Oat flakes[2]
Fat	8.9	8.4
Protein	18	15
Sucrose	2.2	1.0
Total dietary fibre	16	11
whereof β-glucan	7.2	3.5
Ash	3.4	2.1
Digestible carbohydrates	54	63

[1] Oat bran (*Avena sativa*, cv. Sang, batch 1008596, produced 2007 by Lantmännen AB Järna, Sweden)

[2] Oat flakes (*Avena sativa*, cv. Sang, batch 1032156, produced 2008 by Lantmännen AB Järna, Sweden)

DM=Dry matter

Two previous reported studies deal with effect of oats in the wild type strain of C57BL/6 mice (Hundemer *et al.* 1991; Bae *et al.* 2009a). Noteworthy, the early of these studies failed to show effects of oat bran on plasma cholesterol. We have shown that a crucial consideration when evaluating of oats in mice may be the choice of substrain of C57BL/6 (Paper I). The substrain differences have been more properly described by Andersson (2009).

5.4 Evaluation of oat bran preparations in two mouse models

To investigate the role of the MW of β-glucan in the cholesterol-lowering effects of oat products, oat bran was processed to different peak MWs and evaluated in mice (Paper II). Initially, an experiment was performed to elucidate if the cholesterol-lowering effect of a wet-milled, amylase-treated oat bran, not treated with any β-glucanases (1311 kDa), differed from that of untreated oat bran (1800 kDa). From the results of this experiment, it could be concluded that neither the wet-milling nor the starch digestion used affected the cholesterol-lowering effect in the mice (Fig. 8).
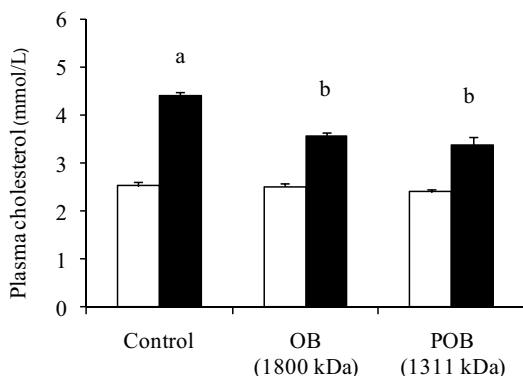
Figure 8. The cholesterol-lowering effect of processed high-MW β-glucan oat bran (POB 1311 kDa) was not significantly different from that of oat bran (OB) in C57BL/6NCrl mice (n = 10 per group). Baseline values (□) were not significantly different between the experimental groups. Groups with different letters had significantly different plasma cholesterol levels ($P<0.05$) after 4 weeks (■). Data are presented as mean values ± SEM.

When β-glucanases were introduced in the production process to reduce the MW of β-glucans in the bran, there was no change in cholesterol-lowering effect from peak MW 1311 kDa down to peak MW 21 kDa. An additional batch of oat bran was produced by using an even more extensive β-glucanase treatment (peak MW <10 kDa). Still, this product was found to lower plasma cholesterol in the same extent as untreated oat bran (Paper II).

Two of the products used in Experiment 1 described in Paper II (peak MW=1311 and 21 kDa) were also evaluated in LDLr$^{-/-}$ mice fed a Western diet, without extra cholesterol or cholic acid. Compared with the control diet, the reduction in plasma cholesterol after 4 weeks was: 32% ($P<0.001$), 27% ($P<0.001$) and 18% ($P<0.05$) for OB (1800 Da), POB (1311 kDa) and POB (21 kDa), respectively (Fig. 9).
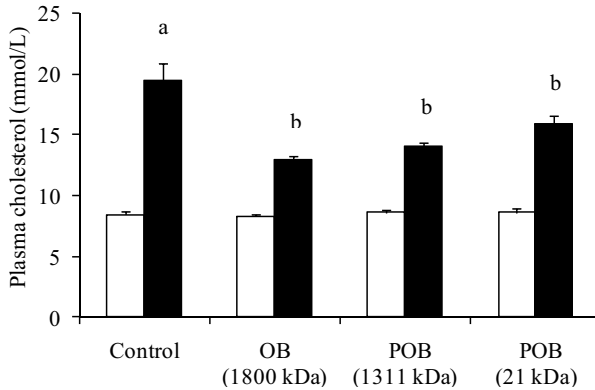
Figure 9. Effects of oat bran (OB) and processed oat bran (POB) with β-glucan of different peak MWs on plasma cholesterol in LDLr$^{-/-}$ mice (n=10 per group). Cholesterol levels in groups with different letters differed significantly (*P*<0.05) after 4 weeks on the experimental diets (■). Baseline values (□) were not significantly different between the experimental groups. Data are presented as mean values ± SEM.

With borderline statistical significance, the OB group differed from the group given POB with the β-glucans of lowest peak MW (21 kDa) (*P*=0.051). In order to establish whether the cholesterol-lowering effect was correlated to the peak MW of the β-glucans in the two mouse models, linear regression was applied to the plasma cholesterol after 4 weeks as a function of the peak MW of β-glucans. In contrast to the C57BL/6NCrl mice (Fig. 10), the correlation was significant in the LDLr$^{-/-}$ mice (Fig. 11), and the relationship was found to be:

Plasma cholesterol after 4 weeks = 16 – 0.0016 • Peak MW   [mmol/L]

The 95% confidence interval for the slope was between -0.0009 (lower limit) and -0.0023 (upper limit) and thus differed significantly from zero (*P*<0.001). This regression model indicates that the cholesterol-lowering capacity increased with increasing peak MW of β-glucan in LDLr$^{-/-}$ mice, although it should be noted that we used oat bran products and not purified β-glucan products in this study.
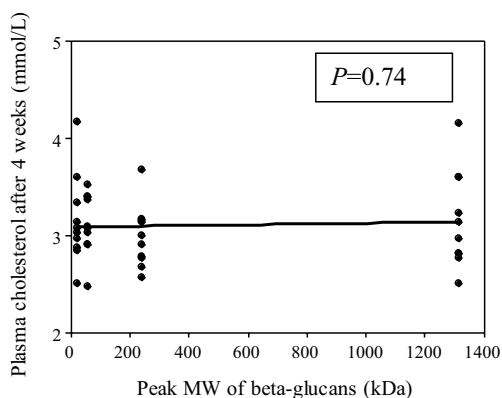
Figure 10. Plasma cholesterol levels after 4 weeks in C57BL/6NCrl mice as a function of the peak MW of β-glucans in the oat bran preparations.
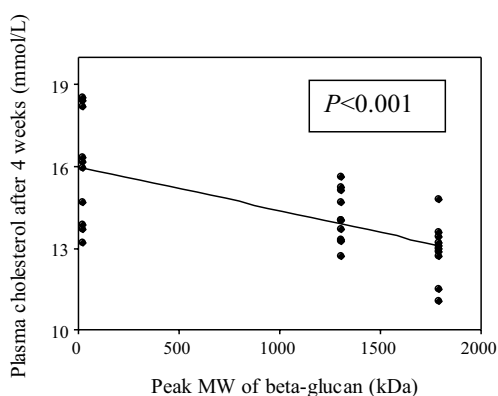
Figure 11. Plasma cholesterol levels after 4 weeks in LDLr$^{-/-}$ mice as a function of the peak MW of β-glucans in the oat bran preparations.

Nevertheless, the results indicate that it may be easier to reveal small differences in the effects on plasma cholesterol in LDLr$^{-/-}$ mice than in the C57BL/6NCrl mice. The fact that the LDLr$^{-/-}$ mice had more than 3 times higher levels of plasma cholesterol at baseline than the C57BL/6NCrl mice is because LDLr$^{-/-}$ mice are genetically modified to have increased plasma cholesterol (Ishibashi *et al.* 1993). In this respect, it is interesting to note that the effects on blood cholesterol demonstrated in man are more pronounced in subjects with higher baseline levels of total cholesterol (Ripsin *et al.* 1992), and is in line with the tendency seen in LDLr$^{-/-}$ mice.

One of the proposed mechanisms for the cholesterol-lowering effect of β-glucans is their ability to increase the viscosity of the intestinal content, which in turn can prevent the absorption of nutrients, cholesterol and bile acids (Kerckhoffs *et al.* 2002).

The processed oat bran products evaluated in the two mouse models differed regarding their viscous properties, and these could be correlated to the peak MW of the β-glucans (Paper III, Fig. 1). Despite their different average MW of β-glucan and viscous properties, they led to equal reductions in plasma cholesterol in C57BL/6NCrl mice. In contrast, the cholesterol-lowering effect in LDLr^{-/-} mice decreased somewhat with decreasing c n v (from 17.7 L/g for 1311 kDa β-glucans to 0.22 L/g for 21 kDa β-glucans. The most important finding from these studies, was that the effect of both untreated oat bran, the high MW product (1311 kDa) and the low MW product (21 kDa) was significant in both mouse models, compared with the control diet (Paper II; Fig. 9 above).

There are indications in the literature that oat components other than β-glucans may have cholesterol-lowering effects (De Groot *et al.* 1963; Welch *et al.* 1988; Illman *et al.* 1991; Yokoyama *et al.* 1998). Therefore, it can not be ruled out that the effects of the various oat bran preparations were partly caused by other components. Attempts were thus made to further elucidate this issue (Paper IV), and the results are discussed in the next section.

5.5 Mechanisms

5.5.1 The role of oat components

The results concerning the cholesterol-lowering effects of various oat components (Table 7), suggest that the effect of oat bran can be ascribed to two complementary fractions produced after ethanol extraction: the oat bran oil (OB oil) comprised of ethanol-soluble components, and the ethanol-extracted bran (EOB) containing e.g. β-glucans (Paper IV). The regression equation obtained between plasma cholesterol after 4 weeks (y) and the intake of EOB (x_1) and the intake of OB oil (x_2) was:

$$y = 4.0 - 0.64\ x_1 - 0.41 x_2\ \text{[mmol/L]},$$

where the coefficients describe the contributions of the two fractions to the effect on plasma cholesterol. Thus, after 4 weeks the effect of the EOB was more pronounced than that of the OB oil. The regression equation after 2 weeks suggested that the two fractions contributed about equally to the effect on plasma cholesterol. Reasons for the somewhat inconclusive results are not known. It should be observed that since approximately 60% of the lipids in the oat bran could be extracted by ethanol extraction, the EOB fraction still contained oat lipids. The most important conclusion from these findings is that the cholesterol-lowering effect obtained by the two fractions of oat bran was statistically significant both after 2 and 4 weeks.

Our finding that an oil fraction from oat bran lowered cholesterol is supported by a preliminary study in rats by De Groot *et al.* (1963), where the addition to the diet of 1.8% oat oil (isolated from rolled oats by ether-ethanol extraction 1:1) reduced plasma cholesterol levels. Another study in rats indicated that a pentane-soluble fraction of oat bran may be of importance (Illman *et al.* 1991). Yokoyama *et al.* (1998) have also reported a cholesterol-lowering effect of an oat fraction that did not contain β-glucans, in hamsters, which further underlines that the effect of oat bran may not only be due to the β-glucan content alone.

Table 7. Cholesterol-lowering effects of oat products in C57BL/6NCrl mice (%)[1].

Oat diet[2]	Oat product		β-glucan content in oat diet (%)	Cholesterol-lowering effect (%)[1]
	B-glucan content (%)	MW_p (kDa)		
Not included in Papers				
Oat flakes (270 g/kg diet)	3.5	1995	1	17
Oat flakes (490 g/kg diet)	3.5	1995	2	32
Paper I				
Oat bran (270 g/kg diet)[3]	7.2	3000	2	19
Oat bran (400 g/kg diet)	7.2	3000	3	24
Paper II				
Oat bran (270 g/kg diet)[4]	7.5	2348	2	14
Processed oat bran (270 g/kg diet)	8.0 - 8.4	1311 / 241 /56 / 21	2	27 / 31 / 28 / 27
Processed oat bran (270 g/kg diet)	6.8	< 10 kDa	2	13
Paper IV				
Oat bran (270 g/kg diet i.e. 246 g DM/ kg diet)	6.3	1749	2	27
Oat bran oil (17 g DM/kg diet)[5]	(0)*	-	0	7
Ethanol-extracted bran (229 g DM/kg diet)[5]	(8)*	nd	2	13
Oat bran (270 g/kg diet)[4]	7.5	2348	2	14 (P=0.06)
Oat β-glucan product	46	356	2	12 (P=0.11)
Oat β-glucan product	97-98	285	2	16

[1] Calculated as the difference between the cholesterol level for the oat diet group and the control group after 4 weeks on an atherogenic diet (0.1 weight-% cholic acid and 0.8 weight-% cholesterol). $P < 0.05$ unless otherwise stated.

[2] The amount of oat product added to the diet is presented within brackets and are expressed as fresh weight unless otherwise stated

[3] Control experiment for Experiments 1 and 2 in Paper II (See Results, Fig. 8), [4] Data for this group are presented in two separate Papers (II, IV)

[5] Two complementary fractions of oat bran (see Paper IV, Fig. 1A), * We have previously found that ethanol-extracted bran based on oats

from the cv. Sang contains about 8% β-glucans (DM) and, therefore, the oil may be assumed to not contain any β-glucans.

DM= dry weight, nd= not determined, MW_p=peak molecular weight of β-glucan

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To elucidate whether the cholesterol-lowering effect of the oat bran oil was due to its unsaturated fatty acid content or not, a new control diet was mixed in order to compensate for the unsaturated fatty acids in the oat bran diet. A mixture of peanut oil, sunflower oil and rapeseed oil was designed to reflect the composition of unsaturated fatty acids in oat bran (Table 8). Thus, when designing the new control diet 2.51% butter in the normal control diet, i.e. the amount of "oat fat" in the oat bran diet, was replaced by the corresponding amount of oil mixture. However, no significant difference was seen in the plasma cholesterol levels between mice given the new oil mixture-based control diet and the normal "butter-only" control diet (3.98 vs. 4.14 mmol/L, respectively). The cholesterol levels in both the oil mixture group and the normal control group were significantly higher than the group given the diet containing 27% oat bran (3.55 mmol/L) (see Discussion in Paper I). Therefore, it can be concluded that the unsaturated fatty acids in the oat bran and the oat bran oil did not contribute to the cholesterol-lowering effect (Paper IV).

Taking into consideration the finding that the effect of oat bran can be partly ascribed to a fraction not containing β-glucans, it is possible that the effect of the depolymerisation of β-glucans by processing of the oat bran (described in the former section; 5.4) was not completely revealed, due to presence of other potentially cholesterol-lowering components derived from the oat bran.

Table 8. Fatty acid composition of an oil-mix reflecting the composition in oat bran fat (weight%)

Fatty acid composition	Peanut oil	Sunflower oil	Rapeseed oil	Oil mixture[1]	Oat bran fat[2]
SAFA	20.0	11.5	7.5	13.8	20.0
MUFA	53.0	27.5	62.5	41.8	36.9
PUFA	27.0	61.0	30.0	44.0	43.1
18:2, n-6	22.0	60.0	21.0	40.5	42.0
18:3, n-3	ni	ni	9.0	1.4	1.6

[1] 35% peanut oil, 49% sunflower oil and 16% rapseed oil

[2] The fatty acid composition was based on data from the Swedish National Food Administration (2010)

SAFA= saturated fatty acids

MUFA= monounsaturated fatty acids

PUFA= polyunsaturated fatty acids

ni= no information

A β-glucan product derived from oats containing 80% β-glucans has been shown to lower blood cholesterol levels in humans (Braaten *et al.* 1994). In the present work it was demonstrated that a diet containing a β-glucan product isolated from oat bran (46% β-glucan content; peak MW=356 kDa) and a diet containing a pure β-glucan product (97-98% β-glucan content; peak MW=285 kDa) did not differ in their cholesterol-lowering properties (Fig. 12). Their effects were also in the same range as that of the oat bran diet (12%, 16% and 14%, Table 7), which contained the same amount of β-glucan as the diets with purified β-glucan products (Paper IV). However, only one of the β-glucan groups (285 kDa) differed statistically from the control group ($P<0.05$) while the oat bran group and one of the β-glucan glucan groups did not ($P=0.06$ and $P=0.11$, respectively) when using Tukey´s test for multiple comparisons.

When another statistical test for multiple comparisons was used, Dunnett's test, based on a comparison against a reference group only, both the OB diet and one of the β-glucan diets (peak MW 285 kDa) showed reduced cholesterol levels compared with the control diet ($P=0.034$ and $P=0.014$, respectively). The other β-glucan (peak MW 356 kDa) was close to significance (in reducing cholesterol levels) ($P=0.063$; Fig. 13).
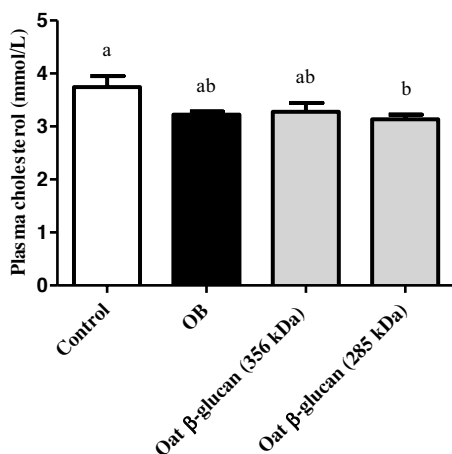
Figure 12. Plasma cholesterol levels after 4 weeks in C57BL/6NCrl mice (n=8-10 per group). Data are presented as means ± SEM. Statistics is analysed by one-way ANOVA, using Tukey´s test for multiple comparisons where each group is compared with the control group. Bars with different letters differ significantly ($P<0.05$). OB=oat bran. (Paper IV).
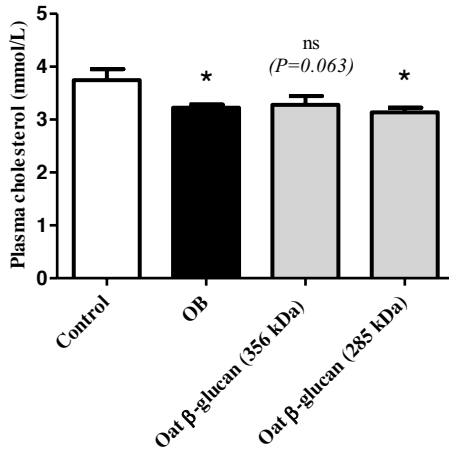
Figure 13. Plasma cholesterol levels after 4 weeks in C57BL/6NCrl mice (n=8-10 per group). Data are presented as means ± SEM. Statistics is analysed by one-way ANOVA, using Dunett´s test for multiple comparisons where each group is compared with the control group (* $P<0.05$). OB=oat bran, ns=not significant.

Moreover, both the oat bran group and the β-glucan groups increased the excretion of bile acids in faeces to about the same extent (see Section 5.5.5). An increased excretion of bile acids is a recognised mechanism for cholesterol-lowering effects. In fact, there are cholesterol-lowering drugs (e.g. cholestyramine) whose functions are to bind bile acids and by that prevent their reabsorption. As a consequence, the bile acid synthesis from cholesterol is upregulated (Chen *et al.* 2008). This, together with the interpretation of results on cholesterol, supports the conclusions that both β-glucan diets and the oat bran diet had effects on plasma cholesterol, when compared with the control diet.

To the best of the author's knowledge, no previous study has demonstrated the effects of a highly purified oat β-glucan (97-98% β-glucan), in animals or in humans. It seems as remaining proteins and arabinoxylans in the purified β-glucan from oat bran (46% β-glucan, 7% other types of dietary fibre, 20% protein and other components), did not play a major role in the cholesterol-lowering effect. This is an interesting aspect as there are indications in literature implying that oat β-glucan may interact with these components (Autio *et al.* 1992; Izydorczyk and MacGregor, 2000). In addition, it can, again, be concluded that processing of oats does not necessarily have an impact on

their cholesterol-lowering properties, as both β-glucan products were isolated using several kinds of treatment, but still had an effect on plasma cholesterol.

To briefly sum up this section about the role of oat components, we have found that both an oat bran oil containing ethanol soluble component(s) and purified β-glucans lower plasma cholesterol in mice. Moreover, the effect of the oat bran oil was not attributed to its content of unsaturated fatty acids. In the future, it would be interesting to further investigate which ethanol soluble component(s) that caused the effect (e.g. avenanthramides, tocotrienols and/or galactolipids).

5.5.2 The role of the physico-chemical properties of β-glucans

Evidence was found in this work that two purified β-glucan products differed with regard to their average MW of the β-glucans and apparently to their viscous properties. Despite these differences, their effects on plasma cholesterol levels in C57BL/6NCrl mice were similar. These findings are in good agreement with the results obtained from the evaluation of various enzyme-treated oat bran products in the same mouse model, where the viscous properties of the solubilised fraction of the products not could be correlated to the cholesterol-lowering effects (Paper II). These results imply that the viscous properties of oat products may not be crucial parameters for the cholesterol-lowering effect, and therefore other mechanisms should be considered (e.g. production of SCFA, see section 5.5.4).

The results obtained from the studies on LDLr$^{-/-}$ mice (see Fig. 9 in section 5.4), on the other hand, indicate that a change in physico-chemical properties of native β-glucans (such as reduced MW) may play a role in the cholesterol-lowering effect. Results from previous studies both agree and disagree with these results. For example, the effects of two kinds of bread containing oat bran with β-glucans of different average MWs (217 and 797 kDa), on plasma cholesterol in man were found not to differ (Frank *et al.* 2004). Similarly, another recent study in humans showed that the cholesterol-lowering effects of a low-MW β-glucan product and a high-MW β-glucan product derived from barley were similar (Keenan *et al.* 2007). A recent study in rats, by Bae *et al.* (2009b), also showed the same effects on total cholesterol in serum of two oat bran concentrates (43% β-glucan content) containing different average MWs of β-glucan (730 kDa and 1450 kDa). It should, however, be noted that the lower MW product in that study resulted in lower levels of VLDL cholesterol in serum than the higher MW product,

while levels of LDL cholesterol did not differ. Moreover, the average MWs of β-glucan in their products, were determined by gel chromatography using dextran standards and therefore may be somewhat overestimated (Beer *et al.* 1997b). In contrast to the above-mentioned studies, it has been reported that a diet containing untreated oat bran lowered plasma cholesterol levels, compared with a diet containing β-glucanase-supplemented oat bran in rats (Tietyen *et al.* 1995) and chicks (Petterson and Åman, 1992).

Studies in rats, hamsters and pigs have shown that the MW of β-glucans in intestinal contents are within the range 30-100 kDa, despite a high average MW in the feed (Wood *et al.* 1991; Johansen and Bach Knudsen 1997; Yokoyama *et al.* 2002). This could mean that the change in viscosity of the intestinal contents may be more dependent on the concentration of β-glucans than the variations in the MW of the β-glucans in the diet. Indeed, it has been proposed that beverages may be a more suitable food matrix than bread when designing cholesterol-lowering oat products (Kerckhoffs *et al.* 2003). These studies suggest that high solubility of β-glucans may play a crucial role in the cholesterol-lowering effect of oat products.

5.5.3 Release of β-glucans in an in vitro model

The release of β-glucans from various experimental diets was evaluated in a simple *in vitro* model, intended to resemble conditions in the upper human gastrointestinal (GI) tract (Paper IV). In agreement with previous studies, the release of β-glucan from an oat bran diet was low (34% after 5 hours'). Higher levels were released from a diet containing purified β-glucan products (285 kDa 76% and 356 kDa 88%). Beer *et al.* (1997a) found that 13-29% of the β-glucans was solubilised from oat bran samples after an *in vitro* digestion composed of a treatment with amylase, pepsin and pancreatin at 37°C, i.e. within the same range as our release of β-glucans from an oat bran diet (34%).

When 5 g (DM) oat bran (2006, cv. Sang, Lantmännen AB, Sweden, particle size <0.8 mm) was treated in the same *in vitro* model of the gastrointestinal tract (GI tract) described in Paper IV, the peak MW of the β-glucans in samples withdrawn after 3 and 5 hours was the same as that in untreated oat bran (3.0 MDa), i.e. the peak MW remained unchanged (Ulmius *et al.* to be published). These findings are in disagreement with *in vivo* animal studies (discussed in section 5.5.2), where the

average MW of β-glucans was found to be greatly reduced in the intestinal contents. On the other hand, the results obtained in the present work agree with another study indicating that β-glucans are not digested at conditions mimicking those in the stomach (Johansson *et al.* 2006).

5.5.4 Production of SCFA

Inhibition of the biosynthesis of cholesterol through the formation of propionic acid (a SCFA formed by the fermentation of fibre in the colon) constitutes one of the proposed mechanisms for the cholesterol-lowering effect of soluble oat β-glucans (Kerckhoffs *et al.* 2002; Theuwissen and Mensink, 2008). The fact that acetic acid is the primary substrate for cholesterol synthesis in the liver (Wong *et al.* 2006) motivates the interest in increasing the ratio of propionic to acetic acid in order to lower plasma cholesterol levels. It is interesting to note that *in vitro* experiments on isolated liver cells (Caco-2/TC-7 enterocytes) have shown that propionic acid and butyric acid reduce the mRNA levels of the rate-limiting enzyme for cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase) by 16% and 33%, respectively (Alvaro *et al.* 2008). Results from catheterised pigs have also shown increased circulation levels of butyric acid when the formation of butyric acid in the colon increased (Bach Knudsen *et al.* 2003). Although 70-90% of the butyrate is metabolised by the colonocytes (Wong *et al.* 2006) it can not therefore be ruled out that the formation of large amounts of butyrate may also have an effect on plasma cholesterol levels. For this reason, the (propionic acid + butyric acid) / acetic acid ratio was also evaluated. Furthermore, the pools of individual caecal fatty acids (μmol/caecal content) were preferred above the levels (μmol/g caecal content), as the size of caecum differed between mice (Papers II and IV).

The effects of untreated oat bran, various processed oat bran products and two purified β-glucan products on the production of SCFAs in the caecum were evaluated in two separate experiments in C57BL/6NCrl mice (Papers II and IV). The two control groups in the two separate experiments showed about the same ratios of major SCFA, as was expected, as they were fed a control diet with the same nutritional composition.

The following proportions of acetic, propionic and butyric acid were formed in the caecum of mice fed oat bran: 59%, 19% and 16%, respectively (Paper IV). These values are in agreement with those found in previously reported studies of caecal

contents in rats fed oat bran (Berggren *et al.* 1993; 65%, 20% and 9% and Bränning *et al.* 2010; 62%, 16% and 16%). The proportion of butyric acid found in the caecum contents of rats fed oat bran in another study was 20% (Drzikova *et al.* 2005), i.e. in the same range as that found in this work (16%).

Untreated oat bran had an effect on cholesterol levels but the ratio of propionic to acetic acid was unaffected compared with the control. Instead, the (butyric acid + propionic acid) /acetic acid ratio was higher (Paper IV). Furthermore, this ratio was found to correlate with the peak MW of β-glucans in processed oat bran preparations, but an increased ratio did not further improve cholesterol-lowering effects (Paper II). However, it can not be excluded that an even higher ratio of (butyric acid + propionic acid)/acetic acid may affect plasma cholesterol levels. Formation of SCFA in caecum, however seems to have played a lesser role in the cholesterol-lowering effects of untreated oat bran in the mice. In a study on ileostomy patients, a cholesterol-lowering effect of oat bran-based bread (29 g total dietary fibre/day) was found compared with a wheat flour bread containing low amounts of fibre (5 g total dietary fibre/day), which supports the view that other mechanisms than fermentation might have been of importance for the effect of the oat bran used (Zhang *et al.* 1992).

Untreated oat bran containing native β-glucans did not lead to an increase in the propionic acid/acetic acid ratio compared with the control group (Paper IV), whereas 3 of 4 processed oat bran preparations did. Interestingly, these preparations contained β-glucans with higher water solubility than untreated oat bran (Paper II), and it may be questioned if this was the reason for the higher ratio of propionic acid to acetic acid. Further, two purified β-glucan products, that contained highly soluble β-glucans increased the ratio of propionic to acetic acid and had an effect on plasma cholesterol (Paper IV). Thus, one might speculate that processed oat products with a high content of soluble β-glucans may increase the ratio of propionic to acetic acid, and by that partly contribute to the cholesterol-lowering effect. If this mechanism is of importance, soluble, non viscous β-glucans (low MW) may be of relevance.

5.5.5 Excretion of faecal bile acids

It has been suggested that soluble oat β-glucans also may lower plasma cholesterol by binding bile acids in the small intestine (Kerckhoffs *et al.* 2002). It is known that most bile acids excreted in intestine are reabsorbed and transported back to the liver. To compensate for the small loss of bile acids in the stools, the liver synthesises new bile acids in the liver from cholesterol (Williams, 1976).

Binding of bile acids (directly or indirectly) leads to a reduced re-absorption from the small intestine by the liver, and as consequence the liver will be forced to produce more bile acids from cholesterol in the blood to compensate for the faecal excretion of bile acids (Kerckhoffs *et al.* 2002; Theuwissen and Mensink, 2008). The mechanism(s) by which oat β-glucans may bind bile acids is still not clear. However, Bowles *et al.* (1996) suggested that barley β-glucans do not bind to specific sites on the bile acid molecules, and speculated whether β-glucans may increase the excretion by binding complete micells or increase the viscosity of intestinal contents by entrapping the bile acids and thus diminishing absorption.

In one of the studies in the present work the oat bran diet was found to double the amount of bile acids excreted in faeces, compared with the control diet (Paper IV). Before, excretion of bile acids has been shown to be an important mechanism for the cholesterol-lowering effect of oat bran in humans (Zhang *et al.* 1992; Lia *et al.* 1997), which is in agreement with our results. We also found that the oat bran group and the two β-glucan groups excreted similar amounts of total bile acids in faeces, although the average MWs differed between the oat products (285, 356 or 2348 kDa) (Paper IV).

A study in ileostomy patients has shown that cholesterol absorption was lower, while the excretion of bile acids was higher after consumption of oat bran diet containing native high MW β-glucans (11.6g β-glucan/day) compared with hydrolysed oat bran (4.5g β-glucan/day) (Elllegård and Andersson, 2007). The study period comprised 2 x 3 days with at least 4 days wash-out between the periods. There was no information about the MW of β-glucan in the hydrolysed oat bran but it was reported that 61% of the β-glucans had been completely digested to glucose. From this study it cannot be concluded that the MW of β-glucans is a crucial parameter in the excretion of bile acids, but nevertheless, mainly the β-glucans in the oat bran seem to give rise to

the increased excretion of bile acids and to the lower absorption of cholesterol. It can, however, not completely be excluded that other dietary fibre of the oat bran contributed to the effect as well, since a mixture of enzymes were used in the production of the hydrolysed oat bran (i.e. hemicellulase, cellulase and β-glucanase).

To summarise this section about the mechanisms of the cholesterol-lowering effects of oats, our results indicate that the mechanisms behind the cholesterol-lowering effect of untreated oat bran include: i) an effect by oat bran oil which contained ethanol soluble components, and ii) an effect by a β-glucan containing fraction. Moreover, the effect of the oat bran oil fraction might have compensated for the low release of β-glucans from the bran during the passage in the GI tract. The results obtained for products highly enriched in oat β-glucan, on the other hand, imply that soluble β-glucans may lower plasma cholesterol by increasing the excretion of bile acids – which is in line with mechanisms proposed for water soluble dietary fibre (Kerckhoffs *et al.* 2002). The viscous properties of β-glucan seemed to play a less role. In the future it would be interesting to investigate if purified β-glucans with even lower average MW than 285 kDa would have the same effect on plasma cholesterol.

6 Conclusions

The work presented in this thesis was focused on isolation and characterisation of oat products and their cholesterol-lowering properties. Another important issue was to find out how processing of oats influenced the effect on plasma cholesterol. The following conclusions were drawn:

- A wild-type mouse strain (C57BL/6NCrl) together with an atherogenic diet was shown to be an appropriate model for the evaluation of the cholesterol-lowering effects of both oat flakes and oat bran products. However, the response to oats was dependent on the mouse substrain (Paper I).

- Various oat bran preparations containing different average MW of β-glucans (2348 kDa to <10 kDa) lowered plasma cholesterol in C57BL/6NCrl mice. The difference in viscous properties and MWs were not reflected in plasma cholesterol levels. The ratio of (butyric acid + propionic acid)/acetic acid formed in caecum was found to be dependent on the average MW. Also in LDLr$^{-/-}$ mice, oat bran preparations with different peak MW of β-glucans (21 kDa, 1311 kDa or 1800 kDa) lowered plasma cholesterol. With this model there was a small, but statistically significant, decline in the cholesterol lowering effects with decreased average MW of β-glucans. Most important, it could be concluded that when oat bran was processed by wet-milling, starch digestion, heat, β-glucanases and freeze-drying to lower average β-glucan MWs and viscous properties, there was no or only marginal loss of the cholesterol-lowering effect in the mouse models (Paper II).

- A combined hot water extraction (3 hours; 100°C) with a thermostable α-amylase resulted in about 80% extractable β-glucan (i.e. maximum extraction yield), while for example an extraction at a lower temperature (48°C) without enzymes for the same time resulted in only 28% extractable β-glucan. Inclusion of a sequential pancreatin treatment before centrifugation did not further improve the amount of extractable β-glucans but increased the purity of the extract. Using papain as a protease source resulted in a product containing low MW β-glucans with low protein content. The side activity of β-glucanases

which was found for papain might partly explain the low MW after purification by this enzyme (Paper III).

- The cholesterol-lowering effect of oat bran could be ascribed to two complementary fractions; an oat bran oil fraction containing ethanol-soluble components and a β-glucan containing fraction. The cholesterol-lowering effect of the oat bran oil was shown not to be attributed to its content of unsaturated fatty acids (Paper IV).

- Two purified β-glucan products with different viscous properties and somewhat different average MWs of β-glucan (285 kDa or 356 kDa) did not differ in their effects on plasma cholesterol in C57BL/6NCrl mice (Paper IV). This implies that the MW and viscous properties of oat products are not necessarily crucial parameters for the cholesterol-lowering effect.

- When oat bran, a purified oat bran β-glucan (46% β-glucan) and a processed commercial β-glucan (97-98% β-glucan) were used in diets balanced to give the same β-glucan content, the cholesterol-lowering properties were the same (Paper IV).

- Noteworthy, since the two purified β-glucan products were produced by extensive processing (Paper IV) it can be concluded that processing is not necessarily detrimental to the cholesterol-lowering effect of oat products, in line with conclusions drawn from Paper II.

In the future it would be interesting to find out which components of the oat bran oil that contributed to the cholesterol-lowering effect. Another interesting issue to elucidate is whether purified β-glucans with even lower average MW (less than 256 kDa) could lead to a loss in the cholesterol-lowering effect; an important issue when developing oat based foods with optimised cholesterol-lowering properties.

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Paper I

Effects of oats on plasma cholesterol and lipoproteins in C57BL/6 mice are substrain specific

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Cholesterol-lowering effects of oats have been demonstrated in both animals and human subjects. However, the crucial properties of oat-containing diets that determine their health effects need to be further investigated to optimise their use. A mouse model would be a valuable tool, but few such studies have been published to date. We investigated the effects of oat bran on plasma cholesterol and lipoproteins in two substrains of C57BL/6 mice. Western diet was made atherogenic by the addition of 0·8 % cholesterol and 0·1 % cholic acid. After 4 weeks on atherogenic diet, total plasma cholesterol had increased from 1·86–2·53 to 3·77–4·40 mmol/l. In C57BL/6NCrl mice, inclusion of 27 and 40 % oat bran reduced total plasma cholesterol by 19 and 24 %, respectively, reduced the shift from HDL to LDL + VLDL and caused increased faecal cholesterol excretion. There was no effect of oat bran on plasma levels of the inflammatory markers fibrinogen, serum amyloid A or TNF-α. Contrary to findings in C57BL/6NCrl mice, there was no sustained effect of oat bran (27 or 40 %) on plasma cholesterol in C57BL/6JBomTac mice after 4 weeks of feeding. Thus, C57BL/6NCrl mice fed an atherogenic diet are a good model for studies of physiological effects of oats, whereas a substrain derived from C57BL/6J, raised in a different breeding environment and likely possessing functional genetic differences from C57BL/6N, is considerably less responsive to oats. The present finding that two substrains of mice respond differently to oats is of practical value, but can also help to elucidate mechanisms of the cholesterol-lowering effect of oats.

β-Glucans: Dietary Fibres: Cholesterol: Lipoproteins: Atherosclerosis

Beneficial effects of oats and oat β-glucans on plasma cholesterol and lipoproteins have been reported in both human subjects[1–5] and animals[6–9]. In 1997, Food and Drug Administration approved a health claim stating that 'soluble fibre from food such as oat bran, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease'[10], and recent meta-analyses of a large number of human studies on oat fibres support the concept that they lower total and LDL-cholesterol in plasma, with no or little effect on HDL or TAG[11,12]. Some trials investigating the hypocholesterolaemic effect of oats and/or oat β-glucans do, however, fail to show significant reduction in plasma cholesterol, and when stating the health claim Food and Drug Administration reviewed thirty-three clinical studies, out of which twenty-one showed significant reduction in blood cholesterol by oats, whereas twelve did not[10]. The concentration and size of β-glucans have been suggested to influence their physiological effects, and furthermore, processing, cooking and storage of oat products can change their physico-chemical properties, causing the cholesterol-lowering effect to be altered or lost[13]. Therefore, the effects of β-glucans may vary depending on the preparation of the

food ingredients[1,2]. An animal model in which effects of defined preparations of oats and/or β-glucans can be systematically investigated would be an important tool for clarifying these issues.

The basis for the cholesterol-lowering effect of oats is not definitely known, although components suggested to be responsible are the soluble fibres, β-glucans, present. These are non-starch polysaccharides composed of β-(1 → 4)-linked glucose units separated by a single β-(1 → 3)-linked glucose unit every two to three units. Several mechanisms of action have been proposed for their effect, including decreased intestinal (re)uptake of dietary cholesterol and bile acids, fermentation in the colon leading to release of carboxylic acids with effects on cholesterol metabolism, as well as effects on glucose uptake and insulin levels[14–20].

The incomplete understanding of the mechanisms by which β-glucans may lead to reduced cholesterol levels contributes to difficulties in interpreting the divergent outcomes of human studies with regard to oat-containing products. It is not clearly elucidated which role the molecular weight (Mp) of β-glucans plays and to which extent other components in oats also contribute to the beneficial effects.

In vitro experiments suggest anti-oxidative and anti-inflammatory effects of phenolic acids, sterols, flavonoids and vitamin E (α-tocopherol) present in oats, but little *in vivo* evidence for this exists[21]. Increased understanding of the relevant parameters may allow the development of new oat-containing foods that are attractive to consumers as well as beneficial to health. It would further be desirable to systematically evaluate new products in an animal model before costly human trials are performed.

Studies in hamsters[8], chickens[22] and rats[6,7,9] have previously evaluated the cholesterol-reducing effect of oats or oat β-glucans. Some of these studies do not compare effects of oat dietary fibre with a control fibre[6,7], which makes it difficult to ascribe the effects to the oat fibres rather than fibres in general. Delaney *et al.*[8] found similar effects of oat and barley β-glucans on plasma cholesterol and lipoproteins in hamster, an animal with a lipoprotein profile similar to human subjects. Different animal models may be appropriate for studies of dietary effects of oats depending on the particular goal. A mouse model would be attractive because of the large number of genetic variants available. These can be used to provoke hypercholesterolaemia and atherosclerosis on either high-fat or normal food formulations and can potentially allow mechanisms of action to be efficiently analysed. Furthermore, mice are common laboratory animals that are cost efficient to keep and consume small amounts of food, a consideration of importance when experimental diets are tested. The inbred strain of C57BL/6 mice develops hypercholesterolaemia and eventually atherosclerosis when fed a high-fat diet containing cholesterol and bile acids (atherogenic diet)[23], and has been used extensively in studies of cholesterol-lowering drugs or dietary components such as soya isoflavones[24], psyllium husks[25], persimmon fruits[26] and taurine[27]. An early study in wild-type C57BL/6 mice did not reveal any cholesterol-lowering effect of oats[28]. Given recent developments in the use of mice in atherosclerosis research, there is, however, ample reason to further consider this species as an experimental model.

The aims of the present study were to evaluate mice as a model for studies of cholesterol-lowering effects of oats. As an initial step, we evaluated the effects of oat bran on plasma cholesterol, lipoproteins and systemic inflammatory markers related to atherosclerosis, and also on faecal cholesterol excretion in C57BL/6 mice. We found that the substrain C57BL/6NCrl (B6NC) readily responds with sustained lowered plasma cholesterol concentration and reduced levels of LDL + VLDL on an oat bran diet, whereas this was not the case with the substrain C57BL/6JBomTac (B6JB).

Materials and methods

Animals

Female C57BL/6NCrl mice were purchased from Charles River laboratories (Sulzfeld, Germany) and C57BL/6JBomTac mice from Taconic (Lille Skensved, Denmark). During an adaptation period of 2 weeks, all mice were fed normal chow (R34 rodent chow, Lactamin, Vadstena, Sweden). At 10–12 weeks of age (body weight 17–21 g), mice were randomly assigned to the experimental groups. The mice had free access to food and water, and were kept in

a temperature-controlled environment and 12 h light cycle environment. All experiments followed national guidelines for the care and use of animals, and were approved by Malmö/Lund regional ethical committee for laboratory animals (M86-05).

Diets

Experimental diets were designed to resemble a human 'Western' diet, in terms of the contribution of fat, protein and carbohydrate to the total energy intake (41, 16 and 43 %, respectively), and were made atherogenic by inclusion of 0·8 % (w/w) cholesterol and 0·1 % (w/w) sodium cholate, except where indicated. In initial experiments, a concentration of 0·5 % cholic acid was used, but this was reduced to 0·1 % in the major part of the study to prevent gallstone formation. Control and oat bran diets were adjusted in order to keep the energy ratios constant; for details, see Tables 1 and 2. DL-Methionine was added to provide a sufficient supply of amino acids and to compensate for differences in methionine contents between oat bran protein and casein[29,30].

The oat bran diets contained 40 or 27 % oat bran (*Avena sativa* cv. Sang, produced 2007, Lantmännen AB, batch 1008596, Järna, Sweden), pre-milled to particle size less than 0·8 mm. The oat bran had a total fibre content of 16 % (oat bran composition analysed by Eurofins Food, Lidköping, Sweden), whereof 7·2 % was β-glucans. The β-glucan

Table 1. Formulation of the atherogenic diets with 0·1 % cholic acid and 0·8 % cholesterol

	Control diet (g/kg diet)	27 % oat bran diet (g/kg diet)
Casein, 80 mesh*	200	146
DL-Met	3	4·5
Maize starch	272	133
Maltodextrin	100	100
Sucrose	100	94
Cellulose	44	0
Butter, anhydrous†	200	176
Maize oil	10	10
Mineral Mix S10026	10	10
Dicalcium phosphate	13	13
Calcium carbonate	5·5	5·5
Potassium citrate·1 H₂O	16·5	16·5
Vitamin Mix V10001	10	10
Choline bitartrate	2	2
Cholesterol†	7·54	7·59
Sodium cholate	1	1
Oat bran	0	270
Oat bran contents (g/270 g oat bran)‡		
Protein		48
Sucrose		6
Starch		139
Fat		24
Dietary fibres (whereof β-glucans)§		44 (19·4)
Ash		9

* Casein is 88 % protein.
† Anhydrous butter has 230 mg cholesterol/100 g. To compensate for this, extra cholesterol was added to the oat bran diet so that total amount of cholesterol was 8 g/kg diet in both diets.
‡ Nutrient contents of oat bran were analysed by Eurofins Food Lidköping, Sweden, 2007.
§ β-Glucan content was analysed in our laboratory, see Materials and methods for details.

Table 2. Macronutrient and energy content of the atherogenic diets

	Control diet	27 % oat bran diet
Protein (% energy)	16	16
Carbohydrate (% energy)	43	43
Fat (% energy)*	41	41
Saturated	29	26
Unsaturated	10	11
Polyunsaturated	2	4
Energy (kJ/g diet)	19	19

* The composition of fat was based on data for maize oil, oat bran and butter from Swedish National Food Administration[39].

content was determined using an enzymatic kit (Megazyme International, Wicklow, Ireland) based on the McCleary[31] method for mixed linkage β-glucans. The diet with 27 % oat bran thus contains approximately 2 % β-glucans and 4·4 % total fibre, whereas the diet with 40 % oat bran contains 3·0 % β-glucans and 6·5 % total fibre. In the control diets, the oat fibres were replaced by 4·4 or 6·5 % microcrystalline cellulose (Avicel® PH 101, FMC Biopolymer, Philadelphia, PA, USA), respectively. Table 1 shows the formula for the 27 % oat bran diet with the respective control diet. The diet with 40 % oat bran and its respective control diet were also made according to the formula in Table 1, but the control diet was compensated for the larger proportion of oat protein, sucrose, starch, fat and fibres present in the test diet. In both cases, the control and oat bran diets were matched with respect to dietary fibre, energy and macronutrient contents. The diets were purchased as premixes from Research Diets Inc. (New Brunswick, NJ, USA), to which melted anhydrous butter, maltodextrin, cellulose and oat bran were added in our laboratory by careful mixing. All experimental diets were fed as powder. Feed consumption was determined per cage over 1-week periods and expressed as g consumed per mouse and day.

Molecular weight of β-glucans in oat bran

Oat bran samples were extracted with 82 % (v/v) boiling ethanol (2 h) to inactivate endogenous β-glucanases. After centrifugation (9300 **g**; 15 min), the supernatant was discarded and the residue oven dried (13 h at 60°C), pulverised and extracted with 0·1 mol/l NaOH for 2 h at room temperature. After centrifugation (15 000 **g**; 10 min), the supernatant was neutralised, diluted ten times and filtered through a nylon filter (0·45 μm; R04SP04700, GE Water & Process Technologies). Peak Mp and Mp distribution of β-glucans were analysed using high-performance size-exclusion chromatography with post-column addition of calcofluor, as described by Tosh *et al.*[32]. The Mp of the β-glucans in the oat bran batches used here was found to be approximately 3·0 MDa.

Experimental protocol

B6NC and B6JB mice were fed diets either with 27 or 40 % oat bran or with their respective control diets. In each study, mice were initially divided into groups that were housed together in cages of seven or ten animals. Following 2 weeks

on regular chow diet, blood samples were drawn to establish baseline values and the different cages were then fed experimental diets. The animals (*n* 82) tolerated the studies well, and after 4 or 5 weeks on the experimental diet, mice were sacrificed by cervical dislocation under isoflurane anaesthesia.

Plasma cholesterol and TAG

At weeks 0 (study start), 1, 2 and 4, blood samples were collected after 4 h fasting, from vena saphena into EDTA-coated microvette tubes. Samples were taken after 4 h fasting to achieve stable conditions without starvation. Plasma was prepared by centrifuging whole blood at 5000 **g** for 10 min at 4°C. Samples were stored at −80°C until assayed. To protect the structure of the lipoproteins during freezing, 10 % sucrose was added to plasma aliquots aimed for lipoprotein analysis. Total plasma cholesterol and TAG levels were determined colorimetrically using Infinity cholesterol/TAG Liquid Stable reagent (ThermoTrace, Noble Park, Vic., Australia).

Plasma lipoproteins

Plasma lipoproteins were separated by electrophoresis in 0·8 % agarose gels in barbital buffer according to the method of Noble[33], using a Sebia Hydragel 7 Lipoprotein(E), K20 chamber (Sebia, France). With this method, apo B-containing lipoproteins (LDL, LDL and VLDL) can be separated from HDL due to their individual charges. After staining of the gels with Sudan black and densitometric scanning (BioRad GS 800 Calibrated Densitometer and Quantity One quantitation software), the relative amounts of HDL and VLDL + LDL were calculated from the intensity of the bands. Values of VLDL and LDL were summed since the bands are not clearly distinguishable. With this method, it is possible to analyse very small amounts of plasma (2–3 μl), but the method only yields relative amounts in each sample, not absolute concentrations of lipoproteins.

Plasma inflammatory markers

To investigate whether oat bran had an impact on low-grade systemic inflammation related to the development of atherosclerosis, plasma from mice fed 27 % oat bran for 4 weeks was analysed with commercially available ELISA kits for the acute-phase proteins fibrinogen (Immunology Consultants Laboratory, Inc. Newberg, OR, USA) and serum amyloid A (Tridelta Development Ltd, Maynooth, Ireland) and for the proinflammatory cytokine TNF-α (R&D Systems, Inc., Minneapolis, MN, USA).

Faecal sampling and analyses

Faeces were collected from each cage during 24 h at baseline and after 4 weeks of experimental diet administration. The collected faeces (from ten or seven animals) were lyophilised and weighed. Lipids were then extracted from the material by a modified version of the protocol by Hara & Radin[34]. Triplicates of 80–150 mg lyophilised faecal material from each sample were extracted in hexane–isopropanol (3:2 v/v) with 0·005 % 2,6-di-tert-butyl-4-methylphenol. A total of

5 ml (2 ml + 3 × 1 ml wash) of the extract was dried under N_2, and the residue was redissolved in 1 ml isopropanol + Triton X-100 1 %[35]. This solution was used in duplicate (2 × 5 μl) for the cholesterol assay, which was the same as the one used for the plasma total cholesterol analysis. The total amount of cholesterol in the sample was divided by the number of mice per cage to yield the average cholesterol excretion in faeces (mg/mouse and day).

Data presentation and statistical analysis

Data are presented as mean values with their standard errors. Significance of differences between means was tested by Student's *t* test for unpaired data in GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). Values of $P < 0.05$ were considered to indicate statistical significance.

Results

C57BL/6 substrains and plasma cholesterol

Inclusion of oat bran in an atherogenic diet prominently attenuated the ensuing hypercholesterolaemia in the B6NC mouse substrain, whereas only a transient effect was seen in B6JB mice (Fig. 1). This difference is of interest with respect to the analysis of genetic effects and mechanisms of action of oats and β-glucans, and is also of obvious practical importance for the testing of dietary components in mice. Since the purpose of the present study was to establish a mouse model for evaluating effects of oats and oat β-glucans, we concentrate here on the B6NC strain for the majority of the results. Data from B6JB mice are shown for comparison. The effects of 27 and 40 % oat bran cannot be directly compared since the total amounts of fibre differ in the two diets and their respective control diet (4·4 *v*. 6·5 %). Both concentrations do, however, lower plasma cholesterol significantly, and the effect is slightly more pronounced with 40 % oat bran (Fig. 1(b)). There was no effect of 40 % oat bran on plasma cholesterol in the B6JB substrain (Fig. 1(d)). The data in Fig. 1(d) were obtained using a concentration of cholate of 0·5 % in the atherogenic diet, whereas the data in Fig. 1(a)–(c) were obtained using 0·1 % cholate. The reason for this change is that we noticed more variation in cholesterol levels, lower weight gain and a tendency to gallstone formation using the higher cholate concentration.

Body weight and feed intake

The mice gained weight throughout the study. When B6NC mice were fed 40 % oat bran, they gained more weight than those fed control fibre (Table 3), which was accompanied by a greater food intake in this group (Table 3). With 27 % oat
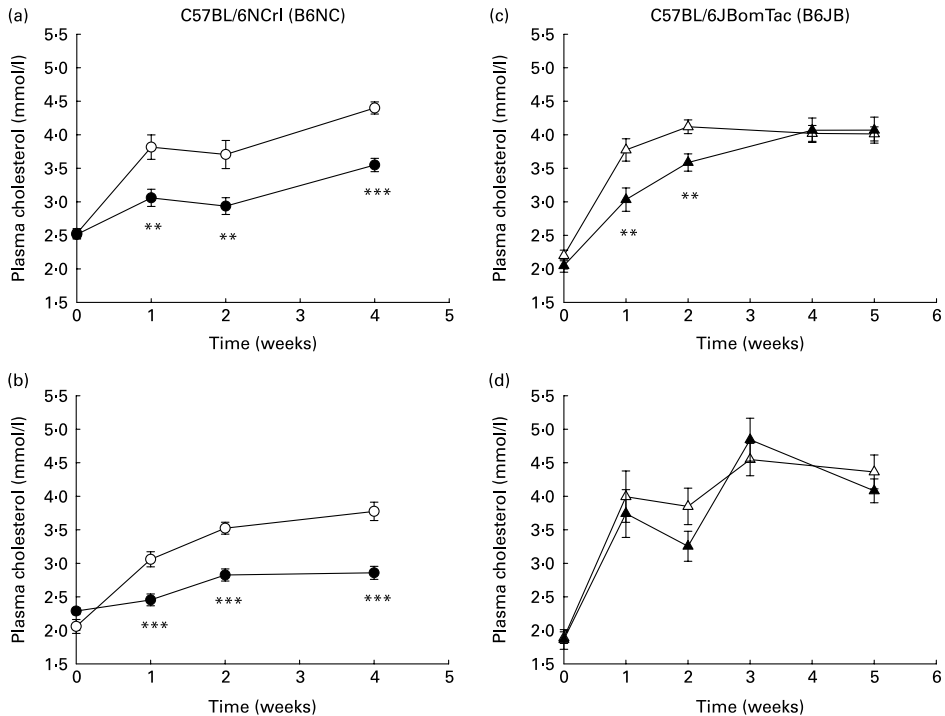
Fig. 1. Effects of oats on plasma cholesterol differ between substrains of C57BL/6 mice. Data show plasma cholesterol at baseline (normal chow, week 0) and after 1–5 weeks on atherogenic diet in C57BL/6NCrl mice with 27 % ((a), *n* 10) or 40 % ((b), *n* 14) oat bran, and in C57BL/6JBomTac mice with 27 % ((c), *n* 10) or 40 % ((d), *n* 7) oat bran. Data are presented as mean values with their standard errors. (●), Oat bran; (○), Control. Statistical analysis was performed with unpaired Student's *t* test. **$P < 0.01$ or ***$P < 0.001$.

Table 3. Initial weight, body weight gain, feed intake and faecal cholesterol excretion in C57BL/6 mice fed atherogenic diets for 4 weeks
(Mean values with their standard errors)

	Initial weight (g)			Body weight gain (g)			Feed intake (g/mouse and day)			Dry faeces (g/mouse and 24 h)		Faecal cholesterol (g/mouse and 24 h)	
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n		n		n
C57BL/6NCrl													
Control (4·4 % total fibre)	18·3	0·3	10	2·6	0·4	10	2·1	0·03	3†	0·17	1†	7·0	1†
27 % oat bran	18·2	0·2	10	2·7	0·2	10	2·1	0·07	3†	0·21	1†	9·1	1†
Control (6·5 % total fibre)	21·5	0·3	14	0·7	0·3	14	2·1	0·03	8†	ND	–	ND	–
40 % oat bran	21·1	0·2	14	1·8*	0·2	14	2·4*	0·06	8†	ND	–	ND	–
C57BL/6JBomTac													
Control (4·4 % total fibre)	19·2	0·3	10	1·7	0·3	10	2·2	0·2	4†	0·19	1†	9·6	1†
27 % oat bran	19·8	0·3	10	2·7*	0·3	10	2·4	0·1	4†	0·21	1†	10·3	1†
Control (6·5 % total fibre)	19·2	0·4	7	−1·0	0·3	7	2·0	0·1	4†	0·29	1†	ND	–
40 % oat bran	18·6	0·5	7	1·3*	0·6	7	1·9	0·1	4†	0·31	1†	ND	–

n, numbers of observations.
Statistics were calculated with Student's unpaired t test.
* Significantly different between oat bran and control ($P < 0.05$).
† The number refers to number of cages (in each cage, an average from seven to ten mice housed together).

bran, there was, however, no difference in body weight and feed intake between the two groups. Mean feed intake for the two B6NC experiments was 2·1 (SEM 0·1) g/mouse and day for control groups, and 2·3 (SEM 0·2) g/mouse and day for oat bran groups, corresponding to an energy consumption of about 40 kJ per mouse and day.

Cholesterol excretion

There were no marked differences in total amount of faeces collected over 24 h between control and oat groups (Table 3). Feeding mice atherogenic diet increased the cholesterol excretion approximately threefold, and, in B6NC mice, oat bran (27 %) caused an increase in cholesterol excretion compared with control diet. In contrast, there was no effect of oat bran on cholesterol excretion in B6JB mice (Table 3).

Plasma lipoproteins

The atherogenic diets induced a dramatic shift of lipoproteins towards a more human-like, atherogenic profile with elevated LDL + VLDL v. HDL: after treatment with the atherogenic diet, about 55 % of the lipids were in the LDL + VLDL fraction, compared with 35 % at baseline. Addition of oat bran resulted in a less atherogenic lipoprotein profile, with LDL + VLDL fractions of 50 and 41 % in the presence of 27 and 40 % oat bran, respectively, both significantly lower compared with control diets (Table 4). In B6JB mice, the effects of the atherogenic diets on lipoprotein profiles were similar to those in B6NC, but oat bran did not reduce the levels of LDL + VLDL in this substrain (Table 4).

Plasma TAG

The atherogenic diet induced a significant reduction in plasma TAG compared with baseline levels (Table 5). This effect has been seen earlier in C57BL/6 mice fed similar diets[36]. Oat bran in the diet did not further decrease the TAG levels, and inclusion of 27 % oat bran actually increased the TAG content significantly (Table 5).

Table 4. Lipoprotein profiles in C57BL/6NCrl and JBomTac mice at baseline (normal chow) and after 4 weeks on atherogenic (Ath) diets
(Mean values with their standard errors of relative amounts of lipoproteins)

	HDL (%)				LDL and VLDL (%)				
	Control		Oat bran		Control		Oat bran		
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	n
C57BL/6NCrl									
Baseline	66·4	1·8	66·1	0·9	33·6	1·8	33·9	0·9	10
Ath diet, 27 % oat bran	44·2††	0·9	49·5*	1·9	55·8††	0·9	50·5*	1·9	10
Ath diet, 40 % oat bran	46·5	1·9	58·9**	2·0	53·5	1·9	41·1**	2·0	14
C57BL/6JBomTac									
Baseline	65·9	1·5	65·7	2·1	34·1	1·5	34·3	2·1	10
Ath diet, 27 % oat bran	44·0†	1·2	40·9	1·1	56·0††	1·2	59·1	1·1	10
Ath diet, 40 % oat bran	37·7	0·9	32·1*	2·2	62·3	0·9	67·9*	2·2	7

Number of animals (n) are shown in the right column. Statistics were calculated with Student's t test.
Mean values were significantly different between oat bran and control: * $P < 0.05$, ** $P < 0.001$.
Mean values were significantly different from baseline levels: † $P < 0.01$, †† $P < 0.001$. (There are no baseline values available for mice fed 40 % oat bran.)

Table 5. Plasma TAG and inflammatory markers in C57BL/6NCrl and JBomTac mice after 4 weeks on atherogenic diets
(Mean values with their standard errors)

	TAG (mmol/l)			Fibrinogen (µg/ml)			SAA (µg/ml)			TNF-α (pg/ml)		
	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*
C57BL/6NCrl												
Baseline	0·83	0·04	20	ND	–	–	ND	–	–	ND	–	–
Control	0·61†	0·03	10	21·3	2·0	10	6·6	0·5	10	30·4	1·4	10
27 % oat bran	0·71*	0·03	10	20·6	2·2	10	6·7	0·8	10	32·0	2·4	10
C57BL/6JBomTac												
Control	ND	–	–	25·1	0·6	10	7·3	0·9	10	29·3	1·5	10
27 % oat bran	ND	–	–	23·6	0·5	10	8·0	0·7	10	32·6	3·7	10

n, numbers of observations; SAA, serum amyloid A.
Statistics were calculated with Student's *t* test.
* Significantly different between oat bran and control ($P < 0.05$).
† Significantly different from baseline levels ($P < 0.05$).

Plasma inflammatory markers

Addition of 27 % oat bran to the atherogenic diet did not affect the levels of fibrinogen, serum amyloid A or TNF-α in plasma in any of the two substrains of mice (Table 5).

Discussion

The present study shows that effects of oats on plasma cholesterol levels can be conveniently evaluated in a mouse model with observation times of weeks. Mice thus provide a useful model to evaluate how different components of oats contribute to the cholesterol-lowering effect and how processing of oats might interfere with these effects. The choice of mice as an animal model makes it possible to study not only cholesterol-lowering but also anti-atherogenic effects of oats in different GM mice available.

The mice tolerated the diet well and after an initial weight loss regained weight to exceed the initial weight at the end of the 4-week period. This is similar to earlier findings in mice fed atherogenic diets[37,38]. A slightly greater weight gain in oat-fed than in control animals has been reported in rats[9] and, over the course of the present study, we found a slightly greater increase in mice fed 40 % but not 27 % oat bran. The diets were adjusted to have the same energy contents, and the food intake was similar. However, we cannot exclude that the oat bran contained minor components that affected the weight gain. We chose to use a natural oat product containing high Mp β-glucans for the present study, in order not to jeopardise the effect of the oat fibres since it has been suggested that processing can alter the structure of the fibres and hence also their bioactive effects[13].

A possible drawback of using oat bran is that it is difficult to properly compensate for its lipid and protein contents. Although the amounts of protein and lipids were adjusted to be exactly the same in both diets, the sources of these components were different. Therefore, for example, the amounts of fatty acids in control and oat bran diets differed slightly as a result of different sources of fat (Table 2). A control experiment was performed to evaluate whether this difference had an impact on plasma cholesterol. In an alternative control diet (oil mix control), the fat in oat bran was replaced by a mixture of peanut oil (35 %), sunflower seed oil (49 %) and rapeseed oil (16 %) instead of butter to achieve an identical fatty acid composition in control and oat bran diets. Results showed that oil mix control and 'butter-only' control did not differ significantly in plasma cholesterol after 4 weeks (4·14 *v.* 3·98 mmol/l, respectively, *n* 9–10), whereas oat bran diet (3·55 mmol/l, *n* 10) differed significantly from both control diets. This demonstrates that the slightly different composition of fatty acids in control and oat bran diets in the present study was not crucial for the cholesterol-lowering effect observed.

Addition of cholesterol and cholic acid to the diet is necessary to induce hypercholesterolaemia in C57BL/6 mice, since this effect is very moderate on Western diet alone[38]. This was supported in a pilot experiment where we fed C57BL/6 mice Western diet. This diet induced only a moderate increase in plasma cholesterol, which made it difficult to register significant effects of oats (data not shown). We found that the addition of 0·8 % cholesterol together with 0·1 % cholic acid is preferable over the commonly used concentration of 0·5 %. In initial experiments in the present study, we fed mice atherogenic diets with 0·5 % cholic acid and found insignificant weight gain, and in some individuals even weight loss, over 4 weeks (Table 5, B6JB, 40 % oat bran). High-fat diets containing added cholesterol and cholic acid are sometimes referred to as lithogenic because of their tendency to induce gallstone formation in susceptible mice strains, including C57BL/6[37,39–41]. In the present study, gallstones were observed in some cases after feeding diets containing 0·5 % cholic acid. This was, however, not seen with 0·1 % cholic acid. From these observations, we conclude that the addition of cholesterol and cholic acid to the diet is a prerequisite to induce sufficient hypercholesterolaemia in C57BL/6 mice, but the concentration of cholic acid should be moderate to prevent gallstone formation.

The atherogenic diet used in the present study induced a dramatic decrease in lipids transported by HDL and increase in lipids transported by LDL and VLDL, as reported before in C57BL/6J mice[6,23,42]. Inclusion of 27 or 40 % oat bran to the diet significantly inhibited the shift from HDL to LDL + VLDL in B6NC mice, and thus contributed to a less atherogenic lipoprotein profile. In the B6JB mice, on the other hand, oat bran in the diet did not inhibit the shift from HDL to LDL + VLDL. Oat bran did not reduce plasma TAG in the C57BL/6 mice. These findings are in line with

the effects of oats in human subjects as reported in the meta-analysis by Kelly *et al.* [12] and in rats[9]. In contrast, some later studies have reported decreased TAG in rats after consumption of oats[6]. Species or strain differences on the effects of oats on TAG levels cannot be excluded.

Studies in various infection models have shown immuno-modulatory effects of β-glucans from oats both *in vitro* and *in vivo* [44], but effects on systemic inflammation in relation to atheroclerosis are less documented. In the present study, we found no prominent effects of 27 % oat bran on the systemic inflammation markers investigated (Table 5). This is in line with two human studies where consumption of oat β-glucan did not have an influence on inflammatory markers such as the acute-phase protein, C-reactive protein[44,45]. Further investigations are, however, needed to clarify whether systemic anti-inflammatory effects of oats would be observable in more advanced atherosclerosis models and if so, which component(s) in oats are effective.

Increased cholesterol excretion in subjects fed oat-based diets has earlier been found in both human ileostomy patients[3] and hamsters[8]. Consistent with this, B6NC mice fed 27 % oat bran excreted more cholesterol in faeces than control mice, whereas this was not the case with B6JB mice, which also did not respond with decreased plasma cholesterol levels. The daily intake of dietary cholesterol was on average 18 mg/mouse, and although other mechanisms may contribute, the observed levels of faecal cholesterol excretion, from 7 (control) to 10 (oat bran) mg/d, are therefore likely to partly account for the reduction of plasma cholesterol in oat-fed mice.

The C57BL/6N and J substrains have been separated in different breeding environments for more than 50 years, and genetic differences between them have been noted in other investigations, notably in their development of alcohol dependence[46] and also in their cardiac responses to anaesthesia[47]. Transcriptional profiling has revealed differences in gene expression in neural tissue from the two substrains[46,48]. Recently, a mutation in a gene (*Nnt*) coding for a mitochondrial enzyme, nicotinamide nucleotide transhydrogenase, has been identified in C57BL/6J, but not in other B6 strains and proposed to account for their decreased glucose tolerance and insulin secretion[49,50]. The C57BL/6JBomTac substrain used in the present study was separated from the original C57BL/6J stock from Jackson laboratories three decades ago, and the *Nnt* mutation does not occur in C57BL/6JBom-Tac[51]. To our knowledge, the substrain difference in response to oats revealed here is a novel finding and further studies are needed to explore the basis of this phenomenon. The difference in response to oats could originate from genetic variations between substrains such as single nuclotide polymorphism, but could also originate from different gene expression patterns (epigenetics) or from environmental factors, such as a divergent intestinal microflora. Composition of the microflora has recently been suggested to influence diet-induced obesity and diabetes in mice[52]. Elucidation of the mechanisms responsible for the substrain difference found here may offer new insight into mechanisms of the cholesterol-lowering effect of oats, and this could help to explain the different outcomes observed in human studies. The C57BL/6 mice have already been used for decades in studies of cholesterol-lowering drugs or dietary components[24–27]. The present finding of the substrain difference demonstrates,

however, that the origin of the C57BL/6 mice used might be of importance for the outcome of the experiments.

Digestion and lipid metabolism differ in several aspects between mice and human subjects. For example, mice are coprophages and have their most fermentative activity in the caecum instead of in the colon as in human subjects, with possible impact on the intestinal microflora and the fermentation of soluble fibres. Mice also lack cholesterol ester transfer protein, which in human subjects is important for delivery of HDL cholesterol to the liver by first transferring the cholesterol from HDL to apoB-containing lipoproteins (LDL, VLDL)[53]. A large number of studies have documented effects of oats and oat fibres on cholesterol levels in human subjects[12]. The value of the mouse model is rather that it permits studies of mechanisms, including impact on atherosclerosis development, not possible to obtain in human studies. Furthermore, the mouse model can serve as a convenient screening system for new diet ingredients before costly human trials are performed.

In conclusion, we show that C57BL/6 mice on an atherogenic diet serve a good model for systematic investigations on cholesterol-lowering effects of oat preparations, but that substrain differences occur in the responsiveness to oats. The substrain difference reported here may possibly be of use in further efforts to define the mechanistic basis for the cholesterol-lowering effect of oats.

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Paper II

Effects of oat bran, processed to different molecular weights of β-glucan, on plasma lipids and caecal formation of SCFA in mice

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In the present study, we evaluated the cholesterol-lowering effects of different oat bran (OB) preparations, differing regarding their peak molecular weight (MW_p) of β-glucans (2348, 1311, 241, 56, 21 or < 10 kDa), in C57BL/6NCrl mice. The diets were designed to be atherogenic (0.8 % cholesterol and 0.1 % cholic acid), and they reflected the Western diet pattern (41 % energy fat). All OB preparations that were investigated significantly reduced plasma cholesterol when compared with a cellulose-containing control diet, regardless of the molecular weight of β-glucan. Moreover, the difference in viscous properties between the processed OB (from 0.11 to 17.7 l/g) did not appear to play a major role in the cholesterol-lowering properties. In addition, there was no correlation between the molecular weight of β-glucan and the amount of propionic acid formed in caecum. Interestingly, however, there was a significant correlation between the ratio of (propionic acid + butyric acid)/acetic acid and the MW_p of β-glucans: the ratio increased with increasing molecular weight. The results of the present study suggest that the molecular weights and viscous properties of β-glucan in oat products may not be crucial parameters for their cholesterol-lowering effects.

Molecular weight: β-Glucan: Cholesterol: SCFA: Mice

The cholesterol-lowering effects of oats have been studied in both human subjects and animals since the beginning of the 1960 s. This effect has mainly been ascribed to its content of the soluble fibre β-glucans, as 80 % purified oat β-glucan has been shown to reduce cholesterol levels in hypercholesterolaemic human subjects[1]. In 1997, the Food and Drug Administration approved a health claim for oat products based on soluble fibres from whole oats (i.e. oat bran (OB), oatmeal or rolled oats, and also whole-oat starch) after a review of thirty-seven clinical studies of the effect of oats on blood lipids[2]. Daily intake of a minimum of 3 g oat β-glucans was deemed necessary to cause a relevant reduction in cholesterol levels. Health claims for β-glucans from barley have subsequently been approved[3]. However, it is not completely understood what molecular structure the β-glucans should exhibit to be physiologically active or to what extent other cereal components, e.g. lipids, antioxidants and other types of dietary fibres, contribute to the effect.

Cereal β-glucans are linear polysaccharides that are present in the cell walls, and they are found in oats, barley, wheat and rye. They are composed of a chain of glucose units connected by β-(1–4) and β-(1–3) linkages. Apart from the β-glucan content, the repeating pattern of these linkages varies between cereals; it has been shown to affect the solubility and gelation properties[4]. Different processing treatments of oats, e.g. bread baking[5] or repetitive freeze–thaw treatments[6], have been shown to change the molecular weight and/or the solubility of β-glucans. Such changes may possibly affect the cholesterol-lowering effects, although our knowledge about the relevant parameters is incomplete. Both the viscosity and the concentration of β-glucans after *in vitro* digestion have been reported to have a significant influence on the glucose response after a meal[6]. Another clinical study of various oat β-glucans found that there was a linear correlation between the change in plasma glucose after a meal and the viscosity of the drink consumed[7]. In contrast to the above-mentioned reports, studies of β-glucans of different molecular weights have shown that there is no difference in cholesterol-lowering effects either in animal models[8,9] or in human subjects[10]. In order to optimise the cholesterol-lowering

effects of oat products, a more detailed understanding of the importance of different physico-chemical properties of β-glucans (including molecular weight) is needed.

Gallaher *et al.*[11] investigated the cholesterol-reducing effects of hydroxypropyl methylcellulose in hamsters, and found a correlation between plasma cholesterol levels and the viscosity of the intestinal contents. Isolated β-glucan (80 % pure) and rolled oats have been shown to increase the viscosity of the small intestinal contents in rats in comparison to rats fed a diet containing cellulose[12]. One might therefore expect that the viscous properties of oat products would play a fundamental role in their biological activity.

It has been suggested that propionate, one of the SCFA produced when β-glucans are fermented in the large intestine, can reduce hepatic cholesterol synthesis[13]. Such effects of intestinal fermentation of β-glucans may thus contribute to their cholesterol-lowering effects. However, to our knowledge, the effect of the molecular weight of β-glucan on the formation of SCFA has not been investigated.

Human trials of oat products are time-consuming and costly to perform. Thus, an animal model (e.g. mice) can often be a suitable tool for screening of new potential food ingredients or to investigate the mechanisms of action. We have recently demonstrated that cholesterol-lowering effects of oats can be evaluated in C57BL/6NCrl mice that are fed an atherogenic diet[14]. This mouse model was used in the present study, where the main objective was to investigate the roles that molecular weight and viscous properties of oat β-glucan play in the cholesterol-lowering effects of oat products. We also wanted to evaluate their effect on the lipoprotein pattern and on TAG, and their effect on the formation of SCFA in the caecum.

Materials and methods

Experimental protocol

A control experiment was performed to investigate whether processing of OB, wet milled and amylase treated but without β-glucanase treatment, affects its cholesterol-lowering properties. After confirming that there was no difference in cholesterol-reducing effects between processed OB (POB; 1311 kDa) and OB, or in gain in body weight, feed intake and dry faeces output (data not shown), two separate experiments were performed to evaluate how different molecular weights of β-glucans in the POB preparations affect plasma cholesterol and production of SFCA in the caecum. In the first experiment, we compared the effect of POB (1311 kDa) used in the control experiment with the effect of three other β-glucanase-treated POB (241, 56 and 21 kDa). In the second experiment, the effect of an additional POB with β-glucans of even lower peak molecular weight (MW_p; < 10 kDa) was compared with the effect of untreated OB.

Animals

Female C57BL/6NCrl mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All mice were fed normal chow (R34 rodent chow; Lactamin, Sweden) during an adaptation period of 2 weeks. At 10–12 weeks of age (body weight 17–21 g), mice were randomly divided into experimental groups, and were housed together in cages with ten animals per cage. The mice were kept in a temperature-controlled room with a 12-h light cycle environment, and they had free access to food and water. All experiments were approved by the Malmö/Lund regional ethical committee for laboratory animals. The animals (*n* 110) tolerated the studies well except for one animal (in Expt 2, fed control diet) that had symptoms of illness and was withdrawn from the study. After 4 weeks on the experimental diet, the mice were killed by cervical dislocation, and the caecal tissue and contents were collected.

Diets

To induce hypercholesterolaemia, an atherogenic diet (0·8 % cholesterol and 0·1 % cholic acid) was designed, which reflected the Western diet with about 41 % energy fat, 16 % energy protein and 43 % energy carbohydrates (Table 1). The different diets were produced in our laboratory from a premix purchased from Research Diets, Inc. (New Brunswick, NJ, USA), in the same way as we have described previously[14]. During preparation of the diets, we assumed that all ingredients were dry, without correcting for traces of water.

In Expt 2, the diet formulae were adjusted to fit the nutrient composition of a new batch of OB (Table 2), in the same way as done when we designed the diets used in the initial control experiment and Expt 1 (Table 1). The experimental diets were fed as powders.

Table 1. Formulation of the atherogenic diets (g/kg diet)*

Ingredients	Control diet	OB or POB diet†
Casein, 80 mesh‡	200	146
DL-Met	3	4·5
Maize starch	272	133
Maltodextrin	100	100
Sucrose	100	94
Cellulose	44	0
Butter, anhydrous‖	200	176
Maize oil	10	10
Mineral mix S10026	10	10
Calcium phosphate	13	13
Calcium carbonate	5·5	5·5
Potassium citrate·1 H₂O	16·5	16·5
Vitamin mix V10001§	10	10
Choline bitartrate	2	2
Cholesterol‖	7·54	7·59
Sodium cholate	1	1
OB or POB¶	0	270
Nutritional composition¶		
Digestible carbohydrates	482	464–483
Fat	210	210–212
Protein	176	176–193
Total dietary fibre	44	41–46
Oat β-glucan	0	19–23
Total energy content (kJ/g diet)	19	19

OB, oat bran; POB, processed OB.
* All values are expressed in fresh weight.
† POB (produced from OB) with β-glucan of different peak molecular weights.
‡ Casein is 88 % protein.
§ Containing 97·8 % sucrose.
‖ Anhydrous butter has 230 mg cholesterol/100 g. To compensate for this, extra cholesterol was added so that total amount of cholesterol in all diets was 8 g/kg diet.
¶ Dry-milled OB (*Avena sativa*, cv. Sang b 1008596) or dry-milled POB, both < 0·8 mm. The nutritional composition of OB and POB is illustrated in Table 2, and it explains the range in nutritional compositon of the OB diet and the POB diets.

Table 2. Nutrient content of experimental processed oat bran (POB)* products and the oat bran (OB)† used as a starting material (g/100 g)‡

	Expt 1					Expt 2	
Nutrient	OB (1800 kDa)§	POB (1311 kDa)	POB (241 kDa)	POB (56 kDa)	POB (21 kDa)	OB (2348 kDa)‖	POB (<10 kDa)
Fat	8·9	9·3	9·4	9·1	9·6	9·5	9·8
Protein	18	24	24	24	24	19·7	19·1
Maltose	ND	28	22	25	28	ND	27
Maltotriose	ND	2·5	2·5	2·2	2·7	ND	2·9
Sucrose	2·2	1·4	1·1	1·5	1·7	1·2	0·7
Glucose	ND	0·0	2·1	0·0	0·0	ND	0·2
Total dietary fibre	16	17	15	15	17	16	8·5¶
Whereof β-glucan	7·2	8·1	8·0	8·0	8·4	7·5	6·8
Ash	3·4	2·6	2·6	2·8	3·0	2·9	3·0
Digestible carbohydrates**	54	47	49	50	47	52	60¶

ND, not determined.
* POB (produced from OB) with β-glucan of different peak molecular weights.
† OB used as a starting material for the production of POB (see Materials and methods).
‡ All values are based on DM.
§ *Avena sativa* (cv. Sang), produced in 2007 by Lantmännen.
‖ *A. sativa* (cv. 43 % Sang, 10 % Kerstin and 47 % mixed oats containing Belinda in large part); produced in 2008 by Lantmännen.
¶ The deviation of total dietary fibre and consequently total carbohydrates between POB (<10 kDa) and OB (2348 kDa) was most probably a consequence of the method used for the total dietary fibre analysis, which is based on an enzymatic digestion of starch and protein followed by precipitation of fibre with 80 % ethanol[22]. However, fibres of less than ten to twenty monomers are not expected to be quantitatively precipitated.
** Calculated by difference: 100 − protein − fat − ash − dietary fibre (for example, starch or minor sugars).

Processing of oat bran

Two batches of OB were used in the different experiments in mice; they were produced in the same mill (Lantmännen AB, Järna, Sweden) but from different cultivation varieties of oats. The nutrient compositions of the two OB were similar (Table 2), and both were used as starting materials to produce five POB products that would differ only with respect to the molecular weights of β-glucans. In the initial control experiment and in Expt 1 (performed in 2008), we used OB based on a Swedish variety of oats named 'Sang' (produced in 2007, batch 1008596). In Expt 2 (performed in 2009), the OB used was based on a mixture of Swedish oat varieties: 43 % Sang, 10 % Kerstin and 47 % mixed oats, mainly Belinda (produced in 2008, batch 1047749). This change was for agricultural reasons. However, we did not see any significant difference in the present results related to the source of the OB (Table 2 and Fig. 2).

Four POB products were produced essentially as described by Triantafyllou Oste[15], and were treated with different amounts of β-glucanase from *Aspergillus* sp. (Biocon, Barcelona, Spain) to obtain different molecular weights of β-glucan. One of the batches was produced without the addition of β-glucanase for comparison with untreated OB. An additional, fifth POB product was produced in the same way except that in the β-glucanase step, excess amounts of a β-glucanase from *Trichoderma longibrachiatum* (Biocon) were used in addition to the β-glucanase from *Aspergillus* sp. The five products were obtained from OATLY AB (Landskrona, Sweden) as liquid suspensions. Before freeze-drying, a solution of maltodextrin (1:3 in water) was added to the liquid suspension to a final concentration of 19 % in an attempt to prevent the formation of insoluble complex that would remain undissolved during the passage through the intestine *in vivo*. The mixture of OB and maltodextrin was placed on trays and stored at −20°C before freeze-drying. DM and minor sugar components were analysed in all suspensions before mixing with maltodextrin.

The freeze-drying was kept constant at −20°C for 162 h, and the temperature was then raised to +5°C (at 4°C per h; Labconco, Ninolab, Upplands Väsby, Sweden). The freeze-dried materials were dry milled to a particle size of less than 0·8 mm (Laboratory Mill 120; Perten Instruments, Huddinge, Sweden).

Analysis of β-glucan in oat products

Samples for molecular weight determination of β-glucan were extracted and analysed as described previously[16]. The POB samples were not extracted with ethanol before extraction with 0·1 M-NaOH since the β-glucanases were assumed to be inactivated by processing. This was confirmed by comparing two samples with and without an ethanol extraction; the results showed that the molecular weights were the same in both cases.

The total β-glucan content of solid materials and liquid samples from viscosity measurements were determined by using a kit, following an enzymatic assay method for mixed linkage β-glucans[17].

Viscosity measurements

The freeze-dried POB products were solubilised in deionised water for 1 h at room temperature under agitation with a magnetic stirrer (approximately 2·8 g POB per 28 g water). At least two replicates were made for each POB product. The samples were centrifuged at 15 000 g for 10 min, after which the amount of the supernatant was weighed. In order to determine the percentage of solubilised DM and β-glucan, a small aliquot of supernatant was taken and stored at −20°C until the analysis was done. The viscosity of the supernatant was measured with a stress-controlled rheometer (StressTech, Reologica, Sweden) with a concentric cylinder (25 mm diameter: CC25) at room temperature.

Solutions with different sucrose concentrations were used to verify the method. In order to operate within a Newtonian region, the supernatant obtained from POB (1311 kDa) was diluted 1:1 with deionised water before the measurement of viscosity. Different shear stresses were used to provide a range of shear rate from 5 to 50 per s.

Analysis of nutrient composition

Protein content was determined with a Kjeltec System 1003 (Tecator AB, Höganäs, Sweden) or with a carbon/nitrogen analyser (Vario Max CN, Elementar Analysensysteme GmbH, Hanau, Germany). Crude oat protein was calculated as nitrogen content × 6·25. Fat content was determined using the conventional styrene − butadiene rubber solvent extraction method based on the work of Schmid[18], Bondzynski[19] and Ratzlaff[20], involving a gravimetric extraction in diethyl ether and petroleum ether (40–60°C, 1:1) after hydrolysis in 7·7 M-HCl and ethanol for 1 h at 75°C. The content of total dietary fibre in the OB samples was determined by Eurofins Foods (Lidköping, Sweden) according to the Association of Official Analytical Chemists (985·29) method of Prosky *et al.*[21], whereas total dietary fibre in POB products was analysed according to the method of Asp *et al.*[22]. Both methods are gravimetric, and are based on the enzymatic digestion of starch and proteins followed by precipitation of the fibre with ethanol. These methods have shown good agreements[23]. The sugar analysis of liquid suspensions was performed by means of HPLC using a Zorbax carbohydrate analysis column (4·6 × 150 mm) from Agilent Technologies, Inc. (Santa Clara, CA, USA); elution was done with acrylonitrile−H$_2$O (63:37) at a flow rate of 1 ml/min and at 35°C. Moisture content was determined by drying the samples for 15 h at 105°C, whereupon the DM that remained was weighed after cooling in a desiccator for 1 h.

Plasma cholesterol, TAG and lipoproteins

At baseline and after 4 weeks, blood samples were collected after 4-h fasting[14]. Total plasma cholesterol and TAG were determined with Infinity cholesterol/TAG liquid stable reagent (Thermo Trace, Noble Park, Vic, Australia). Plasma lipoproteins were electrophoretically separated in agarose gels in barbital buffer according to the method of Noble[24]. The gels were stained with Sudan black, and densitometric scanning (BioRad GS 800 Calibrated Densitometer and Quantity One quantitation software; BioRad, Hemel Hempstead, Herts, UK) of the intensity of the bands revealed the relative lipid distribution between LDL + VLDL *v.* HDL. Data are reported as (LDL + VLDL)/(HDL + LDL + VLDL) × 100. The percentage given for the lipoproteins reflects the lipid distribution among lipoproteins (since Sudan black stains cholesterol, TAG and phospholipids) and it does not exactly correspond to HDL- and LDL-cholesterol.

Caecum

The caecum was removed and weighed. The contents were transferred to a sterile tube and stored at − 80°C until analysis of SCFA. The caecal tissue was washed with PBS (pH 7·4), dried between layers of filter paper and weighed. Caecal content was calculated as the weight of the full caecum minus that of caecal tissue.

SCFA

SCFA (i.e. acetic, propionic, butyric, isovaleric, valeric, caproic and heptanoic acids) in the caecal content were analysed using a GLC method[25]. A sample of caecal content (0·1 g) was mixed with 1 ml of a solution containing 0·25 M-HCl (to protonise SCFA) and 1 mM-2-ethylbutyric acid (as an internal standard). The sample was homogenised for 1 min with an Ultra Turrax T25 basic (IKA-WERKE, Staufen, Germany), and then was centrifuged (MSE Super Minor, Hugo Tillquist AB, Solna, Sweden). Two hundred microlitres of the supernatant were transferred to a micro-insert bottle, and were injected onto a fused-silica capillary column (DB-FFAP 125-3237; J&W Scientific, Folsom, CA, USA; Agilent Technologies Inc.). Caecal pools (μmol) of the different SCFA were calculated as the concentration of each acid (μmol/g caecal content) multiplied by the caecal content. The total SCFA pool was determined as the sum of SCFA in μmol per caecal content, and the proportions of SCFA were determined as the ratio between the amount of acid (μmol) per caecal content and the total SCFA pool.

Calculations and statistical evaluation

For calculations of the nutrient composition of POB products, the results were corrected for the 19 % of maltodextrin by dividing the values by a factor of 0·81.

The concentration-normalised viscosity was calculated as

$$\frac{\eta_{sp}}{c} = (\eta_r - 1) \times \frac{1}{c} = \frac{\eta_s - \eta_0}{\eta_0} \times \frac{1}{c} \quad (l/g), \quad (1)$$

where η_{sp} is the specific viscosity; η_r is the relative viscosity (η_s/η_0); η_s is the viscosity of the solution containing the solute (i.e. the β-glucan in the present study); η_0 is the viscosity in the absence of the solute and c is the concentration of the solute. The concentration of β-glucan in the solution was converted from weight percentage to g/l using the corresponding density for sucrose solutions.

Data were analysed using the Minitab software package version 14.0 (Minitab, Inc., State College, PA, USA). Unless otherwise stated, results are expressed as means with their standard errors. Outliers were identified as samples deviating from the third quartile with more than 150 % of the interquartile range. The Anderson−Darling test was used to determine the normality of the measurements, where $P < 0.05$ rejects the null hypothesis that the data are normally distributed. For normally distributed data, one-way ANOVA was used for multiple comparisons (using the general linear model procedure), where Tukey's test for pairwise comparisons of means was used for the significance of difference ($P < 0.05$). Two sets of data for SCFA were not normally distributed, and therefore median values were calculated and percentiles were presented (Table 5). The non-parametric Kruskal−Wallis test was performed to compare the median values between the groups based on the variance by ranks[26].

Results

Nutrient content of experimental products

The analysis of nutrient content confirmed that the nutrient composition of the POB products was roughly equal to what was obtained for OB, which was used as a starting material (Table 2). A somewhat greater variation in protein content was seen (18–24 %) and consequently also in the content of digestible carbohydrates (46–53 %). There were no significant differences in the levels of minor sugars (i.e. sucrose, maltotriose, maltose and glucose) between the POB products. The total dietary fibre content measured was clearly lower in POB (<10 kDa) than in the starting material (i.e. OB). This was most probably a consequence of the method used for the total dietary fibre analysis, which is based on an enzymatic digestion of starch and protein followed by precipitation of fibre with 80 % ethanol as described by Asp et al.[22]. Following this method, fibre of less than ten to twenty monomers are not expected to be quantitatively precipitated. Thus, a significant amount, approximately 50 % (8·5/16, see Table 2), of the dietary fibre that is present should be composed of less than ten to twenty monomers.

Physico-chemical properties of processed oat bran

The solubility of the β-glucans in the POB products after a standardised dissolving procedure of the β-glucans in the POB products is presented in Fig. 1(A). There were small differences in the amount of solubilised DM (Fig. 1(B)). Generally, β-glucans from products with low molecular weights dissolve to a greater extent than those from products with high molecular weights.

The viscosity of the particle-free supernatant of each product was determined. From the results on viscosity and the β-glucan concentrations, the concentration-normalised viscosity was estimated to compare the thickening efficiency of the different degradation levels of β-glucans. The concentration-normalised viscosity ranged from 1·1 for POB <10 kDa to 17·7 l/g for POB of 1311 kDa (Fig. 1(C)). Solubilisation of dry-milled OB in deionised water, by using the same conditions as for the POB, resulted in approximately 23 % solubilised β-glucan and 5·7 % solubilised DM.

The MW_p value of β-glucan from OB based on 'Sang' oats was determined to be approximately 1800 (SEM 17) kDa, whereas β-glucan from for OB based on 43 % Sang was 2348 (SEM 25) kDa (means with their standard errors for duplicate samples). MW_p values for the different POB products were 1311 (SEM 12), 241 (SEM 5), 56 (SEM 0·0) and 21 (SEM 0·2), respectively (expressed in kDa as means with their standard errors for duplicate samples). The MW_p value of β-glucan for POB product produced using the most extensive enzyme treatment was not determinable, as we obtained a low fluorescence intensity that was close to the background level. It is known that the fluorescing complex between β-glucan and calcofluor is only formed at molecular weights of β-glucan that are greater than approximately 10 kDa[27]. The β-glucans present in the most extensively enzyme-treated POB were therefore most probably equal to or less than about 10 kDa, and thus we refer to this sample as POB <10 kDa.
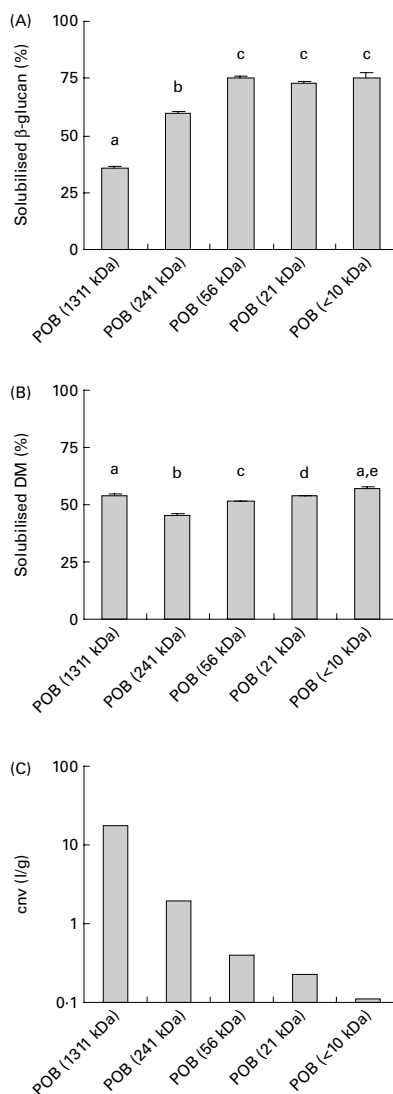
Fig. 1. Physico-chemical properties of processed oat bran (POB) samples with different MW_p of β-glucans. The obtained level of water-soluble β-glucan from POB before viscosity measurement ((A), n 4–5). Solubilised DM from POB before viscosity measurement ((B), n 6). Viscous properties of solubilised fractions of POB products, expressed as the concentration-normalised viscosity (cnv) which is equal to $\eta_{sp}/c_{\beta\text{-glucan}}$ (see equation 1), where $c_{\beta\text{-glucan}}$ in solution was 1·4 g/l for POB of 1311 kDa, 5·5 g/l for POB of 241 kDa, 5·9 g/l for POB of 56 kDa, 5·8 g/l for POB of 21 kDa and 4·9 g/l for POB <10 kDa, respectively (C). The results are presented as mean values. Error bars in (A) and (B) show SEM. [a,b,c,d,e] Mean values with unlike letters were significantly different ($P < 0.05$).

Body weight, feed intake and faeces excretion

As in our previous study[14], the body weight of all mice had increased during the 4 weeks on experimental diets, by an average of 2·8 g per mouse (pooled standard deviation = 0·95).

The mice that were fed oat products generally increased significantly more in body weight than mice that were fed control diet, even though this varied somewhat between the experimental series. Moreover, feed intake and faecal output were similar in the different dietary groups (Table 3).

Plasma cholesterol, lipoproteins and TAG

Distribution plots representing the baseline and the 4-week levels of plasma cholesterol for all dietary groups of mice in each experiment revealed one outlier, which was excluded from further analysis. This mouse belonged to the control group in Expt 2, and had unusually high plasma cholesterol (3·1 mmol/l) at baseline, which became reduced by 0·15 mmol/l after 4 weeks on atherogenic diet.

In the first experiment, we found that all POB products, with β-glucans with different MW_p values (1311, 241, 56 and 21 kDa), lowered plasma cholesterol equally (Fig. 2(A)). In an attempt to reveal a loss in efficiency by further reduction of molecular weight, we prepared another batch of POB with an $MW_p < 10$ kDa (see Results: physico-chemical properties). However, the cholesterol-lowering effects of this product were also not significantly different from those of the unprocessed OB (Fig. 2(B)).

Four weeks on atherogenic diet induced a prominent shift of the lipoprotein profile, where the relative proportion of LDL + VLDL was approximately doubled. No statistically significant reduction in the proportion of LDL + VLDL was found after the addition of OB or any of the POB to the diet (Table 4). Mean TAG levels in Expt 1 were 0·52 mmol/l in control mice after 4 weeks, and they were not significantly affected by the oat products (data not shown), which is in line with the results of our previous study[14].

Caecal content, caecal tissue weight and formation of SCFA

Weight of caecal tissue and the distribution of SCFA in the contents are given in Table 5. Mice fed POB with the highest MW_p value of β-glucan (1311 kDa) had higher caecal content than the control group ($P<0.01$). The weight of caecal tissue was higher for mice fed POB with a low MW_p value, i.e. 21 kDa ($P<0.01$) and 56 kDa ($P<0.05$).

The total pool of caecal SCFA was higher ($P<0.05$) for mice that were fed POB with an MW_p value of 1311 or 21 kDa than for mice that were fed the control diet. Acetic acid was the predominant SCFA in all groups, followed by propionic acid and butyric acid. The mean ratio between these three acids was 64:23:13. The caecal pool of propionic acid was higher ($P<0.05$) for all POB groups than for the control group (2·6–3·3 *v.* 1·4 μmol, respectively). Butyric acid, on the other hand, was formed in higher amounts at the high MW_p value ($P<0.01$) and low MW_p value ($P<0.05$) compared with the control group.

The ratio of (propionic acid + butyric acid)/acetic acid was significantly higher in all POB groups than in the control group, and increased with the MW_p of the β-glucans in the POB products (Table 6).

Discussion

Solubility, viscosity and molecular weight are physico-chemical properties that have been suggested to play crucial roles in the beneficial health effects of β-glucans. It has been demonstrated in human subjects that changes in molecular weight affect the glucose response[28], and also have different effects on gastrointestinal hormones[29]. The importance of the molecular weight of β-glucans for the cholesterol-lowering effects of oat products is not, however, completely understood. To address this, we evaluated the effects of OB, processed to different MW_p values of β-glucan (1311, 241, 56, 21 and <10 kDa), on plasma cholesterol levels, lipoprotein composition, TAG and intestinal production of SCFA in mice.

Table 3. Initial weight, body weight gain, feed intake and dry faeces for mice fed experimental diets for 4 weeks*
(Mean values with their standard errors)

	Intitial weight (g)			Body weight gain (g)			Feed intake (g/mouse and 24 h)			Dry faeces (g/mouse and 24 h)	
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	n
Expt 1											
Control	20·0	0·3	10	1·4^a	0·3	10	2·2^a	0·04	4†	0·21	1†
POB (1311 kDa)	19·5	0·3	10	2·4^b	0·2	10	2·4^a	0·08	4†	0·19	1†
POB (241 kDa)	19·0	0·3	10	2·6^b	0·2	10	2·3^a	0·09	4†	0·21	1†
POB (56 kDa)	19·2	0·3	10	2·9^b	0·3	10	2·1^a	0·1	4†	0·19	1†
POB (21 kDa)	19·1	0·3	10	2·4^b	0·1	10	2·2^a	0·09	4†	0·19	1†
Expt 2											
Control	19·1^a	0·4	8	3·1^a	0·3	8	2·3^a	0·09	3†	0·21	1†
OB (2348 kDa)‡	19·1^a	0·2	10	5·0^b	0·5	10	2·4^a	0·12	3†	0·20	1†
POB (<10 kDa)	19·2^a	0·2	10	2·8^a	0·4	10	2·4^a	0·06	3†	0·20	1†

n, Number of observations; OB, oat bran; POB, processed OB with β-glucan of different peak molecular weight.
^{a,b} Mean values with unlike superscript letters were significantly different between groups within each experiment ($P<0.05$).
* Statistics were calculated with one-way ANOVA for multiple comparisons (Tukey's test for pairwise comparisons of means).
† The number refers to the number of cages (ten mice housed per cage).
‡ OB, used as a starting material for the production of POB (see Materials and methods).
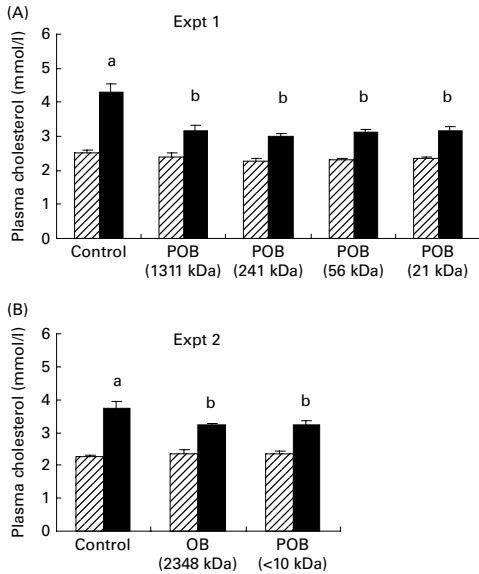
Fig. 2. Oat bran (OB) and processed OB (POB) with β-glucans of different peak molecular weight (MW$_p$) values reduce plasma cholesterol equally. Four POB with different β-glucan MW$_p$ values had a similar effect on plasma cholesterol ((A), n 10), and a third POB with even smaller β-glucans had the same cholesterol-lowering effects as OB ((B), n 8–10). Baseline values (▨) were not significantly different between the experimental groups. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$) after 4 weeks on experimental diets (■). Data are presented as means with their standard errors.

It should be noted that the MW$_p$ value describes the average of the molecular weight distribution of extracted β-glucans, as the curves appeared to be symmetric[16]. The MW$_p$ values for β-glucan from the two batches of OB used were 1800 and 2348 kDa, which is in good agreement with previously reported results[5,14,16]. The analysis used for molecular weight determination revealed that most of the β-glucans present in the sample with the lowest molecular weight had an MW$_p$ < 10 kDa (corresponding to less than sixty-two monomers). Interestingly, results on total dietary fibre indicated that about 50 % of the dietary fibre that is present should be composed of less than ten to twenty monomers. However, we cannot exclude the possibility that cellulose fibre in addition to the β-glucans present was also digested since we used a cellulase during the production of this POB product.

The water solubility of β-glucans increased as the molecular weight decreased, and ranged from 36 to 75 % for POB products (Fig. 1(A)). In comparison, β-glucans from untreated OB (dry milled) dissolved to approximately 23 %.

C57BL/6 mice develop high cholesterol levels when fed an atherogenic diet[30]. All the mice gained weight on experimental diets, and the molecular weight of β-glucan (from 1311 down to 21 kDa) had no influence on the gain in body weight. The cholesterol-lowering properties of OB

preparations were unchanged over the whole range of MW$_p$ investigated (Fig. 2). This suggests that β-glucans with MW$_p$ as low as 10–20 kDa are functional in lowering cholesterol, so that any limit for a loss in efficiency appears at even lower MW$_p$. We cannot, however, exclude the possibility that the cholesterol-lowering effects were partly caused by oat component(s) other than β-glucans, for example, by arabinoxylans, sterols, lipids and/or antioxidants (e.g. avenanthramides and vitamin E). The present findings regarding the effects on plasma cholesterol agree with previous studies on oat or barley β-glucans in animals[8,9] and human subjects[10]. Furthermore, a newly published study by Bae et al.[31] has shown that there is no difference in cholesterol-lowering properties between oat products with different molecular weights of β-glucan (1450–371 kDa) in male C57BL/6 mice, which is in agreement with the results of the present study. The study by Bae et al.[31] used enriched β-glucan at a high concentration (8·6 %), which is hardly attainable in human diets and which may have influenced the nutritional state, as the animals differed significantly in weight gain between the experimental groups.

In the present study, the viscous properties of the five POB were found to vary with MW$_p$ of the β-glucans (Fig. 1(C)). One hypothesis might be that these samples create different viscosities of the absorptive layer in the small intestine, and consequently affect the absorption rate as well as the amount of cholesterol absorbed into the plasma differently. We found, however, no correlation between plasma cholesterol levels and the viscous properties of POB. Thus, other mechanisms for the cholesterol-lowering effects must be considered (e.g. intestinal fermentation). Even so, we cannot exclude the possibility that β-glucans increase the viscosity of the intestinal contents in a way that is not strongly dependent on molecular weight or on the viscous properties of the β-glucans themselves. It has, for example, been suggested

Table 4. LDL + VLDL levels in mice at baseline and after 4 weeks on experimental diets*

(Mean values with their standard errors)

	LDL + VLDL (%)†				
	Baseline		4 Weeks		
	Mean	SEM	Mean	SEM	n
Expt 1					
Control	28·7[a]	2·5	60·4[a]	1·4	10
POB (1311 kDa)	21·7[a]	1·9	53·4[a]	2·5	10
POB (241 kDa)	25·8[a]	2·2	52·9[a]	1·9	10
POB (56 kDa)	22·0[a]	1·8	59·4[a]	2·8	10
POB (21 kDa)	22·6[a]	1·9	58·9[a]	2·7	10
Expt 2					
Control	22·7[a]	2·4	57·9[a]	5·9	8
OB (2348 kDa)‡	26·4[a]	2·0	42·9[a]	4·9	10
POB (<10 kDa)	24·5[a]	1·3	54·2[a]	2·0	10

n, Number of observations; POB, processed OB with β-glucan of different peak molecular weights; OB, oat bran.
[a] Mean values within a column with superscript letter was significantly different between groups within each experiment ($P < 0.05$).
* Statistical analysis was performed by using one-way ANOVA for multiple comparisons (Tukey's test for pairwise comparisons of means $P < 0.05$) on normally distributed data.
† Calculated as (LDL + VLDL)/(HDL + LDL + VLDL) × 100.
‡ OB, used as a starting material for the production of POB (see Materials and methods).

Table 5. Caecal content and tissue, and caecal SCFA in mice fed experimental diets (Expt 1)
(Mean values with their standard errors)

	Control diet			POB (1311 kDa)			POB (241 kDa)			POB (56 kDa)			POB (21 kDa)		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Caecal content (mg)	180[a]	6		270[b]	16	10	230[a]	29	9	240[a]	19	9	250[a]	16	9
Caecal tissue weight (mg)	36[a]	2.2		41[a,b]	3.9	10	47[a,b]	3.3	9	48[b]	2.7	9	51[b]	2.9	10
Total SCFA levels (μmol/g)	43[a]	3		56[a]	5	10	46[a]	3	10	47[a]	2	10	53[a]	3	10
Total SCFA pool (μmol)	8[a]	1		15[b]	1	10	11[a,b]	2	8	11[a,b]	1	8	13[b]	1	9
SCFA pools (μmol/caecal content)*															
Acetic acid	5.2[a]	0.4		8.3[b]	0.7	10	6.2[a]	1.0	8	6.8[a]	0.8	9	8.3[a]	1.0	10
Propionic acid	1.4[a]	0.1		3.3[b]	0.4	10	2.6[b]	0.4	8	2.6[b]	0.3	9	2.7[b]	0.2	10
i-Butyric acid	0.14[a,b]	0.01		0.21[a]	0.03	10	0.16[a,c]	0.03	8	0.21[b]	0.02	9	0.21[a,b]	0.02	10
n-Butyric acid†	0.65[a]	0.45–0.83		2.9[a]	1.9–4.1	10	1.1[a,c]	0.97–1.9	8	0.87[a,c]	0.66–1.3	9	1.5[a,c]	0.99–2.2	10
i-Valeric acid	0.19[a]	0.01		0.31[a]	0.03	10	0.26[a]	0.04	8	0.30[a]	0.02	9	0.30[a]	0.03	10
Valeric acid	0.13[a]	0.01		0.20[a,b]	0.02–1.4	10	0.11[b]	0.03	8	0.17[a,b]	0.02–1.6	9	0.17[a,b]	0.02–1.6	10
n-Hepatonic acid†	0.018[a]	0.014–0.023		0.20[b]	0.18–0.027	10	0.011[b]	0.009–0.014	8	0.015[a,b]	0.015–0.022	9	0.017[b]	0.007–0.021	10

n, Number of observations; POB, processed oat bran with β-glucan of different peak molecular weights.
a,b,c Mean values with unlike superscript letters were significantly different ($P < 0.05$). See Materials and methods for description of statistical procedures.
*The concentration of each acid (μmol/ g caecal content) multiplied with the caecal content.
†Since these data were not normally distributed, a non-parametric test was done (Kruskal–Wallis test). Data are expressed as median and 25th to 75th percentiles.

that increased viscosity of the intestinal contents may be an effect of increased mucus secretion stimulated by the presence of β-glucans. This hypothesis was based on the finding that the small intestinal content was viscous even after a 13-h fasting period, when no β-glucans were detected in the material from the small intestine[32].

The difference in MW_p of β-glucan in the different OB preparations had no statistically significant influence on the measured proportions of LDL + VLDL and HDL (Table 4). The electrophoretic separation of plasma lipoproteins is evaluated by staining of all plasma lipids (cholesterol, TAG and phospholipids) in the bands, and possible changes in HDL *v.* non-HDL cholesterol may therefore be obscured by the contributions from other lipids. In our previous study, the OB group had significantly lower LDL + VLDL values than the control group[14].

β-Glucans generally belong to the group of indigestible carbohydrates, which includes NSP, resistant starch and oligosaccharides. These are not digested and absorbed in the small intestine, but are partially or completely fermented to SCFA in the large intestine. However, animal studies have shown that the molecular weight of β-glucans is reduced during passage through the upper gastrointestinal tract, reaching between 35 and 100 kDa in the small intestinal content of pigs[33], hamsters[8] and rats[34], which suggests that the molecular weight of β-glucans may have been reduced during passage through the gastrointestinal tract in the present study also.

β-Glucans are generally considered to be water-soluble, fermentable dietary fibre. In contrast, cellulose is a water-insoluble fibre and is much more resistant to fermentation, giving low amounts of SCFA[35]. The effects on colonic fermentation of four POB products, containing β-glucans with different MW_p (1311, 241, 56 or 21 kDa), were evaluated in Expt 1 (Table 5). The major fatty acids formed were acetic acid, propionic acid and butyric acid at a mean ratio of 64:23:13. This is in the same range as the ratios found in studies on human intestinal material[36,37] (57:22:21) and on rat caecum[35] (69:21:10).

Butyric acid is usually considered to be important for the health of the colon, and a high degree of butyric acid formation has recently been suggested to have metabolic effects[38–40]. Drzikova *et al.*[41] found that the caecal pool of propionate and butyrate was significantly higher in rats fed an OB-based diet than in those fed a cellulose-containing diet, as we also obtained for the POB product with the highest MW_p of β-glucans.

Propionic acid has previously been suggested to reduce plasma cholesterol levels in human subjects, but the mechanism behind this is not completely understood[13,42–44]. The acetate produced after fermentation of fibres in the intestine is readily absorbed and transported to the liver where it can act as a substrate for acetyl-CoA formation, the precursor for endogenous cholesterol synthesis. It has been suggested that propionate could possibly impair the acetate utilisation, and thereby also cholesterol biosynthesis[42,45,46]. In the present study, we found that all POB gave rise to significantly higher pools of propionic acid compared with the control diet. There was no clear effect of β-glucan MW_p on the pools of either propionic acid or acetic acid and, except for the lowest MW_p, the ratio between propionic acid and acetic acid was significantly higher for all POB groups

Table 6. Ratio between the major caecal SCFA formed in mice (Expt 1)*
(Mean values with their standard errors)

Dietary group/ratio	(PRO + BUT)/ACE		PRO/ACE		n
	Mean	SEM	Mean	SEM	
Control	0·40[a]	0·02	0·28[a]	0·02	10
POB (1311 kDa)	0·73[b]	0·05	0·40[b]	0·02	9
POB (241 kDa)	0·65[b,c]	0·04	0·42[b]	0·03	8
POB (56 kDa)	0·54[c]	0·03	0·39[b]	0·02	9
POB (21 kDa)	0·54[c]	0·02	0·34[a,b]	0·02	10

PRO, propionic acid; BUT, butyric acid; ACE, acetic acid; n, number of observations; POB, processed oat bran with β-glucan of different peak molecular weights.
[a,b,c] Mean values within a column with unlike superscript letters indicate statistical significant difference between groups ($P < 0.05$).
* Statistical analysis was performed by using one-way ANOVA for multiple comparisons (Tukey's test for pairwise comparisons of means $P < 0.05$) on normally distributed data.

v. control group. There was, however, a significant positive correlation between the ratio of (propionic acid + butyric acid)/acetic acid and the MW_p of β-glucans (Table 6). Since this SCFA ratio was dependent on the MW_p of β-glucan but the plasma cholesterol was not, we suggest that caecal formation of specific SCFA may not have been a crucial mechanism for the cholesterol-lowering effects of oats found in the mice. However, it cannot be excluded that the results of SCFA in plasma would have been different.

The caecal content was significantly higher for mice fed β-glucans of the highest molecular weight than for those fed a cellulose-based control diet, which is in agreement with a previous study on rats fed a diet based on OB[41]. The weight of caecal tissue was, however, significantly higher in mice fed low-MW_p oat products. This might be due to an extensive fermentation of β-glucans and utilisation of SCFA. Another potential explanation is that the formation of SCFA is involved in the proposed stimulation of mucus secretion by β-glucans[32,47].

The results of the present study suggest that the molecular weight of β-glucan and the viscosity of oat products may not be crucial parameters for the cholesterol-lowering effects. However, this does not preclude the possibility that the viscosity of the intestinal contents may be of importance through a more complex mechanism, and that this may be affected by β-glucans without there being any strong relationship concerning the viscosity of β-glucans themselves. Binding of bile acids to β-glucans is another possible mechanism of action that may not be strongly dependent on molecular weight. Regarding the formation of SCFA in the caecum, we found that all POB gave rise to a significantly higher caecal content of propionic acid compared with the control group. There was no clear relationship between MW_p and propionic acid content, but the ratio of (propionic acid + butyric acid)/ acetic acid increased with increasing MW_p of β-glucans.

The cholesterol-lowering effects of oats are most likely a result of different mechanisms, and the present study indicates that development of new oat-based products with beneficial health effects can involve incorporation of β-glucans with a wide range of molecular sizes. However, human trials are needed to confirm the validity of the conclusions drawn

from the present study. It would be interesting to define more exactly how individual oat components, such as β-glucans, sterols and various antioxidants, contribute to the cholesterol-lowering effects.

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Paper III

Extraction of β-Glucan from Oat Bran in Laboratory Scale

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ABSTRACT   Cereal Chem. 86(6):601–608

Effects of various enzymes and extraction conditions on yield and molecular weight of β-glucans extracted from two batches of commercial oat bran produced in Sweden are reported. Hot-water extraction with a thermostable α-amylase resulted in an extraction yield of ≈76% of the β-glucans, while the high peak molecular weight was maintained (1.6×10^6). A subsequent protein hydrolysis significantly reduced the peak molecular weight of β-glucans (by pancreatin to 908×10^3 and by papain to 56×10^3). These results suggest that the protein hydrolyzing enzymes may not be pure enough for purifying β-glucans. The isolation scheme consisted of removal of lipids with ethanol extraction, enzymatic digestion of starch with α-amylase, enzymatic digestion of protein using protease, centrifugation to remove insoluble material, removal of low molecular weight components using dialysis, precipitation of β-glucans with ethanol, and air-drying.

Oats is an interesting raw material for development of food products due to its unique nutrient composition of carbohydrates, fat, and protein and its positive physiological effects on controlling blood lipids and glucose levels (Braaten et al 1994; Butt et al 2008). Several studies, both in animals and in humans, suggest that the soluble fibers (β-glucans) may be the most important component in the cholesterol-lowering effect of oats. In 1997, the U.S. Food and Drug Administration (FDA 1997) approved a health claim for the use of oat-based foods that said "A diet high in soluble fiber from whole oats (oat bran, oatmeal or rolled oats and whole oats flour) and low in saturated fats and cholesterol may reduce the risk for heart disease". The FDA also concluded that a daily minimum dose of 3 g of β-glucans is needed to reduce total serum cholesterol levels. FDA based their position on a review of 37 clinical studies on oat products, from which 11 studies showed no significant effect on total cholesterol or lipoproteins (FDA 1996). However, the claim refers only to the quantity of oat β-glucans needed but it ignores the physicochemical properties such as solubility and molecular weight that have been demonstrated recently as extremely important for its physiological effects (Lan-Pidhainy et al 2007; Tosh et al 2008). Frank et al (2004) did not obtain any significant cholesterol-reducing effect in humans after consumption of breads based on oat bran, which could be due to the reduced solubility of the β-glucans.

Extraction of oat β-glucans has been evaluated previously both on a laboratory scale and in a pilot plant where 6 g to 25 kg of oat bran has been used as starting material. The extraction methods include extraction with carbonate buffer (pH 10) at 60°C; hot-water extraction combined with starch hydrolysis at 90°C; and extraction with sodium hydroxide solutions (Wood et al 1989; Wood and Weisz 1991; Jaskari et al 1995; Bhatty 1995; Beer et al 1996, 1997). The highest extraction yield of β-glucans was obtained by Bhatty in 1995, who reported an extraction yield >90% after extraction of oat bran with 1.0*M* NaOH. The molecular weight was 2×10^6 from size-exclusion chromatography using blue dextran as standard. The ratio between oat bran and sodium hydroxide was 1:50, which makes it practically difficult to scale up. Lazaridou et al (2004) used a water extraction of oat flour at 47°C for 3 hr, followed by centrifugation. After treatment with amylase and pancreatin, low molecular weight sugars and amino acids were separated with dialysis and the β-glucans were precipitated in ethanol. The β-glucan content in the isolated concentrates was 90% and the average molecular weight was 203×10^3 or lower by using SEC connected to a RI detector.

Several studies reveal how different isolation techniques affect the structural features and rheological properties of oat β-glucans (Johansson et al 2000; Skendi et al 2003; Papageorgiou et al 2005). However, very few studies, if any, have dealt with extraction techniques aimed at obtaining a high extraction yield while retaining the molecular weight of the native β-glucans present in the starting material.

The objective of this study was to develop a laboratory procedure for enriching β-glucans from oat bran to produce sufficient amounts for physiological studies in mice. The developed process was to provide a product with a high representative amount of β-glucans from the starting material (i.e., a high yield) and keep the molecular weight of β-glucans as high as possible.

MATERIALS AND METHODS

Materials

Commercial oat bran (from *Avena sativa* cv. Sang) obtained from conventional farming sources and produced by Lantmännen AB (Järna, Sweden) was delivered as two different batches. Batch one, produced in 2005, was used as starting material for the experiments in small-laboratory scale, and batch two, produced in 2006, was used for experiments in a large-laboratory scale. The nutritional composition of the two oat brans is given in Table I. Before extraction, the oat bran was dry-milled to <0.8 mm (Laboratory mill 120, Perten Instruments, Huddinge, Sweden).

Enzymes used for purifying β-glucans were a thermostable α-amylase (Termamyl 120L or 300L type DX, Univar, Malmö, Sweden), pancreatin (P-7545, Sigma-Aldrich, Steinheim, Germany) with a protease activity at 200 U.S.P. units, an amylase activity at 200 U.S.P. units, and a lipase activity 16 U.S.P. units

TABLE I
Nutritional Composition of Commercial Oat Bran (g/100 g, db)[a]

Component	Oat Bran by Year	
	2005	**2006**
Total dietary fiber	20.4	19.3
β-Glucan	8.2	7.3
Protein[b]	18.8	22.6
Sucrose	1.7	<0.04
Fat	9.2	9.6
Digestible carbohydrates[c]	48.3	45.0
Total starch[d]	46.6	45.0
Ash	3.4	3.5

[a] Two batches produced from the same cultivar (*Avena Sativa* cv. Sang).
[b] N × 6.25.
[c] Calculated by difference (100 – protein – fat – ash – dietary fiber).
[d] Calculated by difference (digestible carbohydrates – sucrose).

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expressed as mg of pancreatin premix (Reynolds 1989), and papain (EC 3.4.22.2, 5125, Calbiochem, Darmstadt, Germany) with a protease activity at 30,000 U.S.P. units expressed as mg of papain premix. All other chemicals used were of analytical grade.

Small-Laboratory Scale: Evaluation of Extraction Method (Experiments I–VI)

In experiment I, β-glucans were isolated in the same way as described by Lazaridou et al (2004), except that treatment with 2-propanol and oven drying was omitted (Fig. 1). After collecting the β-glucan concentrate, it was air-dried at room temperature (RT) and milled to a particle size <0.5 mm.

Different experimental conditions were used to find the best possible extraction method for extracting β-glucans from oat bran (Experiments I–VI, Fig. 1): 47°C for 3 hr (I), 47°C for 3 hr under ultrasound (II), boiling for 3 hr with or without a subsequent amylase treatment (III:a and III:b), autoclaving for 10 min with or without a subsequent amylase treatment (IV:a and IV:b), a combined hot-water extraction and amylase treatment (V), and, finally, inclusion of a protease treatment (pancreatin) after the combined hot-water extraction with amylase (VI).

After extraction, the mixture was centrifuged and the residue was separated from the supernatant, freeze-dried (HETOSICC freeze dryer type CD, Birkerød, Denmark), ground and dry-milled to a particle size <0.5 mm. The total dry weight of residue was determined by measuring the total dry weight amount before centrifugation minus the dry weight of supernatant. A portion of the supernatant was used for analysis of β-glucan content.

Small-Laboratory Scale: Evaluation of Protein Hydrolysis (Experiments VII and VIII)

In Experiments VII and VIII, ≈70 g of ethanol-extracted oat bran was treated with α-amylase, followed by either pancreatin (0.5 g/100 mL) or papain (0.1 g/100 mL) incubation. Pancreatin was added and agitated for 10 min at 40°C, the mixture was adjusted to pH 7.0 and then incubated for 3 hr under agitation at

40°C. Papain, on the other hand, was dissolved in sodium phosphate buffer ($0.1M$, pH 6.0) containing 2.0 mM EDTA and 5.0 mM cystein (5 min, 37°C). The addition of papain was based on a ratio of 1:10 between papain and protein as described by Nair et al (1976). The mixture was adjusted to pH 6.0 with sodium phosphate buffer ($0.1M$, pH 6.0) and the total volume was adjusted to 1,000× the amount of added papain. The incubation with papain lasted for 24 hr under agitation at 37°C. After protein hydrolysis, the suspension was centrifuged (4,000 × g, 15 min) and the supernatant was dialyzed against distilled water at 5°C with exchange of water four times the first day and then two times per day (SpectraPor 2, 12-14 kDa, Flat Width 45 mm). After dialysis, the suspension was concentrated and the β-glucan was precipitated with ethanol as illustrated in Fig. 1.

Large-Laboratory Scale

Experiment VIII was evaluated in large-laboratory scale (Fig. 2A) and was intended to investigate the influence on molecular weight during ethanol, amylase, and papain treatment. The same conditions as Experiment VIII were used, but scale was 15× larger and included an inactivation of papain at 100°C for 10 min after 18 hr of treatment, to ensure that enzymes were inactivated.

The first step included five separate ethanol extractions. The extracted oat brans were pooled by dry mixing (HCC Hackman Mixo, Wodschow & Co, Brøndby, Denmark), extracted with amylase and papain, and centrifuged (8,000 × g, 1 hr). After α-amylase and papain treatment, samples, together with the insoluble material were stored at –20°C before freeze-drying (–20°C for four days and increased to 20°C at a rate of 3°C/hr, with a Labconco, Ninolab, Upplands Väsby, Sweden). Freeze-dried material was ground and dry-milled to particle size <0.8 mm before analysis of molecular weight.

Analysis of Nutritional Composition

Before analysis of nutritional composition, the product was milled to particle size <0.5 mm (Cyclotec, Tecator AB, Höganäs,
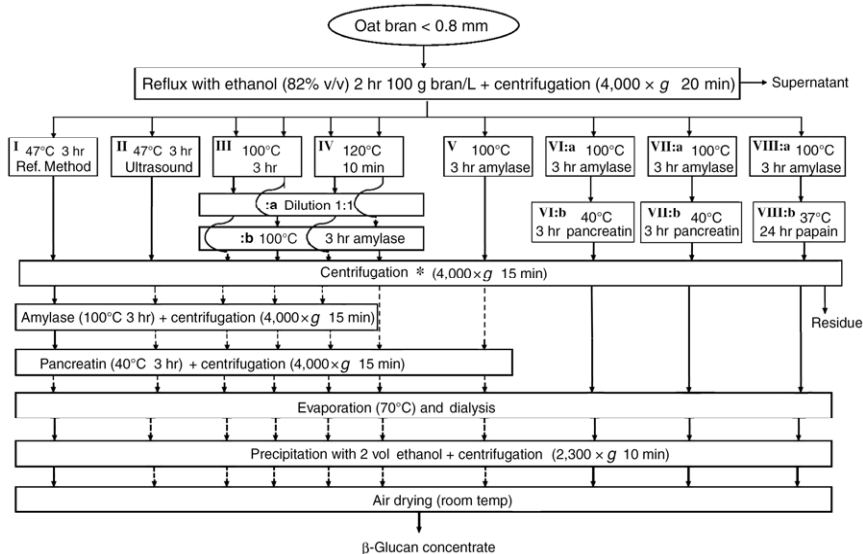

Fig. 1. Simplified flow-sheet for experiments in small-laboratory scale. * Experiments I–VI were to find highest extraction yield after this centrifugation. Experiments VII and VIII were to evaluate protease treatment. Solid arrows indicate how the experiments were performed, whereas dashed arrows indicate how the procedure was intended to end.

Sweden). Fat content was determined gravimetrically using the Schmid-Bondzynski-Ratzlaff (SBR) method. Moisture content was determined by drying the samples for 15 hr at 105°C followed by cooling in a desiccator for 1 hr. Nitrogen content was determined by the Kjeldahl procedure (Kjeltec System 1003, Tecator AB, Höganäs, Sweden). Crude oat protein was calculated as N × 6.25. Total dietary fiber, divided into a soluble and insoluble fractions, was determined according to Asp et al (1983). Total starch was analyzed according to a method described by Björk and Siljeström (1992) or calculated from carbohydrates by difference. The sugar content was determined after solubilization by water extraction at 85°C for 15 min and then analyzed with high-performance anion exchange chromatography (HPAEC) supplied with an electrochemical detector. Ash content was determined by incinerating the sample at 550°C overnight, cooling in a desiccator, and then weighing the sample remaining after incineration.

Papain Activity Analysis

Papain activity was determined according to the method described by Nitsawang et al (2006). The procedure uses casein as a substrate and trichloroacetic acid (TCA) to stop the reaction. The phenolic amino acids produced can be detected at $\lambda = 275$ nm. A blank was included for each set where the sample was added after addition of TCA.

β-Glucanase Activity

The β-glucanase activity in the enzyme preparations (papain and pancreatin) was determined using a Megazyme assay kit (McCleary and Shameer 1987) following the procedure described earlier for hydrolysis of proteins. According to this method, a β-glucan substrate, cross-linked with a dye, is incubated with the enzyme solution. Possible hydrolysis releases water-soluble dyed fragments that could be measured as absorbance at 590 nm. A blank sample without enzyme was included. The difference between sample and blank constituted the level of β-glucanase activity.

Molecular Weight Determination

Samples were extracted in two steps before analysis of molecular weight: step 1) with ethanol (if not already extracted with ethanol) and step 2) with 0.1M NaOH. In step 1, ≈4 g (wb) of sample was mixed with 40 mL of 82% (v/v) ethanol, put into a boiling water bath with reflux, and magnetically agitated for 2 hr. The mixture was cooled to room temperature and centrifuged (9,300 × g, 15 min). The residue was dried for 13 hr at 60°C and then mortared. In step 2, the samples were solubilized in 10 mL of 0.1M NaOH for 2 hr at room temperature using 5 mg of β-glucan/10 mL of NaOH. The samples were then centrifuged at 15,000 × g for 10 min. The supernatant was neutralized to pH 7.0 (±0.3) with 0.1M HCl and diluted totally 10× with nanopure water (except the residue sample was diluted 5×). The samples were filtered through a nylon filter into vials (0.45 μm, 47 mm diameter, R04SP04700, GE Water & Process Technologies) and the molecular weight was determined on the same day as the solubilization. The β-glucan content of the supernatants was analyzed within the next five days.
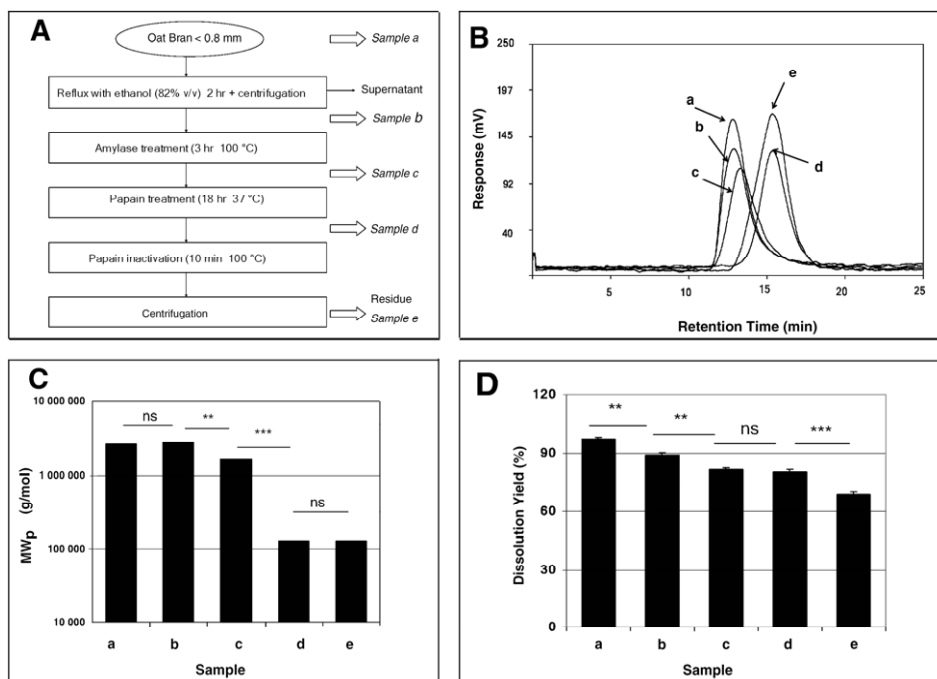
Fig. 2. Results from molecular weight determination of β-glucan for samples isolated during large-scale experiments: a, dry milled oat bran; b, after ethanol extraction; c, after extraction with α-amylase; d, after papain treatment; and e, residue (insoluble material after centrifugation). **A,** Simplified flow-sheet for large-laboratory scale experiment. **B,** Elution profile from size-exclusion chromatography. **C,** Change in peak molecular weight (MW$_p$). **D,** Dissolution yield of β-glucans before molecular weight determination. Statistical analyses by pair-wise comparisons with Tukey's test. Significance is indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Peak molecular weight (MW_p) and molecular weight distribution of β-glucan were analyzed using high-performance size-exclusion chromatography (HPSEC) with postcolumn addition of calcofluor as described by Tosh et al (2008). Five different β-glucan standards with MW_p of 20×10^3 to $1,156 \times 10^3$ were used to make a standard curve (log MW_p vs. retention time). The MW of all standards had previously been determined with HPSEC with refractive index (RI), viscometric and light-scattering detection (Viscotec, Houston, TX).

Total β-Glucans

The β-glucan content of solid materials and supernatants from the isolation schemes was determined with a Megazyme enzyme kit. The assay procedure was based on the McCleary method for mixed linkage β-glucans (McCleary and Codd 1991), where two specific β-glucanases are used (lichenase EC 3.2.1.73 and and β-glucosidase EC 3.2.1.21) to hydrolyze β-glucans completely to glucose. This method is decribed in Approved Method 32-32 (AACC International 2000) and AOAC Official Method 995.16.

Dissolved β-Glucan Before Analysis of Molecular Weight

The dissolved samples for molecular weight determination were also analyzed with flow-injection analysis (FIA) as described by Jørgensen (1988). In FIA, the sample is mixed with calcofluor in tris buffer (pH 8.0) and the complex of β-glucan-calcofluor is detected by fluorescence. Six standards with known β-glucan concentrations (10–100 µg/mL) were used to determine the concentrations of the samples. The dissolution yield of β-glucans could be calculated from the dissolved amount of β-glucan divided by the amount of β-glucan in the starting material determined with the McCleary method (described above). The FIA method correlates well with the AACC enzymatic method but does not analyse β-glucans <9,200 (Gomez et al 2000; Kim et al 2008). The samples from the large-scale experiment were not filtered (i.e., >0.45 µm) before analysis with FIA, due to the high viscosities obtained after filtering the solutions.

Viscosity

Rheological measurements were made on a stress-controlled rheometer (StressTech, Reologica, Lund, Sweden) using a concentric cylinder (25 mm diameter, CC25). Two sucrose solutions (46 and 56 g/100 mL) were used to calibrate the rheometer.

A pure oat β-glucan was obtained from Megazyme International in Ireland. According to the supplier, the molecular weight of the β-glucan was ≈272×10^3 and the viscosity was 72.1 mPa.sec at a concentration of 1 g/100 mL (determined with an Ostwald viscosimeter). A solution of β-glucan (0.5 g/100 mL) was prepared by solubilizing the polysaccharide into Millipore water or sodium phosphate buffer (0.1M, pH 6.0) for 10 min at boiling temperature (according to the product specification). Four drops of toluene was added per 100 mL of solution to prevent growth of microorganisms during viscosity measurements. The

viscosity of the prepared β-glucan solution was measured every 5 min for 24 hr at 37°C using a constant shear rate of 50/sec. Two other experiments were made in a similar way but with proteases added to the β-glucan solution. The same experimental conditions (i.e., temperature and ratio of protease to β-glucan) were used as for the protease treatment in Experiments VII and VIII. After adding EDTA, cystein, papain, or pancreatin to 25 mL of prepared β-glucan solution, the enzyme was solubilized for 5 min before measuring the viscosity.

Viscosity properties of the isolated β-glucan concentrates were determined after solubilizing the sample at ≈0.5 g/100 mL. The sample (225 mg) was mixed with 1.5 mL of 95% (v/v) ethanol and 18.5 mL of Millipore water and kept in a boiling water bath for 10 min. After cooling to room temperature, the suspension was diluted to 25 mL and centrifuged ($15,000 \times g$, 10 min). The supernatant was weighed before viscosity measurement and a small sample of supernatant was frozen at –20°C until analysis of β-glucan content. Different shear stresses were used to give a change in shear rate from 5 to 50/sec.

Statistical Analyses

A software package (v.14.0, Minitab, State College, PA) was was used to perform the statistical evaluation. The results were expressed as mean values and standard error of the mean (SEM). A two-sample t-test was used for statistical evaluation of the β-glucanase activity, based on triplicate determinations. All other data was analyzed with one-way ANOVA for multiple comparisons (using general linear model procedure), where Tukey's test for pairwise comparisons of means was used for the significance of difference ($P < 0.05$). Duplicate samples were run for determination of molecular weight and three or four replicates for determination of dissolution yield.

RESULTS AND DISCUSSION

Evaluation of Extraction Method on Small-Laboratory Scale

The laboratory-scale extraction method was performed in the three steps typically used for extraction of β-glucans from cereals: 1) inactivation of endogenous enzymes with β-glucanase activity, 2) extraction of β-glucans, and finally 3) precipitation of the β-glucans in ethanol (Brennan and Cleary 2005).

Experiments I–VI evaluated the yield of different extraction conditions (Fig. 1): 47°C for 3 hr (I); 47°C 3 hr under ultrasound (II); boiling for 3 hr with or without an amylase treatment (III:a and III:b); autoclaving for 10 min with or without an amylase treatment (IV:a and IV:b); a combined hot-water extraction and amylase treatment (V); and finally, inclusion of a protease treatment (pancreatin) after the combined hot-water extraction with amylase (VI). Results are summarized in Table II, where mass-balance from each experiment is listed for different parameters. The most important parameter in this context is extractable β-glucans, which refers to distribution of soluble β-glucans present in the aqueous phase between the supernatant and the residue.

$$Extractable \ \beta-glucan = \frac{c_{\beta-glucan, aq\ phase} \cdot V_{aq\ phase}}{c_{\beta-glucan, start\ material} \cdot m_{start\ material}} \quad (1)$$

The mass balance for the extraction method in Experiment I (47°C for 3 hr) showed that the extraction yield of β-glucans was only 28% and that 73% of the β-glucans were in the residue isolated after centrifugation (Table II). Further purification (Fig. 1) resulted in a final product that consisted of 55% β-glucans and 19% protein (db); this is very different from the purity (>90%) reported by Lazaridou et al (2004). It is likely that the starting material may have played an important role in the purity of the product; oat flour was used in their study, while we used oat bran. For our purpose, a high yield of β-glucans was more important than a high β-glucan content in the final product.

TABLE II
Mass Balance of β-Glucan in Oat Bran Using Different Extraction Methods[a]

	I: Ref Method	II	III: a	III: b	IV: a	IV: b	V	VI
Extractable[b]	28	3.1	50	90	53	71	76	73
In supernatant[c]	24	1.9	27	72	36	41	60	64
In residue[d]	73	95	63	35	54	61	38	37

[a] Single extractions from ethanol-extracted oat bran. Extraction methods (I–VI) described in Fig. 1. Results in % of total amount of β-glucan from starting material.

[b] (Amount in supernatant plus amount present in aqueous phase of residue) /amount in starting material.

[c] Recovered in supernatant/amount in starting material.

[d] Determined on freeze-dried and milled residue.

Experiment II evaluated the possibility of improving the extraction efficiency in Experiment I by treatment with ultrasound, a technique known to increase the diffusion of molecules at the boundary between a solid and a liquid (Sanchez et al 1999). Using ultrasonic sound without mechanical agitation resulted in a lower extraction yield of β-glucans. In Experiments III and IV, the extraction of β-glucans was intended to be increased by using different methods for gelatinizing starch and starch hydrolysis, either by boiling (III) or through autoclaving (IV). Both methods, 3 hr of boiling and autoclaving for 10 min, resulted in a viscous gel that was further treated in two different ways before centrifugation: dilution with one volume of water (III:a) or incubation with α-amylase under reflux (95–100°C, pH 4.5) (III:b). Experiment III:b gave the most promising result, with an extraction yield of 90%. From this basis, Experiment III:b was further extended to Experiment V, which included both boiling and starch hydrolysis in the same step instead of two sequential steps as in Experiment III:b. The sample was adjusted to pH 6.0 with sodium phosphate buffer (0.1M, pH 6.0) instead of pH 4.5 to work closer to pH 7, the optimum for the α-amylase used in this study. The extraction yield of β-glucans in Experiment V reached 76%, compared with 90% in Experiment III:b. However, we cannot state that this difference is significant because the recovery differed between the two experiments (107% vs. 98%). The type of α-amylase used in this study has been proven previously to also result in high-extraction yields of β-glucans. Jaskari et al (1995) reported that ≈83% of the β-glucans can be extracted from an oat bran using the same type of α-amylase (95°C, 25 min), which is consistent with our findings. An addition of pancreatic enzymes after α-amylase treatment in Experiment VI did not further increase the extraction yield. After further isolation with dialysis and ethanol precipitation, Experiment VI provided a product with a β-glucan content of 54% (Fig. 1).

Evaluation of Protein Hydrolysis Step

In Experiment VII, the amount of added pancreatin was reduced from 2 g/100 mL to 0.5 g/100 mL, which resulted in a final yield of 66%, comparable with the 64% obtained in the supernatant for Experiment VI. Moreover, the purity was not changed much (from 54% to 57%) by reducing the concentration of pancreatin with 75%. In Experiment VIII, we replaced pancreatin with a protease isolated from papaya fruit, so-called papain. Papain (EC.3.4.22.2) contains mainly endo-peptidases and is well known for broad specificity and stability (Rawlings and Barrett 1993; Ménard and Storer 1998). Thus, it is an attractive choice when looking for a protease to be used for solubilization and hydrolysis of protein. This also has been demonstrated previously in literature (Nair et al 1976; Nkonge and Ballance 1984). We found that the papain treatment reduced the protein content from 17.6% to 5.5% (Table III). Moreover, the two products did not differ in β-glucan content or in total yield of β-glucans (66% vs. 63%). We also found that the β-glucans constituted the greatest part of the determined soluble dietary fiber. Lipids were not analyzed. How-

ever, the extraction yield of lipids from the initial ethanol extraction indicated that a notable amount of the lipids still remained in the extracted bran. Pancreatin, which is a mixture of different pancreatic enzymes, does contain lipases (16 U.S.P. units/mg of pancreatin) (Reynolds 1989). Papain, on the other hand, is an extract essentially containing proteolytic enzymes. Thus, pancreatin, but maybe not papain, may hydrolyze and facilitate the removal of the remaining lipids in the extracted bran, accounting for the difference in mass balance (Table III).

However, papain gave rise to a low viscous residue that was difficult to separate from the supernatant. The residue was washed with 2 volumes of Millipore water followed by centrifugation. The resulting supernatant was collected and separated from coarse particles using vacuum filtration.

In addition to the poor separation after centrifugation, papain produced a product with lower MW_p and viscosity of β-glucans compared with pancreatin (Table IV). It should be noted that the MW_p value represents only the average molecular weight of the distribution, calculated from the retention time from the maxima of the peak, which can be done only in a symmetrical profile of the peak. Dissolution yield of β-glucan before molecular weight and viscosity measurements was determined mainly to confirm that a large amount of the β-glucans present in the sample had been dissolved. The dissolution yield was similar for the two β-glucan concentrates. The obtained difference in viscosity between the two samples was also in agreement with the results for the MW_p values (Table IV).

Evaluation of Extraction Method on Large-Laboratory Scale

Experiment VIII was evaluated in large-laboratory scale (Fig. 2A). The influence on the distribution of molecular weight and MW_p of β-glucans was investigated by sampling after each step of the isolation: from dry-milled oat bran (a), after ethanol extraction (b), after amylase treatment (c), after treatment with papain (d), and after centrifugation of the insoluble material (e). These samples were then freeze-dried (only c, d. and e), milled, and extracted with ethanol before extraction with NaOH to inactivate any possible β-glucanases in the samples.

The first step of the experiment (Fig. 2A), the ethanol extraction of oat bran, was necessary to deactivate endogenous enzymes and to extract lipids. The amount of extracted lipids was assumed to be equal to the dry matter of the discarded ethanol extract multiplied by the total amount of ethanol extract. The extraction yield of lipids from the five ethanol extractions was 68.7 ± 1.5% ($n = 5$, mean ± SEM). An alternative method to inactivate endogenous β-glucanases is to use a treatment with strong alkaline (1.0M NaOH) or acidic conditions (0.1M HCL, 55 mM NaCL) (Lazaridou et al 2007). However, these methods may also reduce the molecular weight of β-glucans (Beer et al 1997). The pH level during α-amylase treatment was kept constant but reduced during papain treatment from pH 6.2 to 5.1–5.2. After heat treatment (10

TABLE III
Nutritional Composition of Two β-Glucan Concentrates (g/100 g, db)[a]

Component	VII[a]	VIII[b]
Protein[c]	17.6	5.5
Soluble fiber	61.8	63.4
β-Glucan	57.1	57.0
Insoluble fiber	1.7	1.7
Total starch	11.7	6.5
Ash	2.0	1.8
Not determined	5.2	21.1

[a] Isolated with pancreatin (0.5 g/100 mL).
[b] Isolated with papain (0.1 g/100 mL).
[c] N × 6.25.

TABLE IV
Physicochemical Properties of Two β-Glucan Concentrates[a]

Properties	VII (Isolated with Pancreatin)	VIII (Isolated with Papain)
Molecular weight		
Dissolution yield (%)[b]	92.6 ± 1.0 (4)	94.6 ± 0.3 (4)
MW_p (×10^{3})	908 ± 12 (2)	56 ± 0.0 (2)
R_t[c] (min)	13.8 ± 0.0 (2)	16.0 ± 0.0 (2)
Viscosity		
Dissolution yield (%)	77.4 ± 1.1 (5)	82.5 ± 0.8 (5)
Viscosity (mPa.sec)[d]	39.5	3.1

[a] Mean ± SEM. Number of replicates in brackets.
[b] Yield of solubilized β-glucan before analysis.
[c] Retention time.
[d] Viscosity data presented as mean values at shear rates 5–50/sec. Results showed Newtonian behavior for both samples using this shear rate.

min at 95–100°C) and cooling to room temperature, it had increased to pH 6.2. Measurements of papain activity showed that the absorbance between sample and blank were equal for the sample taken after the heat treatment, which means that the activity was zero.

The influence on molecular weight of β-glucan during the experiment was investigated (Fig. 2A). The elution profile showed that all peaks were symmetrical (Fig. 2B). The MW_p for the peak was determined from the retention time of the maxima. Reflux with 82% (v/v) ethanol did not alter the MW_p of extracted β-glucans (Fig. 2C). These results are in good agreement with literature results. For example, Åman et al (2004) obtained β-glucans with a molecular weight of 2.2×10^6, when treating oat bran from Swedish oats with a thermostable α-amylase before molecular weight determination. Another example is Wood and Weisz (1991), who reached MW_p close to 3.0×10^6 after solubilization of β-glucans from Canadian oat bran with sodium carbonate (60°C, 2 hr).

We also observed that the α-amylase-treated sample caused a reduction in MW_p during α-amylase treatment from 2.8×10^6 to 1.6×10^6 and during papain treatment from 1.6×10^6 to 0.13×10^6 (Fig. 2C). The reduction in MW_p during α-amylase treatment may be due to a significantly lower population of solubilized high molecular weight β-glucans from the α-amylase-treated sample compared with the ethanol-treated sample (b and c in Fig. 2D). The reduction in MW_p may also be caused by the heat treatment or by enzymes from α-amylase, even though a large amount should have been inactivated by the pretreatment of the α-amylase (30 min, 95°C). Beer et al (1997) reported an MW_p of 1.8×10^6 for an α-amylase-treated oat bran, although only 64% of the β-glucans were extracted. Nevertheless, it can be concluded that the papain treatment seems to be detrimental for high molecular weight β-glucans. The β-glucans from insoluble material (Fig. 2A) obtained after centrifugation exhibited a low molecular weight (Fig. 2C). However, only 69% of the β-glucans were dissolved (Fig. 2D). The remaining β-glucans may be trapped in the botanical structure of insoluble fibers and thus not affected by the hydrolysis effects of the enzymes.

β-Glucanase Activity

Results of the molecular weights show that the papain used in this study caused a drop in MW_p of the β-glucans. Papain has been used previously to also purify β-glucans from oat bran (Delaney et al 2002). However, the molecular weight of β-glucans in

Fig. 3. Viscosity as a function of time: (▲) β-glucan solution (Megazyme, 0.5 g/100 mL) at 37°C; (■) β-glucan solution with added pancreatin (0.3 g/100 mL) 40°C; and (●) β-glucan solution with added papain (0.1 g/100 mL) 37°C.

the purified material was not reported in that study, but the β-glucan concentrate had a positive effect on blood cholesterol in hypercholesterolemic hamsters.

Some possible explanations to the drop observed in MW_p during papain treatment can be the presence of β-glucanases, other enzymes from the papain preparation that can break down the β-glucans, a breakdown of possible interactions between proteins and β-glucans, or possibly contamination with microorganisms. However, digestion of polysaccharides may also be caused by acid hydrolysis. Johansson et al (2006) observed no digestion of β-glucans after 12 hr of incubation at pH 1 (0.1M HCl) and 37°C. Hence, acid hydrolysis was ignored in this context due to the high pH level during papain treatment (pH > 5).

Measurement of the viscosity of a solution of a pure β-glucan is a common method used in the industry to test whether there is β-glucanase activity in enzyme preparations for example (McCleary 2008). This method was first described by Bamfort et al (1982). An experiment was designed to test whether the papain preparation could possibly contribute to a reduced molecular weight of a pure β-glucan, hence a reduced viscosity. The purified β-glucan that was used for viscosity measurements contained >97% β-glucan and 0.3% protein. The viscosity of a solution of β-glucan, without added enzymes, was constant in the first 12 hr but then viscosity increased 6% at 12–24 hr (Fig. 3). The increase in viscosity is most likely due to an aggregation of β-glucans, although a slight evaporation of water may also contribute. We can also conclude that shear forces such as those during the measurement did not break down the polymers.

Addition of papain reduced the viscosity of a β-glucan solution (solubilized in sodium phosphate buffer at pH 6.0) by 64% during 24 hr (Fig. 3). The drop in viscosity can be calculated to a theoretical drop in molecular weight by using Mark-Houwink's equation, which correlates the molecular weight and the intrinsic viscosity for polymers

$$[\eta] = K \cdot M_w^{\alpha} \qquad (2)$$

By using the change in intrinsic viscosity between two measurements (1 and 2), the equation obtained was

$$\left(\frac{[\eta_2]}{[\eta_1]} \right)^{1/\alpha} = \frac{Mw_2}{Mw_1} \qquad (3)$$

A number of different values for the exponent α for oat β-glucans have been suggested in a number of studies (Vårum and Smidsrød 1988; Roebroeks et al 2001; Wang et al 2003). If the exponent α is set to 0.62, MW_1 to 272×10^3 (the molecular weight according to the supplier), $[\eta_1]$ to 15 dL/g (initial intrinsic viscosity) and $[\eta_2]$ to 4.0 dL/g (intrinsic viscosity after 24 hr), MW_2 could be estimated to 34×10^3, which is ≈13% of the initial molecular weight (MW_1). Pancreatin, on the other hand, caused a drop in viscosity with only a 3% reduction after 3 hr and an 18% reduction after 24 hr. The type of pancreatin used in this study has previously not shown any β-glucanase activity (Lazaridou et al 2004). However, some other factors have not been simulated in the viscosity experiments that might affect the result, such as contamination of bacteria, etc. Another method to test β-glucanase activity is an assay procedure developed by Megazyme, which was used in this study to confirm presence of β-glucanase activity in our papain preparation. By using this method, a β-glucan substrate was incubated either with or without a solution of the enzyme. Possible hydrolysis caused a release of water-soluble dyed fragments during incubation that could be measured by absorbance. The apparent β-glucan hydrolytic activity was significantly higher for papain (Fig. 4). We can conclude, at least, that papain possesses a significant amount of β-glucanase activity. It should also be noted that both the viscosity reduction and the absorbance (based on the Megazyme assay) was measured after 24 hr of in-
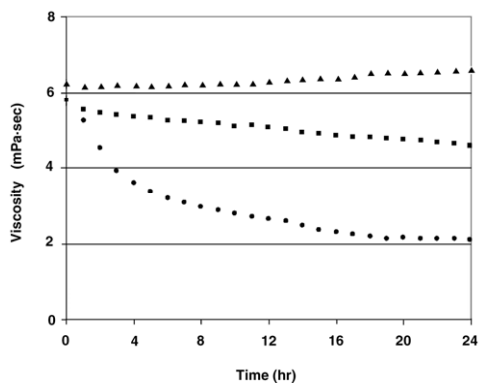
cubation for both enzymes, although 3 hr of incubation with pancreatin was used for isolation of β-glucans in small-laboratory scale.

Together, our results from viscosity reduction and the method based on absorbance demonstrate that the papain preparation used in this study do display enzymatic side activities that may explain our results for MW_p and viscosity from the small- and large-laboratory scale. Papaya fruit utilizes cellulases and other cell-wall-degrading enzymes during fruit ripening (Paull et al 1999), the presence of which may alter the molecular weight results of β-glucans. However, we cannot exclude the possibility that proteases from the papain preparation may have reduced the interaction between protein and β-glucans. Autio et al (1992) found that the viscosity of the β-glucan sample was reduced after treatment with trypsin, an indication that interaction between β-glucans and proteins may exist.

CONCLUSIONS

Comparison of different extraction methods showed that a hot-water extraction combined with a thermostable α-amylase gave the highest extraction yield of β-glucans (76%) from ethanol-extracted oat bran. On the basis of these findings, we developed a sequential procedure including protein hydrolysis after extraction and treatment with amylase. We also evaluated pancreatin against the protease papain; both enzymes have been used previously to purify β-glucans from oat bran. We found that papain (used as a premix) was efficient in digesting oat protein but it also possesses enzymes with β-glucanase activity. If the protease papain should be used as a protease for isolation of β-glucans, care must be taken to minimize the risk for side activities of β-glucanases by evaluating different types of papain for β-glucanase activity. With pancreatin, on the other hand, we obtained a product with high molecular weight β-glucans (908×10^3). The isolation scheme we developed includes removal of lipids with ethanol extraction, enzymatic digestion of starch with α-amylase, enzymatic digestion of protein using pancreatin, centrifugation to remove insoluble material, removal of low molecular weight components using dialysis, precipitation of the β-glucans with ethanol, and air drying. This procedure will be used to produce a β-glucan concentrate for physiological studies in mice. Even though the process is time-consuming, it provides a product of high quality. More studies are needed to find out how different drying techniques may improve the solubility of β-glucans, another property of potential importance for cholesterol-lowering effects.
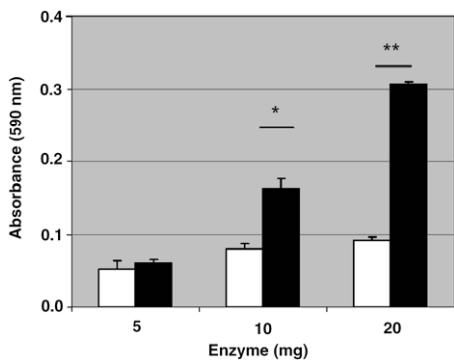

Fig. 4. β-Glucanase activity of proteolytic enzymes measured after 24 hr of incubation: papain (black bars); pancreatin (white bars). Error bars show SEM. Statistical analysis by a two-sample *t*-test. Significance is indicated as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

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Paper IV

Cholesterol-lowering effects of oat components

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Abstract

We have performed two separate experiments in mice to further elucidate whether oat components other than β-glucans have cholesterol-lowering properties, and to compare the cholesterol-lowering effects of two differently purified oat β-glucans with known peak molecular weights (MW_p). The cholesterol-lowering effect of oat bran was found to be due to two complementary fractions: an oil fraction, containing ethanol soluble component(s), and another fraction containing β-glucans.

In the second experiment, two β-glucan products (β-glucan content 46% and MW_p=356 kDa; β-glucan content 97-98% and MW_p=285 kDa) did not differ significantly regarding their cholesterol-lowering properties when compared with the cellulose-containing diet. The effect was also similar to that of oat bran. *In vitro* digestion of experimental diets showed that the release of β-glucan was higher ($P<0.001$) in diets containing purified β-glucan products (356 kDa 88% and 285 kDa 76%) than that of the oat bran diet (~34%) after 5 hours' simulation of the passage through the gastrointestinal tract, and was not correlated with effects on cholesterol levels but to the ratio of propionic acid/acetic acid in the caecum.

Introduction

The cholesterol-lowering effects of oat products are well-documented and have been suggested since the 1960s (De Groot *et al.* 1963; Fisher and Griminger 1967; Ripsin *et al.* 1992; Brown *et al.* 1999). Products highly enriched in oat β-glucan (80% or 54% β-glucan content) have been demonstrated to have significant cholesterol-lowering effects in humans (Braaten *et al.* 1994; Queenan *et al.* 2007, respectively)**.**

Recently, the European Food Safety Agency (EFSA) allowed the following claim for food products containing β-glucan: "Regular consumption of β-glucans contributes to maintenance of normal blood cholesterol concentrations" (EFSA, 2009). This claim may be used if the food product contains β-glucan from barley, barley bran, oats or oat bran, or mixtures of non-processed or minimally processed β-glucans. The EFSA also concluded that an intake of at least 3 g β-glucan per day was necessary to have any effect; the same level as previously deemed as necessary by the US Food and Drug Administration (FDA), although no lower limit was set for the β-glucan content in the product. In contrast to the decision by the FDA in 1997, the EFSA approved health claims for foods fortified with "minimally processed" β-glucans, and not only for oat products containing oat bran, oatmeal or whole oat flour. However, the concept "minimally" processed has not yet been defined. One issue yet to be resolved is the role of the molecular weight (MW) of β-glucans in the cholesterol-lowering effect of oat products. The average MW of β-glucans varies between different oat products, with lower MWs in products such as bread and drinks and higher MWs in, for example, pure oat bran or oat flakes (Åman *et al.* 2004). A β-glucan preparation made from oats (22% pure) with an average MW of 80 kDa was shown to have cholesterol-lowering effects in humans (Naumann *et al.* 2006). The fact that oat β-glucans can interact with other oat components (Autio *et al.* 1992; Izydorczyk and MacGregor, 2000), may affect their conformation and in turn their bioactivity. This underlines another issue remaining to be resolved – the role of the degree of β-glucan purification in the cholesterol-lowering effects. Furthermore, in one of our recent studies we found that processing of oat bran, down to an average MW of 21 kDa and even <10 kDa, did not change its cholesterol-lowering properties in mice (Immerstrand *et al.* 2010). It is however, not clear if other components in the oat bran preparations contributed to the observed effects.

Apart from β-glucans, oats contain other interesting components that may contribute to the cholesterol-lowering effect of oat products. Arabinoxylans, for example, are another type of dietary fibre found in oat bran. Westerlund *et al.* (1993)

found that 13-15% of the total arabinoxylans of oat bran are water-soluble, while in rye flour, more than 50% of the arabinoxylans are in water-soluble form (Cyran *et al.* 2003). The cholesterol-lowering effects of dietary fibre have generally been mainly attributed to the soluble fibres (e.g. β-glucans) since insoluble fibres (e.g. cellulose) have been shown not to be as effective (Chen *et al.* 2008; Theuwissen and Mensink 2008). Welch *et al.* (1988) failed to find a cholesterol-lowering effect of an insoluble fraction of oat bran in chickens, which implies the importance of soluble oat fibres in the lowering of cholesterol levels.

Previous animal studies have shown a discrepancy regarding whether oat lipids contribute to the cholesterol-lowering effect or not (De Groot *et al.* 1963; Welch *et al.* 1988; Illman *et al.* 1991; Petterson and Åman 1992; Yokoyama *et al.* 1998). The unsaturated fatty acids of oats consist mainly of oleic (18:1) and linoleic acid (18:2, n6). Besides triacylglycerols, the other groups of lipids are partial acylglycerides, phospholipids, galactolipids, free sterols, sterol esters and free fatty acids (Peterson, 2002). Dietary phospholipids and galactolipids are well-known for their emulsifying properties of fat droplets in the small intestine, and may play a role in delaying the intestinal fat uptake (Haenni *et al.* 2009).

Oats also contain several antioxidants: vitamin E, phytic acid, phenolic acids, avenanthramides (AVAs), sterols and flavonoids. AVAs are a group of polyphenolics that are present in oats but not in other cereals. The main AVAs found in oats are three "class 2" AVAs, namely amides of 5-hydroxy-anthranilic acid, with caffeic (AVA 2c), p-coumaric (AVA 2p), and ferulic acid (AVA 2f) (Bratt *et al.* 2003). Interestingly, the structures of AVAs exhibit some similarities to that of Tranilast (INN)[1], a drug used to prevent restenosis (Rosanio *et al.* 1999; Tamai *et al.* 2002). Studies *in vitro* have suggested that AVAs may counteract the development of atherosclerosis through different mechanisms (Nie *et al.* 2006). The direct effect of AVAs on plasma cholesterol levels is, however, not yet known. Interestingly however, a polyphenol-containing red grape juice has been reported to significantly lower LDL cholesterol levels in humans (Castilla *et al.* 2006). The main objective of the present study was to determine whether other components apart from β-glucans contribute to the cholesterol-lowering effect of oats, and to what extent. We also investigated what role the degree of β-glucan purification play in the cholesterol-lowering effect. A mouse model was used for the investigations (Andersson *et al.* 2010).

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[1] INN=International Non proporatory Name

Material and methods

Experimental protocol

Two separate experiments were performed using C57BL/6NCrl mice. In Experiment 1, the cholesterol-lowering effect of two complementary fractions derived from oat bran (OB): an oil fraction (OB oil) and the remaining ethanol-extracted oat bran (EOB) were compared with the effects of untreated OB. The main aim of Experiment 2 was to compare effects of two purified β-glucan products from oats, with different β-glucan contents, on plasma cholesterol, faecal bile acids and short chain fatty acids (SCFA) in caecum, and compare these effects with that of oat bran. Data for the control and oat bran groups in Experiment 2 are from a previous study (Immerstrand *et al.* 2010) but are reproduced in this study for clarity (Tables 4, 5 and Fig. 4A and 4B).

Isolation of OB oil and EOB from oat bran

Three ethanol extractions were performed to produce the oil used in the mouse study referred to as Experiment 1 (Fig. 1A). Dry milled oat bran (batch 1013485; 2007; < 0.8 mm) was extracted with 82% (v/v) ethanol for 2 hours under agitation and reflux at boiling temperature. One litre of ethanol (82% v/v) was used per 100 g oat bran during the extraction. After extraction, the suspension was cooled for 30 minutes under agitation using a water bath at 10-15°C. After centrifugation (4000 *g*; 15 min) the residue was washed twice with 600 ml 95% (v/v) ethanol. The dry matter (DM) content of the supernatant was determined from the dry weight of the supernatant. The OB oil was obtained from the supernatant by evaporation under vacuum at 50°C (Heidolph, Type VV60, Germany). The temperature was reduced to 45°C when about 500 g remained. Evaporation was stopped when there was no ethanol odour. The isolated OB oil was a yellow fluid emulsion. The DM content of the three batches of OB oil produced, after evaporation, was determined and found to be 22.3, 23.1 and 21.4 percent, respectively. The remaining liquid consisted of water with possibly small amounts of ethanol. The OB oil was stored at -80°C for about 3 weeks until the OB oil diet was prepared.

The residue was dried by evaporation (Heidolph, Type VV60, Germany) using streaming air at room temperature for 2 hours, followed by vacuum drying until constant weight was obtained. The amount of butter and DM of EOB, OB and OB oil added to the diets were adjusted in order to ensure that the diets in Experiment 1 only differed with respect to the composition of lipids (see Diets below & Table 1).

Isolation of β-glucan from oat bran

The β-glucans were isolated using a method based on the extraction techniques described in Immerstrand *et al.* (2009). However, precipitation with ethanol was omitted in order to improve the solubility of the β-glucans. The β-glucans were instead freeze-dried directly after dialysis.

Ethanol extraction of oat bran was carried out as described above for the extraction of lipids. The air-dried, ethanol-extracted oat bran from four batches was pooled by careful dry mixing and used as the starting material to isolate the β-glucans. The procedure for isolation of β-glucans is illustrated in Fig. 2A and was repeated four times. Ethanol-extracted bran (250 g DM) was treated with an α-amylase (pre-heated for 30 min at 90°C) for 3 hours at 95-100°C (pH 6.0). After 3 hours, the suspension was cooled to room temperature under agitation, and the pH was adjusted to 7.0 with 2 M NaOH. Pancreatin was solubilised for 1 hour at room temperature (11.25 g/250 ml sodium phosphate buffer, pH 7.0, 0.1 M), and then centrifuged (3000 *g*; 10 min) to allow the insoluble material to be discarded. After the addition of the solubilised pancreatin, the mixture was incubated for 3 hours at 40°C, whereupon the suspension was heat-treated for 10 min at 92°C to inactivate the enzymes. After cooling to room temperature the oat suspension was centrifuged (12000 *g*; 15 min) and the residue was washed once with 250 ml Millipore water. The supernatant was kept at 5°C overnight and then dialysed for 3 days against 52 l deionised water at 5°C; the water being exchanged 5 times per day (Spectrapor 2, Flat Width 45 mm, 12-14 kDa). The residue and a sample of supernatant were frozen and then freeze-dried in order to determine the extraction yield of β-glucan.

After dialysis, the amount of DM remaining was determined and the suspension was mixed 1:1 with maltodextrin (based on DM) to increase the solubility of β-glucans after freeze-drying (Fig. 2B). Maltodextrin was added as a solution in Millipore water (1:3), mixed with the oat suspension, frozen and freeze-dried (4 days at -20°C; the temperature was then increased to 5°C in steps of 4°C/h). The texture of the material after freeze-drying was similar to cotton and was therefore difficult to grind using a pestle and mortar. To obtain a more easily ground material it was re-suspended in a water solution of maltodextrin and sucrose (at a ratio of 64:36 w/w) at a mechanical agitation rate of 100 rpm for 2 h at room temperature. The ratio between the β-glucan product (DM) and the added mixture of sucrose and maltodextrin (DM) was 1:1, while the ratio between DM and Millipore water was 1:15. After solubilisation the suspension was frozen for one day and freeze-dried (6 days at -20 °C, then increasing temperature to 5°C at 7°C/h). The dried β-glucan product was frozen for 1 hour at -

20°C in order to make the grinding procedure easier. The final composition was intended to be 11% β-glucan, 57% maltodextrin and 18% sucrose (DM). The β-glucan content of 11% was confirmed by analysis, and the contents of dietary fibre, protein, fat, ash and minor sugars were also determined. This β-glucan product is referred to as β-glucan (356 kDa) below.

Preparation of the β-glucan reference product

A purified oat β-glucan containing >97% β-glucan, 0.3% protein, 1.7% ash and <0.5% arabinoxylan (based on DM) was purchased from Megazyme (High viscosity, Lot 80608, Megazyme, Bray, Ireland). The product was mechanically solubilised in a solution consisting of maltodextrin, sucrose and deionised water for 2 hours at 100°C under reflux at 200 rpm (Fig. 2C). The ratio between the amounts of DM and deionised water was 1:20. The same ratio between β-glucan, maltodextrin, and sucrose (1:5:1.5) was used as for the preparation of the β-glucan from oat bran. Four batches were made and the pooled solution was then frozen on trays for approximately one day, before being freeze-dried (6 days at -20°C, and the temperature increased to 5°C at 7°C/h), frozen for one hour at -20°C and ground using a pestle and mortar. The final product was intended to contain 13% β-glucan, 67% maltodextrin and 20% sucrose. The β-glucan content of 13% was confirmed by analysis. This β-glucan product is referred to as β-glucan (285 kDa) below.

Analysis of nutritional composition

The content of total fibre in the oat bran samples was determined by Eurofins Food (Lidköping, Sweden) according to the AOAC 985.29 method (Prosky *et al.* 1985). The total dietary fibre in the β-glucan product isolated from oat bran was analysed according to Asp *et al.* (1983). Both methods are based on the enzymatic digestion of starch and proteins followed by precipitation of the fibre with ethanol, with corrections for the ash and protein remaining in the fibre residue. These methods have shown good agreement (Spiller, 2000). The composition of the isolated fibre residue was analysed using GLC of the neutral sugars as their alditol acetates (Theander *et al.* 1995). Uronic acids were not analysed as previously studies have reported low contents in oat bran dietary fibre (<5%; Marlett *et al.* 1989; Theander *et al.* 1995). The total starch content in oat bran samples was estimated by difference, i.e. digestible carbohydrates minus minor sugar components, while the total starch in the fibre residue was determined according to the method described by Björk and Siljeström (1992).

The DM contents of the residues and supernatants from the four batches were determined using a vacuum oven at 70°C for 16 hours (Forma Scientific, Marietta, Ohio, USA) to prevent browning reactions which may occur due to the high content of minor sugars and the presence of free amino acids. For other samples, the DM content was determined by drying the sample in an oven at 105 °C overnight, after which the sample was weighed after cooling for 1 hour in a desiccator. The ash content was determined in similar way, but at an oven temperature at 550 °C.

The nitrogen content (N) was determined either with the Kjeldahl method (Kjeltec system 1003, Tecator AB, Höganäs, Sweden) or with a carbon/nitrogen analyser (Thermo Scientific Instruments). The protein content was calculated as N x 6.25. The fat content was determined using the gravimetric SBR method, which is based on hydrolysis in 7.7M HCl and ethanol for 1 hour at 75°C, followed by extraction with diethyl ether and petroleum ether (40-60° C, 1:1). For the determination of minor sugars, the sample was extracted in water at 85°C, after which the solubilised sugars were analysed with high-performance anion exchange chromatography and an electrochemical detector at Eurofins Food (Lidköping, Sweden).

Analysis of avenanthramides

The AVA contents of the oat products used in Experiment 1 were analysed as described below.

Extraction

Oat bran (2 g) and EOB (2 g) were analysed in triplicate samples whereas one replicate was used for the OB oil (4 g). Each replicate was extracted with 3 x 20 ml of 80 % (v/v) aqueous ethanol (pH 2) in a water bath at 50 °C for 20 min. After centrifugation at 2500 rpm for 10 min, the supernatants were collected from each replicate and combined. The pooled supernatants from each replicate were then dried to apparent dryness under vacuum with a rotary evaporator. The residues were suspended in 2 ml methanol (the oil residue in 25 ml) and the suspensions were transferred to Eppendorf tubes and centrifuged at 13000 rpm for 10 min. The extracts were then subjected to high performance liquid chromatography (HPLC) analysis.

Separation of AVAs with HPLC

The AVAs were separated on a reversed-phase C18 column (Hypersil ODS 4 mm*125 mm, particle size 5 µm) in an Agilent 1100 system equipped with a photodiode array detector. Ten µl was injected and the compounds were eluted with a linear gradient of

5 to 43 % acetonitrile in 0.01 M formic acid (pH 3) in 40 min, using a flow rate of 1 ml/min. The compounds were detected at 340 nm. External standards of synthetic avenanthramides were used to identify and quantify the compounds (Kindly provided by Dr Kerstin Sunnerheim, Mid University, Sundsvall, Sweden).

Solubility of β-glucan products

In order to determine whether the water solubility differed between the two different β-glucan products in Experiment 2, a dissolution test was performed. The samples were solubilised in Millipore water (25 mg β-glucan per 25 ml), at room temperature for 60 minutes using a magnetic stirrer (550 rpm). After centrifugation (15 000 g, 10 min) the supernatant was weighed and frozen until analysis of β-glucan content and DM.

Analysis of β-glucan contents

The β-glucan content was determined using an assay based on Megazyme's assay kit for mixed-linkage β-glucans (McCleary and Codd, 1991). For *in vitro* samples, the assay was modified to adjust the pH of the sample (5 ml) and the concentration of β-glucan using 0.1 ml ethanol (50 v/v %) and 0.1 ml lichenase. For samples drawn during the first hour, 3.6 ml sodium phosphate plus (0.3 M) and 0.35 ml NaOH (1M) was added to adjust the pH to 6.5 prior to incubation with lichenase. Samples drawn during the next 4 hours were treated with 3.6 ml sodium phosphate (20 mM) and 0.05 ml 1.0 M HCl. After incubation with lichenase, 2 ml sodium acetate buffer (1 M, pH 4) was added in order to adjust the pH to 4 before incubation with β-glucosidase. No other changes were made to the procedure described by McCleary and Codd (1991).

Molecular weight determination of β-glucan in oat products

Four replicates (i.e. extractions) were made for each sample. The sample was solubilised in 25 ml 0.1 M NaOH for 2 hours using a magnetic stirrer (700 rpm; room temperature) at a β-glucan concentration of 0.8 mg/ml, and was then centrifuged (15 000 g; 10 min). The supernatant (15.625 ml) was neutralised with 1.0 M HCl and 0.1 M HCl to pH 7, and diluted to 25 ml with deionised water, i.e. a total dilution of the supernatant by 1.6 times with deionised water. Supernatants that appeared milky were centrifuged again, using an Eppendorf centrifuge (5700 g; 10 minutes). Before injection, the supernatants were filtered into vials (0.45 μm, Chromatil® Buch & Holm). The mobile phase used consisted of a 0.1 M sodium phosphate buffer

(Na$_2$HPO$_4$, sodium phosphate dibasic Sigma-Ultra, Sigma-Aldrich) at 0.5 ml/min. Calcofluor (Fluorescent Brightener 28, Sigma) at a concentration of 60 mg/l was pumped in at a rate of 0.5 ml/min. Two reversed phase columns in series were used (column 1: PL aquagel OH mixed 8 μm, Agilent and column 2: OH Pak, SB 804 HQ, Shodex). Five (1→3)(1→4) β-glucan standards were used (40, 123, 183, 245 and 359 kDa; Megazyme) to construct a calibration curve, where the logarithm of the molecular weight was plotted against the retention time. The MW of samples with a retention time within the limits set by the calibration curve was calculated using linear interpolation. For samples with a retention time outside this interval (i.e. collected supernatants and residues from the isolation scheme), the molecular weight was obtained by extrapolation, which makes these results somewhat more uncertain.

The analysis of MW was performed according to the same principles as described recently (Immerstrand *et al.* 2009).

Viscosity of β-glucan products

The β-glucan product (containing 0.125 g DM of β-glucan) was mixed with 1.5 ml ethanol (95 v/v %) and 18.5 ml deionised water and was kept for 10 min in a boiling water bath. Duplicate samples were made for each β-glucan product. After cooling to room temperature, the suspension was diluted to 25 ml, centrifuged (10 min, 15 000 *g*), and the supernatant was weighed and its viscous properties determined using a stress-controlled rheometer (StressTech, Rheologica, Lund, Sweden) with a concentric cylinder (25 mm diameter: CC25) at room temperature. The supernatant from the isolated β-glucan product (356 kDa) was diluted 1:1 with deionised water in order to work within the Newtonian region. Different shear stresses were used to obtain a change in shear rate between 5 and 50 per sec. An aliquot of the supernatant was stored at -20°C until the β-glucan analysis was performed.

Animals

Female C57BL/6NCrl mice, 7-8 weeks old (n=80) were purchased from Charles River Laboratories (Sulzfeld, Germany). They were housed in a temperature-controlled, 12 h light cycle environment, with 10 mice per cage. The mice were fed normal chow (R34 rodent chow, Lactamin, Vadstena, Sweden) during an adaptation period of two weeks, and were then divided into experimental groups (10 mice/group) and fed the experimental atherogenic diets (see below). All experiments were approved by the Malmö/Lund Regional Ethical Committee for Laboratory Animals and followed national guidelines for the care and use of animals.

Diets

All mice were fed an atherogenic diet which resembled a human "Western" diet (~ 40 energy-% fat), to which 0.8 (w/w) % cholesterol and 0.1 (w/w) % cholic acid was added. From the estimated DM content of the ingredients and the determined DM content of the diets, the energy ratios of macronutrients based on 1 kg DM diet could be calculated to: 15-16 energy-% protein, 41-42 energy-% carbohydrates and 43 energy-% fat (Table 1 and 2). We used microcrystalline cellulose (Avicel® PH 101; FMC Biopolymer, Newark, NJ, USA) in order to obtain equal contents of total dietary fibre in all diets. The diets were prepared in our laboratory using a careful dry-mixing procedure (HCC Hackman Mixo; A/S Wodschow and Co., Brøndby, Denmark). Melted butter (50°C), maltodextrin, cellulose, sucrose and OB, OB oil, EOB or β-glucan products were added to a purchased premix from Research Diets Inc. (New Brunswick, NJ, USA). All diets were fed as powders.

Similar to our previous studies, the OB diets contained 270 g oat bran per kg diet, based on fresh weight (FW) (Andersson *et al.* 2010; Immerstrand *et al.* 2010). Two different batches of oat bran were used in this study, produced in the same mill (Lantmännen AB, Järna, Sweden) but in different years and from different varieties of oats. In Experiment 1 (performed in 2008) we used oat bran based on 100% Sang (batch 1013485; produced in 2007). In Experiment 2 (performed in 2009) we used oat bran (batch 1047749; produced in 2008) based on mixed varieties of oats: 43% Sang, 10% Kerstin and 47% mixed varieties, composed mainly of Belinda.

To the OB oil diet 17 g OB oil was added, i.e. the amount of dry matter extracted using ethanol extraction from 270 g FW OB (246 g DM). Equal amounts of DM from the three extraction batches of OB oil were added together with the melted butter oil.

The EOB diet contained 229 g DM EOB per kg diet, i.e. the amount of OB added per kg OB diet (i.e. 246 g DM) minus what could be extracted from OB using ethanol extraction of the 246 g OB (i.e. 17 g DM). Analysis of the OB oil fraction showed that it not only consisted of lipids but also contained about 15% minor sugars (i.e. glucose, fructose, sucrose and maltose) based on DM. This resulted in minor differences in the lipid content of the two diets (<1.3 %).

Analysis of plasma cholesterol and lipoproteins

Blood samples were collected after 4 h fasting at weeks 0 (baseline), 2 and 4. Total plasma cholesterol was determined with Infinity cholesterol liquid stable reagent (Thermo Trace, Noble Park, Vic., Australia). Plasma lipoproteins were electrophoretically separated in agarose gels in barbital buffer as previously described

(Andersson *et al.* 2010). Results are presented as (LDL+VLDL)/(HDL+LDL+VLDL) x 100.

Analysis of total bile acids in faeces

In week 4 all the faeces excreted during a 24-h period were collected from each cage (3-4 mice/cage). The faeces were stored at -20°C, and lyophilized and weighed prior to analysis. Two samples (0.1 g) from each cage were analysed for total bile acids content. The stool was minced and extracted in 5 ml of a 75 % ethanol solution at 50°C for 2 h (Yu *et al.* 2000). The extract was centrifuged and the supernatant diluted 1:10 in water before total bile acids were measured enzymatically in a 96-well plate using a kit (Diazyme Colorimetric Total Bile Acids Assay Kit, Diazyme Labs, Poway, California, USA).

Isolation of caecum contents and caecum tissue

The caecum was weighed full with its content. Caecum tissue was washed with PBS (pH 7.4), dried between layers of filter paper and then weighed. The caecum content was stored at
-80°C until analysis of the SCFAs and the weight was calculated from the weight of the full caecum minus the weight of the caecum tissue.

Analysis of SCFAs

The SCFAs in the caecum contents were analysed using a GLC method (Zhou *et al.* 2006). The sample preparation was performed as described recently (Immerstrand *et al.* 2010).

***In vitro* digestion of oat diets by simulation of the gastro-intestinal tract**

Release profiles of β-glucan from the different oat diets studied in Experiment 2 were obtained using a simple simulation of the gastrointestinal (GI) tract (Ulmius *et al.*, to be published) in a dissolution tester. Simulated gastric fluid was prepared with pepsin (800-2500 U/mg, Sigma-Aldrich Co., St Louis, MO, USA) according to the United States Pharmacopeia (USP) (United States Pharmacopeial Convention Inc., 2006)**.** A simulated intestinal boost was added to the simulated gastric fluid. By that means, the conditions of the USP intestinal fluid with pancreatin (activity at least equal to USP, Sigma-Aldrich Co.) could be reached and was then modified to contain 0.3 g bile acid and 0.2 g lecithin per gram fat from the experimental diets. The intestinal boost

contained NaOH to reach a final pH of 6.8, potassium phosphate, bile acid and lecithin, while pancreatin was added after pH adjustment. All chemicals used were of analytical grade (Merck KGaA, Darmstedt, Germany or Sigma-Aldrich Co.)

Simulated gastric fluid (400 ml) was heated to 37°C in 6 different containers in a dissolution tester (Dissolutest, Prolabo, Paris, France), and 10 g DM of the experimental diets were added to duplicate containers. Gastric simulation was allowed to progress for 60 min, and duplicate 7-ml samples were withdrawn after 15, 30 and 60 min. A simulated intestinal boost (100 ml) was added and the pH adjusted to 6.8. After 20 min, pancreatin was added and the intestinal simulation was allowed to progress for 4 hours; duplicate 7-ml samples being withdrawn after 2 and 4 h. All samples were hand centrifuged (rotor 1014, Hettich, Germany) for 30 s to remove insoluble particles. The supernatant was collected and kept in boiling water for 5 min to inactivate the enzymes, and then centrifuged (1600 g, 10 min, Beckman GPR, Fullerton, CA, USA) to remove any remaining particles and denatured proteins. The supernatant was stored at -20°C until β-glucan analysis.

Calculations and statistical evaluation

The concentration-normalised viscosity was calculated using the equation:

$$\frac{\eta_{sp}}{c} = (\eta_r - 1) \cdot \frac{1}{c} = \frac{\eta_s - \eta_0}{\eta_0} \cdot \frac{1}{c} \qquad (l/g) \tag{1}$$

where η_{sp} is the specific viscosity, η_r is the relative viscosity (η_s/η_0), η_s is the viscosity of the solution containing the solute (i.e. the oat product in this study), η_0 is the viscosity in the absence of the solute, and c is the concentration of the β-glucan (g/l).

The release of β-glucan during *in vitro* digestion of the diets was determined after certain times (t) using the following equation:

$$C_{\beta-glucan,R}(t) = \frac{C_{\beta-glucan}(t)}{C_{\beta-glucan}(0)} \cdot 100 \qquad (\%) \tag{2}$$

For conversion of the β-glucan concentration from weight percent to g/ml we used the density of sucrose at 3.3% or 3.9% DM.

The extraction yield of lipids was assumed to be equal to (the amount of DM extracted from oat bran minus its contents of minor sugars) divided by the amount of lipids in the starting material x 100.

The MW distribution of ß-glucans was determined by conversion of the plot of the intensity as a function of retention time to a plot of the intensity as a function of MW

of ß-glucans (Fig. 3A and Fig. 3B). These calculations were based on the regression equation obtained from the calibration curve of ß-glucan standards ($Log\ MW = -0.1 \cdot R_t + 8.4$, with MW in Da and R_t in minutes).

All data were analysed using Minitab® software package version 14.0 (Minitab Inc, State College, PA, USA). All data, apart from the data on bile acids and data with duplicates, were checked to determine if the residuals (i.e. the deviation from the mean) were normally distributed using the Anderson-Darling test. Outliers were identified by distribution plots, and were defined as samples deviating from the third quartile by more than 150% of the interquartile range (using Minitab®). For normally distributed data, the means of the different groups were compared pairwise using one-way ANOVA with Tukey's test for multiple comparisons ($P<0.05$). One data set on caecal tissues was apparently not normally distributed and therefore the non-parametric Kruskal-Wallis test was used to compare the median values between the groups based on the variance by ranks (Siegel and Castellan, 1988).

In Experiment 1, multiple regression was used in order to consider the composition terms:

OB= EOB + OB oil   [3]

The response (y) was set to the plasma cholesterol level after 4 weeks whereas x_1 (the EOB intake) and x_2 (the OB oil intake) were defined as the transformed predictor variables. For the control group we set $x_1=0$; $x_2=0$, for the EOB group $x_1=1$; $x_2=0$, for the OB oil group $x_1=0$; $x_2=1$ and for the OB group: $x_1=1$; $x_2=1$.

Results

Experiment 1

Extraction yield of lipids

The OB oil was obtained by ethanol extraction followed by centrifugation and evaporation of ethanol (Fig. 1A). The isolated OB oil was a yellow emulsion, containing 15% low MW sugars (based on DM). The remaining 85% of the OB oil was assumed to be lipids. The extraction yield of total lipids was estimated to be 58% (see Methods); the more non-polar lipids remaining in the EOB.

Content of avenanthramides in oat diets

The total content of AVAs in dry-milled OB was determined to be about 43 mg/kg OB (AVA 2c: 13.8 ± 0.3; AVA 2p: 14.8 ± 0.4 and AVA 2f: 14.7 ± 0.5) (means and SEM n=3, based on DM). The AVA content, expressed as mg per kg diet, is presented in Table 3. The total contents of AVAs in the OB oil diet were 13 mg/kg diet and 11 mg/kg in the OB diet. It should be noted that the levels of AVA in the OB oil diet are somewhat more uncertain since we made only one replicate by analysing the OB oil sample. The EOB diet contained 19% of the total AVAs (AVA 2c + AVA 2p + AVA 2f) found in the oat bran diet (Table 3).

Body weight, faeces and food intake

All mice gained in body weight when fed the experimental diets (Table 4); on average 3.1 ± 0.90 g/mouse (mean and standard pool deviation). Mice in the group given OB oil gained more body weight than those in the control group and OB group ($P<0.05$) but not more than those in the EOB group. The feed intake and the faecal excretion were similar in all groups.

Caecum content and caecum tissue

The caecum content in the mice fed OB and EOB was higher than in those fed the control diet ($P<0.05$). Furthermore, mice fed OB had a greater caecum content than mice fed OB oil. The weight of caecum tissue did not differ significantly between the mice in the various dietary groups (Table 4).

Plasma cholesterol and lipoproteins

A distribution plot of plasma cholesterol levels at baseline and at 4 weeks revealed one outlier. This mouse belonged to the EOB group, and had an unusually low plasma cholesterol level at baseline (2.08 mmol/l) and was therefore not included in the analysis of the data. There were no significant differences in plasma cholesterol levels between the dietary groups at baseline and the level was on average 2.54 (±0.23) mmol/l (± standard pool deviation).

In an attempt to find out how EOB and OB oil contributed to the cholesterol-lowering effect of whole OB a regression analysis was performed on the plasma cholesterol data. A statistically significant regression equation was found between the cholesterol level after 4 weeks using EOB and OB oil as predictors:

$$y = 4.0 - 0.64x_1 - 0.41x_2,$$

where y represents the cholesterol level after 4 weeks, x_1 = the EOB intake, x_2 = the OB oil intake, and the constant is the plasma cholesterol level after 4 weeks in the control group when x_1=0 and x_2=0. The regression equation is illustrated as a surface plot of the mean of the dietary groups in Fig. 1B. There was no significant interaction between x_1 and x_2. From the regression equation it can be concluded that the cholesterol-lowering effect of oat bran was partly caused by the oil and EOB fractions; the EOB fraction appearing to have the strongest influence (0.64 [$P<0.001$] for EOB fraction vs. 0.41 [$P<0.02$] for the OB oil fraction).

Interestingly, the correlation was significant already after 2 weeks on experimental diets:

$y = 4.0 - 0.34x_1 - 0.33x_2.$ However, the coefficients were quite similar (0.34 (P=0.024) for EOB fraction vs. 0.33 (P=0.027) for the OB oil fraction). No significant differences in lipoproteins were seen after 4 weeks of the atherogenic diets (Table 5).

Experiment 2

The reproducibility of β-glucan extraction from OB

A mass balance of the β-glucan extracted from EOB was made for each of the four batches illustrated in the isolation scheme in Fig. 2A. Three parameters were evaluated: extractable β-glucan, β-glucan in the supernatant and the β-glucan remaining in the residue. The parameter "in supernatant" is an estimate of the practical extraction yield of β-glucan, and was on average 80 % with a SEM of 2% for the four batches. Recovery of the mass balances was greater than 90% for each of the four batches (Table 6).

Good reproducibility was found in the purity of the β-glucan isolate produced as described in Fig. 2A (Table 6). The peak MW of β-glucan (MW_p) in the residue separated after centrifugation was determined to be 431 ± 9 kDa (n=11), while the MW_p of the supernatant was lower (P=0.001) 384 ± 2 kDa (n=8). After isolation (see Fig. 2A and Fig. 2B), the final ß-glucan product contained ß-glucans with a MW_p of 356 ± 4 kDa ($\pm$ SEM; n=4) Thus, we can conclude that there was no hydrolysis of the β-glucans during dialysis (Table 6), although it cannot be excluded that the effect of hydrolysis was compensated for by a loss of β-glucans with low MW (i.e. up to the MW cut-off of the dialysis bag, 12-14 kDa).

Molecular weight, viscosity and water solubility of β-glucan products

The MW_p of β-glucans in the oat bran (2348 kDa) used as starting material to enrich β-glucans in the present study has been reported previously (Immerstrand *et al.* 2010).

The MW distribution of the β-glucans in the two β-glucan products used in Experiment 2 overlapped to some extent (Figs 3A and 3B). However, the MW_p differed significantly between the products: 356 ± 4 kDa vs. 285 ± 4 kDa ($\pm$ SEM; n=4). The MW distribution of the β-glucan reference product (Megazyme) was not symmetrical, and therefore MW_p is not the most appropriate way to describe the average MW (Fig. 3B). However, the value of MW_p obtained for this product was in good agreement with the information obtained from the manufacturer (272 kDa). The MW_p of this β-glucan remained unchanged by processing involving solubilisation of the β-glucan with accompanying freeze-drying, with the MW_p before preparation (289 $\pm$ 2) kDa (n=4). Thus, it seems that neither boiling nor freeze-drying necessarily affects the average MW or the MW distribution of β-glucan.

The viscosity of the two β-glucan products was measured after dissolution to solubilise most of the β-glucans present in the products (see Methods). Approximately the same proportion of β-glucans could be solubilised from both β-glucan products (Table 7), but their viscous properties differed (Fig. 3C). The viscosity of the higher MW β-glucan (356 kDa) product, decreased with increasing shear rate, and thus behaved as a non-Newtonian fluid. In order to work within the Newtonian region, the sample was diluted 1:1 with deionised water. The β-glucan (356 kDa) had a concentration-normalised viscosity about 60% higher than that for the β-glucan (285 kDa).

The water solubility of the β-glucan products was evaluated using a different dissolution method from that used for the viscosity measurement. Here, the products were solubilised in deionised water at room temperature for one hour (see Methods).

The water solubility of the β-glucan (356 kDa) was somewhat higher than that of the β-glucan (285 kDa): 99 and 88%, respectively.

The amount of solubilised β-glucan from the untreated purchased β-glucan (285 kDa) was apparently lower (51 ± 3, n=2) than the material solubilised after freeze-drying in presence with maltodextrin and sucrose (88 ± 0.5, n=2 Table 7). The difference may be due to the creation of a glassy state by maltodextrin (consisting of 7 glucose monomers on average) during freeze-drying. The formation of a solid solution in the glassy state is known to increase re-solubilisation after drying (Roos, 1995).

Nutritional composition of β-glucan products

Extractions of β-glucans from oat bran (Fig. 2A) resulted in a product that contained 46% β-glucan (dry-weight basis) and 7% other types of dietary fibre, 16% fat, 20% protein, 1% maltose, 5% ash and 5% which could not be explained by analysis. After the addition of maltodextrin (Fig. 2A) the purified cotton-like β-glucan product was about 23% pure with respect to β-glucan content.

The isolated fibre residue from total fibre analysis contained no total starch. Therefore, it can be concluded that the isolated β-glucan product from OB did not contain any resistant starch. The dietary fibre polysaccharides in the β-glucan product isolated from OB were mainly composed of glucose (90%), the remaining being arabinose (4.2%), xylose (2.6%), galactose (2.3%) and minor amounts of rhamnose and mannose (<1%) (Table 8). The OB used as starting material to isolate the β-glucan product consisted of glucose (71%), arabinose (12%), xylose (13%), rhamnose and mannose (<2%). The content of arabinoxylans in the OB fibre was about 19% (estimated as the sum of arabinose and xylose monomers), corresponding to a total arabinoxylan content in OB of approximately 3.1%. This is somewhat lower than previously reported values for oat bran (4.3% Theander *et al.* 1995; 4.8% Westerlund *et al.* 1993).

Body weight, feed intake and faeces

All mice gained weight during the four weeks on the experimental diets. The OB group increased significantly more than both the control and β-glucan (285 kDa) groups. There were no differences in feed intake or faeces excretion (Table 4).

Plasma cholesterol and lipoproteins

Distribution plots of plasma cholesterol levels at baseline and after 4 weeks revealed two outliers at baseline, but none at 4 weeks. One of the outliers belonged to the control group and had an unusually high plasma cholesterol level (3.13 mmol/l), while the other belonged to the oat β-glucan group (285 kDa) and instead showed an unusually low plasma cholesterol level (1.89 mmol/l). Data from these mice were therefore not included in the evaluation. Plasma cholesterol at baseline was on average 2.38 (± 0.28) mmol/l (± standard pool deviation), with no significant differences between the dietary groups.
After 2 weeks, none of the three oat dietary groups differed in cholesterol level from the control group (3.11-3.35 mmol/l vs. 3.40 mmol/l). Neither were there any differences between the dietary groups.

After 4 weeks, the β-glucan group (285 kDa) had a lower plasma cholesterol level than the control group ($P<0.05$), whereas the oat bran group and the second β-glucan group (356 kDa) did not ($P=0.06$ and $P=0.11$, respectively). Furthermore, there were no significant differences in plasma cholesterol between the oat dietary groups (Fig. 4A). Furthermore, no significant differences in the levels of LDL + VLDL were seen between the dietary groups after 4 weeks (Table 5).

Bile acids in faeces

The excretion of bile acids was increased by 88% ($P<0.01$), 76% ($P<0.01$) and 124% ($P<0.001$) in oat bran, β-glucan (356 kDa) and β-glucan (285 kDa) groups, respectively, compared with the control group. No statistically significant difference was found between the oat bran and β-glucan groups (Fig. 4B).

Weight of caecum tissue and caecum content

Mice fed the β-glucan diet (356 kDa) had a higher caecum content than mice fed the control diet ($P<0.01$). No significant differences were observed regarding the weight of the caecum tissue between the dietary groups (Table 4).

Short-chain fatty acids in the caecum

Results regarding the distribution of SCFA in the caecum are summarised in Table 9. The total pool of SCFA was higher in mice fed the oat β-glucan diet (356 kDa) than with the control diet ($P<0.01$). The pool of acetic acid did not differ significantly between the dietary groups, while the pool of propionic acid and butyric acid was higher in both β-glucan groups compared to the control group ($P<0.05$).

The ratio of (propionic acid + butyric acid) to acetic acid was higher in all the oat or β-glucan dietary groups than in the control group ($P<0.05$). In addition, the ratio was 32% higher in the low-MW β-glucan group (285 kDa) than in the OB group ($P<0.05$). Furthermore, the ratio of propionic acid to acetic acid was higher in both β-glucan groups than in the control group ($P<0.001$). The ratio of propionic acid to acetic acid in the OB group was found to be 28% lower than that of the β-glucan group (285 kDa) ($P<0.05$) (Table 10).

Release of β-glucans during in vitro digestion of experimental diets

The release of β-glucans from the OB diet was about 34% after 5 hours. The release from the β-glucan diets was more than twice as high as that from the OB diet ($P<0.001$), and remained high (>64%) during the simulated passage along the GI tract (Fig. 5). Furthermore, after 300 minutes the β-glucan diet with the higher MW (356 kDa) caused about a 16% higher release of β-glucan than the other β-glucan diet containing the lower MW β-glucans (285 kDa) ($P<0.01$).

Discussion

Several studies have shown that the inclusion of oat bran in the diet lowers plasma cholesterol levels in humans (Ripsin *et al.* 1992; Brown *et al.* 1999). Reports on the cholesterol-lowering effects of highly purified β-glucan preparations (> 50% pure) are however limited (Braaten *et al.* 1994; Queenan *et al.* 2007). Before new, health-promoting oat-based foods with improved cholesterol-lowering effects can be developed; several issues remain yet to be resolved. The objective with the current study was to elucidate whether other components of oats, apart from β-glucans, are of importance for the cholesterol-lowering effect and what role processing and the degree of β-glucan purification play in this context. Two separate experiments in mice have been performed with this intention.

Evaluation of OB oil and EOB (Experiment 1)

In the first experiment, the cholesterol-lowering effects of two complementary fractions isolated from oat bran: an oil fraction (OB oil) and the remaining ethanol extracted oat bran (EOB), were evaluated. A statistically significant regression equation was found after both 2 and 4 weeks between the plasma cholesterol level (y) and the two variables: the EOB intake (x_1) and the OB oil intake (x_2). From the regression equation after week 4: $y = 4.0 - 0.64x_1 - 0.41x_2$ (Fig. 1B), we conclude that the EOB fraction (x_1), containing all the components (including the β-glucans) except for the separated ethanol soluble fraction, contributed more to the cholesterol-lowering effect than the OB oil. However, this result was not consistent with the regression equation after week 2, where the OB oil and the EOB fraction contributed about equally to the cholesterol-lowering effect. Most important to point out is that our results indicate that other oat components than β-glucans may contribute significantly to the cholesterol-lowering effect of oat products, which is also supported by previous studies (De Groot *et al.* 1963; Welch *et al.* 1988; Illman *et al.* 1991; Yokoyama *et al.* 1998).

The OB oil diet contained about 5 times more of the oat-specific polyphenolics (AVAs) than the EOB diet (13 mg vs. 2.2 mg/kg, respectively). The analysis of AVAs in this study was based on ethanol extraction in order to solubilise the AVAs in the products, which explains our low contents of AVAs in the EOB fraction (Table 3). The total content of AVA in untreated oat bran (43 mg/kg) was in fair agreement with previously reported results: 20 and 13 mg/kg (Bryngelsson *et al.* 2003; Mattila *et al.* 2005). Polyphenol-containing extracts derived from other plant sources have been

found to lower plasma cholesterol levels in both humans (Castilla *et al.* 2006) and animals (Auger *et al.* 2002). Apart from AVAs, minor sugars and DM, no further analysis of the OB oil was made and, therefore, we can not conclude which component(s) of the OB oil that contributed to the reduction of plasma cholesterol levels. We have previously found that ethanol-extracted bran based on oats from the cv. Sang contains about 8% β-glucans (based on DM) and, therefore, the oil may be assumed to not contain any β-glucans.

The OB and OB oil diets contained slightly more unsaturated fatty acids than the control diet and one might question if these fatty aids contributed to the cholesterol-lowering effect. A control experiment, also discussed in one of our previous publications (Andersson *et al.* 2010), did however reveal that the small difference in fatty acid composition between oat bran and control diets did not contribute to the cholesterol-lowering effect of oat bran.

If effects of unsaturated fatty acids are excluded, other potentially active components of the OB oil must be considered. Proteins can be excluded as these generally are regarded to be insoluble in ethanol. The OB oil in this study was an apparently stable emulsion suggesting that the oil may contain polar lipids such as galactolipids. In this respect it is interesting that a patented product, Fabuless[TM] (an emulsion of 40% palm oil and 2.5% oat oil in water), has been shown to reduce food intake and increase satiety in humans, although results vary (Haenni *et al.* 2009; Logan *et al.* 2006). The proposed mechanism is that lipid droplets from the emulsion, consisting of a core of palm oil coated with galactolipids from the oat oil, are more slowly digested to free fatty acids and glycerol due to the coating of oat galactolipids (Diepvens *et al.* 2008). One might therefore speculate that the cholesterol-lowering effect of the OB oil in the present study was a consequence of reduced intestinal uptake of lipids and possibly also cholesterol, due to a similar coating effect of butter droplets by galactolipids and perhaps other polar lipids from the OB oil. However, we observed no effect on feed intake with the OB oil diet, but it should be borne in mind that the measurement of feed intake was performed on per cage basis and not on specific animals.

A tocotrienol-enriched fraction from palm-oil has been proven to lower cholesterol in humans (Qureshi *et al.* 1991). Petterson and Åman (1992) reported that ethanol extracted oat bran contained considerably lower contents of tocotrienol when compared with untreated oat bran and therefore it seems possible that the OB oil fraction (and OB) also contained higher levels of tocotrienols than the EOB fraction. Moreover, dietary intakes of plant sterols have been proven to reduce blood

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cholesterol in humans (Law, 2000). Määtä *et al.* (1999) found that the sterol contents in seven cultivars of oats grown in Sweden varied between 350 and 491 µg/g dehulled oats and Normén *et al.* (2002) reported that the sterol content in oat bran was 460 µg/g. In a study in mice, 2% plant sterol esters were found not to reduce plasma cholesterol in wild-type mice after 4 weeks on a normal chow diet, compared with a sterol-free diet (Weingärtner *et al.* 2008). Based on the data by Normén *et al.* (2002) the plant sterol content originating from oats can be estimated to be about 0.01% plant sterol in our current diets. Taking this into consideration, the contents of sterols in oat bran, however, seem to be too low to cause an effect in mice.

Evaluation of purified β-glucan products from oats (Experiment 2)

The aim of Experiment 2 was to compare the effect of two β-glucan products from oats, purified to different degree of β-glucan, on plasma cholesterol levels, but also on the production of SCFA in the caecum and the excretion of total bile acids in faeces. One of the products was a commercial β-glucan product (β-glucan 285 kDa), which contained 97-98% β-glucan (based on DM, see Methods) and to the best of our knowledge, no other study has been conducted in humans or animals to investigate the cholesterol-lowering effect of such a pure β-glucan product derived from oats. Indeed, this is an interesting aspect, as there are reported studies indicating that there exist interactions between β-glucans and other components of oats (Autio *et al.* 1992; Izydorcyk and MacGregor, 2000) and one may assume that these interactions may affect the conformation of β-glucan molecules and potentially also the bioactivity in the GI tract.

The second β-glucan product (β-glucan 356 kDa) was isolated from oat bran in order to obtain a material representing the same structure of β-glucans as that found in oat bran. The peak MW of β-glucan was lower than what we found previously for the same batch of oat bran used as starting material (2348 kDa) (Immerstrand *et al.* 2010). About 80% of the β-glucan could be extracted from oat bran and the reproducibility was good between batches. Analysis of the β-glucan content showed that the product was 46% pure before the inclusion of maltodextrin and sucrose in the process (Fig. 1A and 1B). The remaining 54% comprised 7% other types of dietary fibre, 16% fat, 20% protein, 1% maltose, 5% ash and 5% which could not be explained by analysis. The dietary fibre in the extracted β-glucan product consisted of 90% glucose, which could be derived from β-glucans and perhaps glucomannan and minor amounts of cellulose. The isolated β-glucan product contained about 6% arabinoxylans and 82% β-glucan (based on the dietary fibre content), while the oat bran used as starting material

contained about 3% arabinoxylans and 7.5% β-glucan. Since only a minor part of the arabinoxylans could be derived by extraction, these results suggest that the most of the arabinoxylans in the oat bran used are water-insoluble fibres – in line with previously reported results (Hashimoto *et al.* 1987; Frølich and Nyman, 1988; Nyman and Asp, 1988; Westerlund *et al.* 1993).

Both oat bran and the two different oat β-glucans lowered plasma cholesterol compared with the control diet containing cellulose. The effect was only significant for one of the β-glucan diets (285 kDa), but a tendency was observed for the oat bran diet and the second β-glucan diet (356 kDa) (P=0.06 and P=0.11, respectively). The bile acid excretion increased significantly in all oat bran or β-glucan groups. An increased excretion of bile acids with concurrent increase in bile acid synthesis from cholesterol has been shown to be an important feature of the cholesterol-lowering effects of oats in humans (Anderson *et al.* 1984; Zhang *et al.* 1992; Marlett *et al.* 1994; Lia *et al.* 1997; Ellegard and Andersson, 2007). Thus the increased bile acid excretion supports the finding that all three diets also had an effect on plasma cholesterol, although there was a lack of statistical power for the oat bran group and for one of the β-glucan groups due to the multiple comparisons of groups (Fig. 4A). Moreover, the plasma cholesterol level after 4 weeks did not differ between the two β-glucan groups (P=0.89). This was also found after 2 weeks (P=0.49). Thus, it seems that the degree of β-glucan purification did not play a crucial role in the cholesterol-lowering effect.

Apart from the degree of β-glucan purification, the two β-glucans also differed with regard to their average MW and apparently to their water solubility of β-glucan and viscous properties. A difference in viscous properties was expected as molecules with higher MW occupy larger volumes in solution. Possibly, interactions between β-glucan and oat protein or between β-glucan and arabinoxylans in the less purified β-glucan product (46%) may partly also explain its higher viscosity after solubilisation. Further, it can not be excluded that the molar ratio between the two predominant blocks in the β-glucan chain (i.e. cellotriose/cellotetraose) differed between the two β-glucans, and thus explain their different viscous properties. Despite the difference in viscous properties, both β-glucans, however did not differ in their cholesterol-lowering properties (Fig. 4A), which agrees with one of our previous studies where oat bran preparations with different viscous properties lowered plasma cholesterol to the same extent (Immestrand *et al.* 2010)

To address how the solubility of β-glucans under physiological conditions may interfere with cholesterol-lowering properties in the current study, we compared the release of β-glucans from the oat diets using a model of the GI tract (see Methods).

We observed a slow release of β-glucan from the OB diet, resulting in 34% release after 5 hours, in fair agreement with previous studies. For example, Beer *et al.* (1997) reported 13-29% release of β-glucan from oat bran samples after an *in vitro* treatment with amylase, pepsin and pancreatin at 37°C. In contrast to the oat bran diet, the release of β-glucan from the diets containing purified β-glucan products was above 80% already after 15 minutes, and remained high during the simulation. After 5 hours, the release from the β-glucan diet with the higher MW (356 kDa) was 16% higher ($P<0.01$) than that from the lower MW (285 kDa) diet, in agreement with data on water solubility (Table 7).

However, despite the large difference in release profiles between the oat bran diet and β-glucan diets, the diets did not significantly differ in cholesterol-lowering properties. The fact that the two β-glucan diets resulted in high release of β-glucan and also had an effect on plasma cholesterol level support the hypothesis that the fraction of the β-glucans solubilised in the small intestine from the oat product is of importance. One may however question by which mechanism oat bran lowers plasma cholesterol, as the release of β-glucans from the OB diet was clearly lower than those from the β-glucan diets. From the results in the first experiment of this study we concluded that the effect of oat bran was partly caused by an oil fraction containing ethanol-soluble components (not including β-glucans). Accordingly, the effect of the OB oil may have compensated for the loss of "non-released β-glucans" in the bran.

Intestinal production of propionate and the subsequent inhibition of cholesterol synthesis is another mechanism that has been suggested as an explanation of the cholesterol-lowering effects of oat β-glucans (Kerckhoffs *et al.* 2002). However, the intestinal formation of acetic acid may counteract the positive effect of propionate by increasing cholesterol synthesis (Wong *et al.* 2006). The ratio of propionate to acetate is therefore of greater interest than the levels of the individual acids regarding the effects on plasma cholesterol. In the present study, the ratio of propionic acid/acetic acid, was significantly higher in β-glucan groups but not in the OB group, when compared with the control group. This may be due to the higher solubility of β-glucans *in vivo*, based on results from our simulation model of GI tract (Fig. 5). Furthermore, the ratios of (propionic acid +butyric acid) to acetic acid were significantly higher in all the oat and β-glucan dietary groups than the control group.

Conclusions

The findings of this study indicate that the cholesterol-lowering effect of oat bran can be explained by two complementary fractions, i.e. an ethanol-soluble fraction and a β-glucan containing fraction. Moreover, oat bran and two purified β-glucan products (β-glucan content: 46% or 97-98%) had about the same effects on plasma cholesterol. In addition, the difference in average MW and viscous properties did not seem to play a role in the cholesterol-lowering effect. It is also important to point out that despite the fact that both β-glucan products had been extensively processed, they were still able to lower the plasma cholesterol level. These findings are in disagreement with the EFSA's recent statement that only foods containing "minimally processed" β-glucan may be associated with claims of cholesterol-lowering properties (EFSA, 2009). Before, an 80% pure oat β-glucan product has been shown to lower the cholesterol level in humans (Braaten *et al.* 1994), but to the best of our knowledge, this is the first time a cholesterol-lowering effects has been demonstrated for a product containing almost only β-glucans (97-98%, based on DM). The fact that the release of β-glucan from the β-glucan diets was more than twice as high as that of the oat bran diet may explain why ingestion of the β-glucan products led to a significantly increased ratio of propionic acid/acetic acid in the caecum, compared with the controls.

In the future, it would be interesting to define the cholesterol-lowering components of the oat bran oil fraction used in this study more precisely, and to further investigate effects of pure β-glucans with different average MWs on cholesterol levels.

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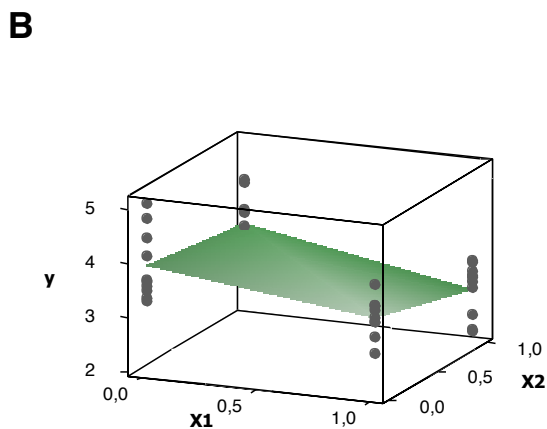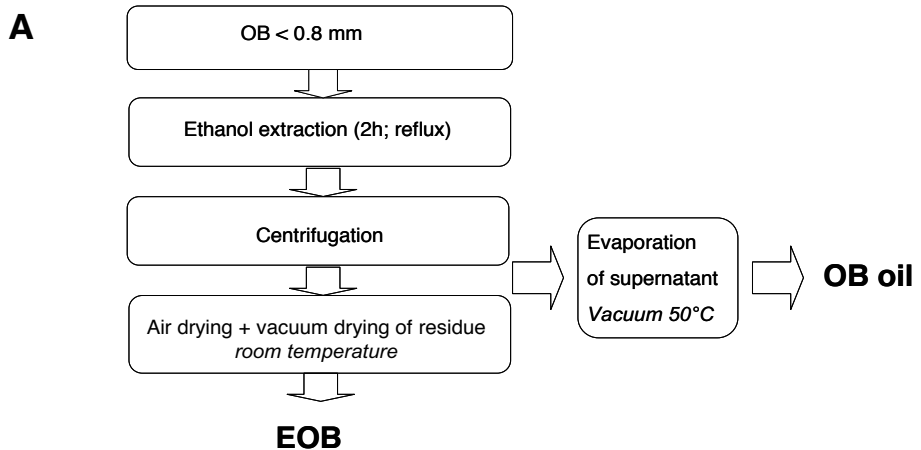
Figure 1. (A) Schematic flow-sheet describing the isolation of oat bran (OB) oil and ethanol extracted oat bran (EOB) from OB used in Experiment 1, and **(B)** EOB and OB oil contribute to the cholesterol-lowering effect of OB according to the significant regression equation: $y = 4.0 - 0.64x_1 - 0.41x_2$, where y is the plasma cholesterol levels after 4 weeks (mmol/l) in mice, x_1 is the intake of EOB and x_2 is the intake of OB oil. For the control group: $x_1=0$; $x_2=0$, for the EOB group: $x_1=1$; $x_2=0$, for the OB oil group $x_1=0$; $x_2=1$ and for the OB group: $x_1=1$; $x_2=1$. The dots represent the cholesterol levels of the individual mice after 4 weeks.
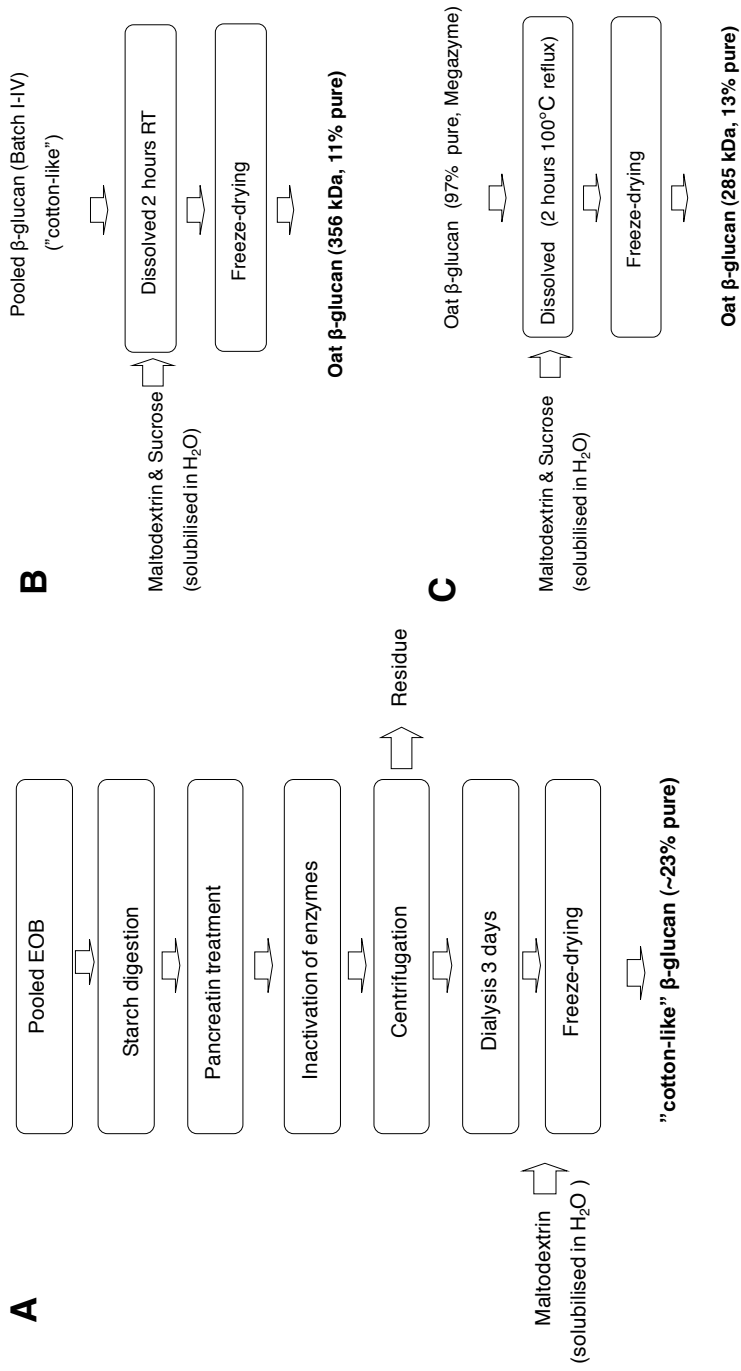
Figure 2. Preparation of the two oat β-glucan products used in Experiment 2 (**A**) Isolation of a β-glucan product from oat bran (Batch I-IV), and (**B**) re-suspension of freeze-dried material to produce a material easier to grind (**C**) Preparation of the β-glucan reference product. RT = room temperature, EOB = ethanol-extracted oat bran.
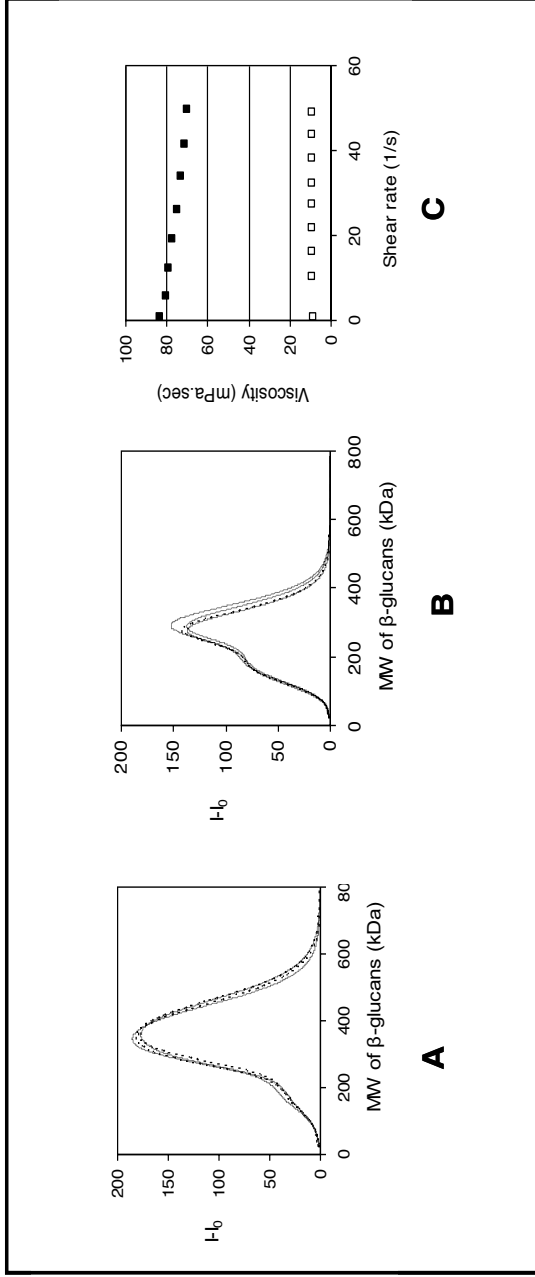
Figure 3. Physico-chemical properties of two β-glucan products used in Experiment 2. (**A**) Molecular weight (MW) distribution of the β-glucan product isolated from oat bran with peak molecular weight (MW_p) = 356 kDa (n=4) and (**B**) MW distribution of the β-glucan reference product (production described in Fig. 2C) with MW_p = 285 kDa (n=4). (**C**) The viscosity as a function of shear rate for β-glucan with MW_p 356 kDa (■) and β-glucan with MW_p of 285 kDa (□). I=intensity.
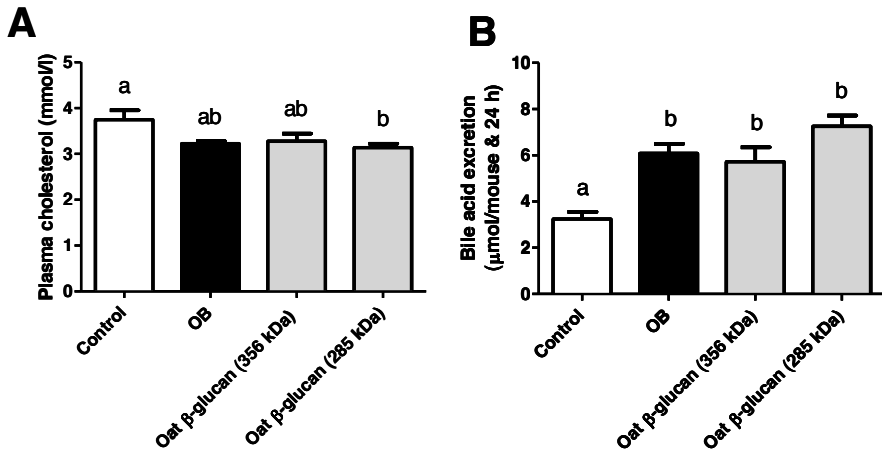
Figure 4. Results from Experiment 2. **(A)** Plasma cholesterol after 4 weeks on atherogenic diets, where the statistics are based on Tukey's test for multiple comparisons (n=8-10 mice per group). **(B)** Bile acids in faeces (n=6 per group). Data are presented as means ± SEM. Groups with different letters were significantly different (*P*<0.05) after 4 weeks on experimental diets. OB=oat bran.
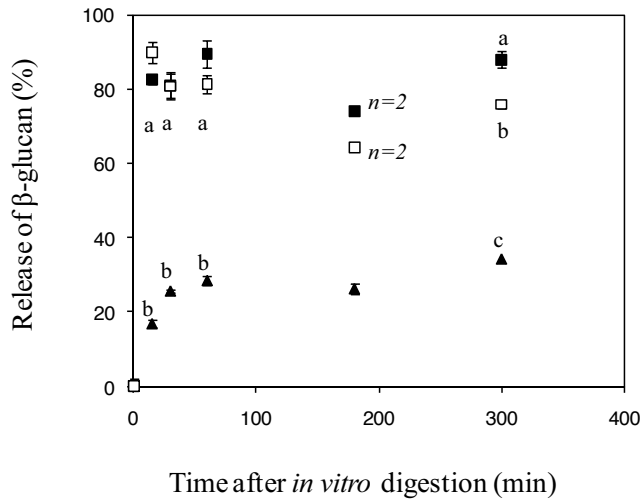
Figure 5. Release of β-glucan from oat diets used in Experiment 2 (n=4 mice per group) in the simulation of the GI tract: (▲) oat bran diet, (■) β-glucan (356 kDa) diet, and (□) β-glucan (285 kDa) diet. Groups with different letters were significantly different (*P*<0.05). The first 60 minutes constitute the simulation in gastric juice and the next four hours simulation in the small intestine. For definition of the release of β-glucan, see equation [2] in Methods.

Table 1. Fomulation of the diets used in experiment 1 (g/kg diet). All values are based on FW unless otherwise stated.

Ingredients	Control	OB	EOB	OB oil
Casein, 80 Mesh[1]	200	123	123	200
DL-Methionine	3.0	5.0	5.0	3.0
Maize Starch	275	155	155	275
Maltodextrin	100	100	100	100
Sucrose	100	95.8	95.8	100
Cellulose	45.1	-	-	45.1
Butter, Anhydrous[2]	200	175	192	183
Maize Oil	10	10	10	10
Mineral Mix S10026	10	10	10	10
Calcium Phosphate	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H_2O	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10
Choline Bitartrate	2	2	2	2
Cholesterol[2]	7.54	7.60	7.60	7.54
Sodium Cholate	1	1	1	1
OB[3]	-	270 (246 DM)	-	-
EOB	-	-	261 (229 DM)	-
OB oil[4]	-	-	-	103 (17 DM)
Contents of OB (g/270g OB)				
Protein		60		
Sucrose		3.8		
Starch		110		
Fat		23		
Dietary fibres (where of β-glucans)		41 (15)		
Ash		8.3		
Water		24		
DM content of diets (%)	94	94	94	90
Energy contents of diets[5]	% energy	% energy	% energy	% energy
Protein	16	15	15	16
Fat	43	43	43	43
Carbohydrates	41	42	42	41
Energy density (kJ/g dry diet)	19.5	19.3	19.4	20.3

OB= oat bran, EOB= ethanol-extracted oat bran, DM=dry matter, FW=fresh weight

[1] Casein is 88% protein, [2] Anhydrous butter has 230 mg cholesterol/100 g. To compensate for this, extra cholesterol was added so that total amount of cholesterol in all diets was 8 g/kg diet, [3] Dry-milled oat bran (< 0.8 mm)

[4] Containing 15% minor sugars (based on DM) - see Results, [5] Estimated after correections for DM of the ingredients, based on 1 kg diet (DM)

Table 2. Fomulation of the diets used in experiment 2 (g/kg diet). All values are based on FW unless otherwise stated.

Ingredients	Control	OB	Oat β-glucan (356 kDa)	Oat β-glucan (285 kDa)
Casein, 80 Mesh[1]	200	140	190	200
DL-Methionine	3.0	4.5	3.0	3.0
Maize Starch	277	139	274	277
Maltodextrin	100	100	-	-
Sucrose	100	96.7	68.4	70.1
Cellulose	44	0	21	24.1
Butter, Anhydrous[2]	200	174	193	200
Maize Oil	10	10	10	10
Mineral Mix S10026	10	10	10	10
Calcium Phosphate	13	13	13	13
Calcium Carbonate	5.5	5.5	5.5	5.5
Potassium Citrate, 1 H_2O	16.5	16.5	16.5	16.5
Vitamin Mix V10001	10	10	10	10
Choline Bitartrate	2	2	2	2
Cholesterol[2]	7.54	7.60	7.54	7.54
Sodium Cholate	1	1	1	1
OB[3]	-	270 (246 DM)	-	-
Oat β-glucan product[4]	-	-	175 (163 DM)	150 (143 DM)
Contents of oat products		OB (g/270 g)	β-glucan (g/175 g)[5]	β-glucan (g/150 g)[5]
Maltodextrin		-	92.9	95
Sucrose		3.0	29.3	28
Maltose[5]		nd	0.5	-
Protein		48.4	8.3	-
Starch		124	nd	-
Fat		23	6.7	-
Dietary fibre (where of β-glucans)		40 (19)	21 (19)	19 (19)
Ash		7.1	2.0	-
Water		24	12	7
Not determined		-	2.3	1
DM content of diets (%)	94	93	93	94
Energy contents of diets[6]	% energy	% energy	% energy	% energy
Protein	16	16	16	16
Fat	43	43	43	43
Carbohydrates	41	41	41	41
Energy density (kJ/g dry diet)	19.6	19.6	19.6	19.5

OB= oat bran, nd=not determined, DM=dry matter, FW=fresh weight

[1] Casein is 88% protein, [2] Anhydrous butter has 230 mg cholesterol/100 g. To compensate for this, extra cholesterol was added so that the total amount of cholesterol in all diets was 8 g/kg diet [3] Dry-milled oat bran (< 0.8 mm)

[4] For more detailed information about the β-glucan products see material and methods

[5] Calculated as total maltose content in the diet minus maltose content in maltodextrin

[6] Estimated after correections for DM of the ingredients, based on 1 kg diet (DM)

Table 3. AVA content of oat diets in experiment 1 (mg/kg diet, based on DM).

Diet \ AVA	2c	2p	2f	Total
OB diet	3.6	3.9	3.9	11
EOB diet	0.9	0.7	0.6	2.2
OB oil diet	4.0	4.7	4.6	13

AVA=avenanthramide

DM=dry matter

OB=oat bran

EOB=ethanol-extracted oat bran

Table 4. Initial weight, body weight-gain, feed intake, dry faeces, caecum contents- and caecal tissue weight for mice fed experimental diets for 4 weeks.

	Initial weight (g)			Body-weight gain (g)			Feed intake (g/mouse & day)			Dry faeces (g/mouse & 24 h)			Ceacum- content (mg)			Ceacum- tissue (mg)		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Experiment 1																		
Control	19.5^{ab}	0.16	10	2.4^b	0.30	10	2.1^a	0.07	4	0.12	-	$1^{§}$	160^a	12	10	$47^{\#\#}$	(40-49)	10
OB	19.7^a	0.23	10	2.9^b	0.19	10	2.2^a	0.04	4	0.14	-	$1^{§}$	240^{bc}	19	10	$50^{\#\#}$	(44-53)	10
EOB	19.6^{ab}	0.25	9	3.2^{ab}	0.27	9	2.2^a	0.09	4	0.14	-	$1^{§}$	310^b	30	9	$44^{\#\#}$	(40-52)	9
OB oil	18.9^b	0.17	10	4.0^a	0.36	10	2.4^a	0.06	4	0.13	-	$1^{§}$	200^{ac}	7	10	$37^{\#\#}$	(34-39)	10
Experiment 2																		
Control*	19.1^a	0.38	8	3.1^a	0.29	8	2.3^a	0.08	3	0.21^a	0.01	$3^{§}$	160^a	7	8	24^a	2	8
OB *	19.1^a	0.24	10	5.0^b	0.49	10	2.4^a	0.12	3	0.20^a	0.02	$3^{§}$	190^{ab}	11	10	25^a	1	10
Oat β-glucan (356 kDa)	19.1^a	0.35	10	3.8^{ab}	0.27	10	2.4^a	0.06	3	0.17^a	0.01	$3^{§}$	230^b	16	10	27^a	2	10
Oat β-glucan (285 kDa)	18.9^a	0.29	9	3.0^{ac}	0.27	9	2.5^a	0.10	3	0.19^a	0.01	$3^{§}$	200^{ab}	9	9	24^a	1	9

OB=oat bran, EOB=ethanol extracted oat bran, nd=not determined, n=number of observations

Data are shown as mean values ± SEM. Statistics were calculated with one-way ANOVA for multiple comparisons (Tukey´s test for pairwise comparisons of means).

Mean values with unlike superscript letters were significantly different (P< 0.05).

* These data are reproduced from Immerstrand *et al. (2010)* and are included from comparison purposes.

§ The number refers to number of cages (10 mice/cage in experiment 1 and 3; 3-4 mice/cage in experiment 2)

Since these data were not normal distributed a non-parametric test was made (Kruskal-Wallis test). Data are expressed as median and 25th to 75th percentiles

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Table 5. LDL + VLDL levels in mice after 4 weeks on experimental diets.

	LDL + VLDL (%)[1]		
Experiment 1	Mean	SEM	*n*
Control	57.0[a]	2.6	*10*
OB	53.8[a]	2.7	*10*
EOB	49.2[a]	2.6	*9*
OB oil	47.7[a]	3.8	*10*
Experiment 2			
Control[2]	57.9[a]	5.9	*8*
OB[2]	42.9[a]	4.9	*10*
Oat β-glucan (356 kDa)	47.7[a]	4.4	*10*
Oat β-glucan (285 kDa)	43.3[a]	4.3	*9*

OB=oat bran, EOB=ethanol-extracted oat bran

n= number of observations.

Data are shown as mean values ± SEM. Mean values in the same column with unlike superscript letters indicate statistical significant difference between groups within each experiment($P<0.05$).

Statistical analysis was performed by using one-way ANOVA for multiple comparisons

 (Tukey´s test for pairwise comparisons of means P< 0.05) on normal distributed data.

[1] Calculated as (LDL+VLDL)/(HDL+LDL+VLDL) x 100

[2] These data are reproduced from Immerstrand *et al. (2010)* and are included for comparison purposes.

Table 6. Results on the reproducibility of the extraction of β-glucan from oat bran.

	Batch			
	I	II	III	IV
Mass balance of β-glucan (%)				
Extractable[a]	84	81	88	84
In supernatant[b]	76	78	85	80
In residue[c]	22	16	14	17
MW_p of β-glucans[d] (kDa)				
Supernatant	380 ± 1 (2)	385 ± 3 (2)	381 ± 1 (2)	389 ± 6 (2)
Residue	444 ± 11 (5)	385 ± 12 (2)	419 ± 1 (2)	454 ± 15 (2)
Final β-glucan content[e] (%)	22.8 ± 0.19 (4)	23.0 ± 0.15 (4)	22.6 ± 0.27 (4)	22.6 ± 0.14 (4)

[a] (The amount in supernatant + the amount present in the aqueous phase of residue)/ the amount in starting material [b] Recovered in supernatant/ amount in start material [c] Determined on freeze-dried and milled residue

[d] The results are shown as means ± SEM with the number of replicates within brackets

[e] After isolation described in Fig. 2a, but before re-solubilisation according to Fig 2b.

Table 7. Physico-chemical properties of β-glucan products used in experiment 2

	Oat β-glucan (356 kDa)			Oat β-glucan (285 kDa)		
	Mean	SEM	n	Mean	SEM	n
Viscosity properties						
Dissolution yield (%)[1]	86.6	0.6	2	82.5	2.7	2
Viscosity (m.Pa.sec)[2]	9.2[§]	0.2	2	10.0	0.1	2
Concentration (g/l)[3]	2.4	0.0	2	4.1	0.0	2
C n v (l/g)[4]	3.5	0.1	2	2.2	0.1	2
Water-solubility (%)[5]	99	0.7	2	88	0.5	2

c n v= concentration normalised viscosity

[1] Yield of solubilised β-glucan after boiling 10 min, before determination of viscosity

[2] Viscosity data presented as mean values at shear rates 5-50/sec

[3] Concentration of β-glucan when measuring the viscosity

[4] See methods, equation [1]

[5] Measured after solubilisation in deionised water for 1 hour at room temperature at 25 mg β-glucan/25 ml (see methods page 8).

[§] Viscosity of the solubilised fraction of oat β-glucan (356 kDa) was diluted 1:1 with deionised water before viscosity measurement in order to work within a Newtonian region. For comparison see results for the non-diluted sample in Fig 3c.

Table 8. Composition of dietary fibre (g/100g, based on DM)

Component \ Oat product	OB[1,2]	Oat β-glucan (356 kDa)[1]
Rhamnose	0.3	0.4
Arabinose	8.8	3.6
Xylose	9.9	2.2
Mannose	1.1	0.4
Galactose	1.6	2.0
Glucose	53.7	77.6
Klason lignin + uronic acids[3]	24.6	13.8

OB=oat bran, nd=not determined, DM=dry matter

[1] Products used in experiment 2

[2] Used as starting material when isolating the β-glucan product (356 kDa)

[3]Calculated by difference: total dietary fibre determined according to Asp *et al.* (1984) minus

total amount of monosacharides detected with GC

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Table 9. Results on caecal SCFA in mice fed experimental diets (experiment 2).

	Control diet			OB			Oat β-glucan (356 kDa)			Oat β-glucan (285 kDa)		
	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Total SCFA levels (µmol/g)	36[a]	2.9	8	37[a]	2.2	10	42[a]	2.8	10	40[a]	2.9	9
Total SCFA pool (µmol/caecal content)	5.8[a]	0.5	8	6.9[ab]	0.7	10	9.7[b]	1.3	10	8.1[ab]	0.7	9
SCFA pools (µmol/caecal content) [1]												
Acetic	3.8[a]	0.4	8	4.1[a]	0.5	10	5.2[a]	0.7	10	4.2[a]	0.4	9
Propionic	0.9[a]	0.1	8	1.3[ac]	0.1	10	1.9[b]	0.3	10	1.7[bc]	0.1	9
i-butyric acid	0.10[a]	0.01	8	0.10[a]	0.01	10	0.14[a]	0.02	10	0.13[a]	0.01	9
n-Butyric acid	0.7[a]	0.1	8	1.1[ac]	0.2	10	2.0[b]	0.3	10	1.6[bc]	0.2	9
i-Valeric acid	0.12[a]	0.01	8	0.14[ab]	0.02	10	0.20[b]	0.02	10	0.17[ab]	0.02	9
Valeric acid	0.08[a]	0.01	8	0.09[a]	0.01	10	0.15[b]	0.02	10	0.12[ab]	0.02	9
n-Hepatonic acid	0.08[a]	0.01	8	0.08[a]	0.01	10	0.09[a]	0.01	10	0.09[a]	0.01	9

OB=oat bran

n= number of observations

Groups with unlike superscript letters were significantly different (P<0.05). See material and methods for description of statistical procedures.

[1] The concentration of each acid (µmol/ g caecal content) multiplied with the caecal content

Statistical analysis was made by using one-way ANOVA for multiple comparisons (Tukey´s test for pairwise comparisons of means P<0.05) on

normal distributed data

Table 10. Ratio between the major caecal SCFA formed in mice (experiment 2).

Dietary group/Ratio	(PRO+ BUT)/ACE		PRO/ACE		
	Mean	SEM	Mean	SEM	*n*
Control	0.43[a]	0.02	0.25[a]	0.02	*8*
OB	0.60[b]	0.05	0.32[ac]	0.03	*10*
Oat β-glucan (356 kDa)	0.75[bc]	0.04	0.37[bc]	0.02	*10*
Oat β-glucan (285 kDa)	0.79[c]	0.04	0.41[b]	0.02	*9*

PRO=propionic acid

BUT=butyric acid

ACE= acetic acid

OB=oat bran

n=number of observations.

Data are shown as mean values ± SEM. Mean values in the same column with unlike superscript

letters indicate statistical significant difference between groups within each experiment(P< 0.05).

Statistical analysis was performed by using one-way ANOVA for multiple comparisons

(Tukey´s test for pairwise comparisons of means P< 0.05) on normal distributed data.