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Generic tools to assess genuine carbohydrate specific effects on in vitro immune modulation exemplified by β-glucans

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ABSTRACT

Even if carbohydrate preparations from plant/fungal sources have a high degree of purity, observed immune-stimulation may be caused by minute sample contaminations. Using the example of different β-glucans we present a range of analytical tools crucial for validation of possible immune-stimulatory effects. Two yeast (MacroGard and Zymosan) and one cereal β-glucan (CBG40) increased IL-8 secretion by HT-29 cells considerably. Degradation of the β-glucan samples with β-glucan specific enzymes did hardly influence the effect of Zymosan and CBG40 but significantly decreased the effect of MacroGard. Stimulation of IL-8 secretion by CBG40 and Zymosan was hence not due to their β-glucan content. Instead, the effect of the CBG40 sample was due to low levels of LPS despite the inability of the known LPS inhibitor Polymyxin B to supress its stimulatory effect. We conclude that targeted enzymatic degradation of samples is a powerful validation tool to investigate carbohydrate specific immune-modulation.

1. Introduction

Dietary components that may affect the immune system have become increasingly popular in health promotion and as functional food ingredients (Volman, Ramakers, & Plat, 2008; Wichers, 2009). The immune-modulating properties of food or medicinal plant components are often initially assessed by in vitro systems to identify the beneficial potential and to investigate structure–functional relationships and mechanisms of actions. However, this requires extremely pure preparations since trace amounts of bacterial contaminants like endotoxins, also called lipopolysaccharides (LPS), can lead to confounding results. LPS is for example known to increase the activity of transcription factor NF-κ-B, which regulates a number of pro-inflammatory genes, increase secretion of pro-inflammatory cytokines and to increase phagocytosis and nitric oxide production by e.g. macrophages (Murphy, Travers, & Walter, 2008; Sun et al., 2012). Nevertheless, observed biological effects of a test preparation are often related to the quantitatively dominating component, but this can be misleading as the example of Zymosan shows. This cell wall preparation from *Saccharomyces cerevisiae* consists mainly of yeast β-glucan, which has been ascribed various immune-modulating properties (Hong et al., 2004; Soltanian, Stuyven, Cox, Sorgeloos, & Bossier, 2009; Stuyven et al., 2009; Tsukada et al., 2003). However, it has been shown that other components in the Zymosan preparation like mannan and endotoxin-like substances also may have immune-modulating properties (Ikeda, Adachi, Ishibashi, Miura, & Ohno, 2005; Ikeda et al., 2008; Taylor et al., 2004). Since preparation of genuinely pure test samples is often difficult and sometimes even impossible to achieve, proper validation methods are needed to assess the specific immune-modulating effects of different polysaccharide preparations or food constituents in general.

β-Glucan polysaccharides found in the cell walls of cereal grains and fungi (yeasts and mushrooms) are one such group of dietary components which have shown a potential to modulate the immune system (Rieder & Samuelsen, 2012; Soltanian et al., 2009).

Abbreviations: M_w, weight average molecular weight; MW, molecular weight; DP, degree of polymerization; LPS, lipopolysaccharide; TLR, toll like receptor; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; LAL, *limulus amoebocyte* lysate; CBG40, cereal β-glucan with weight average molecular weight of 40 kDa; CBG359, cereal β-glucan with weight average molecular weight of 359 kDa; G4G3G, β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,3)-D-Glc*p*; G4G4G3G, β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,3)-D-Glc*p*.

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β-Glucans from different sources share the common structure of β-linked glucose monomers. However, while cereal β-glucans are linear polymers consisting of blocks of β-(1,3)-linked cellotriosyl and cellotetraosyl units, fungal β-glucans are branched type polysaccharides with a backbone of β-(1,3)-linked glucose monomers and β-(1,6)-linked side chains of varying length and distribution (Ohno, 2007; Wood, Weisz, & Blackwell, 1994). Fungal β-glucans exist in soluble and insoluble (particulate) forms, whereas cereal β-glucans can usually be solubilized by boiling in water.

While research has generally focused on fungal β-glucans, both cereal and fungal β-glucans have been found to enhance the effect of antitumor monoclonal antibodies (Cheung, Modak, Vickers, & Knuckles, 2002; Hong et al., 2004; Modak, Koehne, Vickers, O'Reilly, & Cheung, 2005). Fungal β-glucan preparations have been demonstrated to increase resistance to viral, parasitic, bacterial and fungal pathogens (Soltanian et al., 2009). The structurally different cereal β-glucans were reported to be able to activate complement and increase cytokine secretion and phagocytic activity in different cellular test systems (Czop & Austen, 1985; Estrada et al., 1997; Samuelsen, Rieder, Grimmer, Michaelsen, & Knutsen, 2011; Yun, Estrada, Van Kessel, Park, & Laarveld, 2003). In vivo, cereal β-glucans showed possible protective effects against an intestinal parasite (Yun et al., 1997), bacterial (Yun et al., 2003) and viral infections (Davis et al., 2004) in mice.

We have previously reported markedly increased IL-8 secretion by the human intestinal epithelial cell line HT-29 in response to a cereal β-glucan preparation of 40 kDa weight average molecular weight (M_w) (Rieder, Grimmer, Kolset, Michaelsen, & Knutsen, 2011). This increase was not influenced by the addition of the known LPS inhibitor Polymyxin B and the increased cytokine secretion was consequently attributed to stimulation from the β-glucan molecules. The same study also found a slight increase in IL-8 secretion by a 359 kDa M_w cereal β-glucan preparation, while preparations with 123 and 245 kDa M_w showed no effect on IL-8 secretion.

In the present study the carbohydrate specific effects of the two active cereal β-glucans (CBG40 and CBG359) and three yeast β-glucan preparations were further assessed using different validation methods including hydrolysis of β-glucans by different highly specific enzymes. The presented set of validation methods gives examples on how to be more certain about claimed immune modulation by specific carbohydrates or food components in general.

2. Material and methods

2.1. β-Glucan samples

Cereal β-glucans with 40 (CBG40) and 359 kDa M_w (CBG359) were obtained from Megazyme ((1,3)(1,4)-β-glucan molecular weight standards, Megazyme International Ireland Ltd., Bray, Republic of Ireland). Three different fungal β-glucan preparations from *S. cerevisiae* were used: a crude cell wall preparation, Zymosan (Zymosan A, Y4250-1G, Lot Number 50209025, Sigma–Aldrich, St. Louis, MO, USA), a highly purified soluble β-glucan, soluble Wellmune (Biothera, Eagan, MN, USA) and a particulate feed ingredient, MacroGard (Biotec ASA, Tromsø, Norway). The β-glucans were either solubilized by boiling for 15 min (cereal β-glucans) or suspended (Zymosan, MacroGard and Wellmune) in distilled water at a concentration of 1 mg/mL for all experiments if not otherwise stated. The soluble Wellmune sample formed a cloudy solution which, in contrast to Zymosan and MacroGard showed no signs of precipitation 30 min after preparation.

2.2. Enzyme treatment of β-glucan samples

A cellulase preparation (endo-1,4-β-D-glucanase, E-CELTR, Megazyme) and a preparation of 1,3-β-glucanase and β-glucosidase (exo-1,3-β-D-glucanase,β-glucosidase, E-EXBGOS, Megazyme) were removed for low molecular weight constituents using centrifugal filter devices (Millipore centrifugal filter devices, 15 mL, 10 kDa cut off, regenerated cellulose membrane, Millipore Corporation, Billerica, MA, USA). The enzyme solutions were first mixed with buffer (100 mM sodium acetate, pH 4.0 for cellulase or 200 mM sodium acetate, pH 5.0 for β-glucanase, respectively) and then 4 times with MilliQ water. Centrifugal steps consisted of 5 min at 4860 g (Hereaus Multifuge 4KR, DJB Labcare Ltd., Newport Pagnell, UK). The retentate solution was made up to 500 μL with Milli Q water and retained enzymatic activity of the purified solutions was confirmed by the release of sugars/oligosaccharides from a standard β-glucan substrate solution by HPAEC-PAD (see below). Cereal β-glucan preparations of 1 mg/mL were treated with 0.4 U lichenase (endo-1,3(4)-β-glucanase, E-LICHN, Megazyme) or the cleansed cellulase solution (1 U/mg β-glucan). Enzyme substrate solutions were incubated in a water bath at 40 °C (lichenase) or 50 °C (cellulase) for 3 h. One part of the lichenase treated solutions was further processed by adding β-glucosidase (0.04 U/mg β-glucan, E-BGLUC, Megazyme) at 50 °C for 2 h to further depolymerize the oligosaccharides to glucose. The yeast β-glucan samples were treated with lyticase (L2524, Sigma–Aldrich), a preparation with activity solely defined by its ability to disrupt yeast cell walls, and exo-1,3-β-glucanase plus β-glucosidase (Megazyme) in separate experiments. The frozen lyticase solution was thawed and diluted with phosphate buffer pH 7.5 to 500 U/mL and immediately added to yeast β-glucan suspensions (10 mg β-glucan/mL) in the same buffer (37.5 U/mg β-glucan). The suspensions were incubated 6 h in a water bath at 50 °C. For the second experiment yeast β-glucan preparations were suspended in 50 mM sodium acetate buffer pH 4.5 at 1 mg/mL, boiled for 15 min, prior to addition of 0.5 U 1,3-β-glucanase and 0.3 U β-glucosidase per mg β-glucan. Samples were incubated over night at 40 °C and all enzyme reactions above were finally boiled for 5 min.

Oligosaccharide content and composition of the different enzyme treated solutions were determined by HPAEC-PAD and aliquots of the different solutions were freeze dried for cell culture experiments. Samples with water or buffer containing the same amount of enzyme solution β-glucan samples were used as controls. Controls were treated equally as the samples used for the cell culture experiments (see section below).

2.3. Determination of generated glucose and oligosaccharides with HPAEC-PAD

Chromatography was essentially as previously described (Radva, Knutsen, Kosáry, & Ballance, 2012) with minor changes. A linear gradient of acetate was prepared from three different eluents 200 mM NaOH (A), 100 mM NaOH + 500 mM sodium acetate (B) and pure water (C) at a flow rate of 1 mL/min. Each run started with an equal proportion of eluents A and C followed by an increasing proportion of eluent B from 0 to 40% over 40 min. Consequently the concentrations of eluents A and C were reduced to 30% each. The column was then washed with 100% eluent B for 8 min and reconditioned with starting conditions for 7 min. For identification of the different oligosaccharides released from β-glucan during enzymatic treatment standards for β-D-Glc*p*-(1,3)-D-Glc*p* (laminaribiose), β-D-Glc*p*-(1,4)-D-Glc*p*, (cellobiose), β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,3)-D-Glc*p* (G4G3G), β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,3)-D-Glc*p* (G4G4G3G), β-D-Glc*p*-(1,3)-β-D-Glc*p*-(1,4)-D-Glc*p* (G3G4G), and β-D-Glc*p*-(1,4)-β-D-Glc*p*-(1,3)-β-D-Glc*p*-(1,4)-D-Glc*p* (G4G3G4G) were obtained from Megazyme. For quantification, solutions of the above mentioned oligosaccharides and glucose (Sigma–Aldrich) were prepared in water after pre-drying over P_2O_5 under vacuum in concentrations between

0.15625 and 25 μg/mL, containing 10 μg/mL melibiose as internal standard.

2.4. Preparative size exclusion chromatography (P-SEC)

For size exclusion chromatography the cereal β-glucan (M_w = 40 kDa) sample was dissolved in Milli-Q water by boiling at a concentration of 20 mg/mL. The chromatography system consisted of a pump (Pump P-50, GE Healthcare, St. Giles, UK, formerly Amersham Biosciences) delivering the eluent (Milli-Q water) at a flow rate of 1 mL/min, a Sephacryl S-300 high prep column (GE Healthcare) and an RI-detector (Shimadzu RID-6A, Shimadzu Europa, Duisburg, Germany). Injection volume was 5 mL and fractions were collected manually after the following time points (in minutes): 60–100 (F1); 100–140 (F2); 140–180 (F3); 180–220 (F4); 220–260 (F5); 260–300 (F6); 300–340 (F7), 340– 380 (F8) and 380–400 (F9). Fractions were freeze dried and weighed to calculate β-glucan recovery before their use in cell culture experiments (F3–5, F7 + F8) or NMR analysis (F4, F7 + 8). The lack of any salt in the eluent allowed this quantification of the β-glucan in the fractions by weighing only. Water was chosen as eluent in order to avoid an excessive salt load in the different fractions after freeze drying which would not make them suitable for the cell culture test systems. Furthermore, water as the mobile phase may increase the retention or binding of ionic impurity components to the column, while the elution of the non-charged β-glucan was assumed to be less affected.

Cell culture experiments (for details see below) were performed with fractions containing more than 4 mg β-glucan (F3–5) by dissolving 4 mg of the freeze dried material in cell culture medium. Two fractions containing no β-glucan (RI-trace back to baseline and very low weights of freeze dried fractions), F7 and F8, were tested in cell cultures by adding 4 mL of culture medium directly to the freeze dried tubes.

2.5. NMR

For NMR analysis, 0.5 mL 99.9% D_2O (Apollo Scientific LTD, Bredbury Stockport, UK) was added directly to freeze dried P-SEC fractions 7 and 8. Freeze dried P-SEC fraction 4 and the original 40 kDa cereal β-glucan were dissolved in 99.9% D_2O at a concentration of 3 mg/mL.

All NMR spectra were recorded at 25 °C on a BRUKER Avance 600 spectrometer equipped with 5 mm z-gradient CP-TCI cryoprobe, using TopSpin 2.6. The NMR data were processed and analyzed with TopSpin 3.0. The following spectra were recorded for the above mentioned samples: 1D proton with excitation sculpting to suppress the water resonance, 2D in-phase correlated spectroscopy (IP-COSY) (Xia et al., 2005), and 2D ^{1}H, ^{13}C heteronuclear single quantum coherence (HSQC).

2.6. MALDI–TOF

Enzymatic degradation of 40 and 359 kDa β-glucan solutions (5 mg/mL) was performed as described above. Borohydride reduced xyloglucan heptasaccharide (O-X3G4R, Megazyme) was added as internal standard after enzymatic degradation to a final concentration of 0.75 mg/mL in all samples. The MW of this internal standard was 1064.94 g/mol, which lies between the MW of DP6 and DP7 oligosaccharides. The internal standard was used for semi-quantitative comparison of different enzyme degraded β-glucan samples. Two microliter of a 9 mg/mL mixture of 2,5-dihydroxy-benzoic acid (DHB; Bruker Daltonics, Bremen, Germany) in 30% acetonitrile was applied to a MTP 384 target plate ground steel TF (Bruker Daltonics). One microliter sample was then mixed into the DHB droplet and dried under a stream of air. The samples were analyzed with an Ultraflex MALDI-tandem time-of-flight mass spectrometer with the LIFT module (Bruker Daltonics). The data were collected from averaging 250 laser shots, with the lowest laser energy necessary to obtain sufficient spectra intensity.

2.7. Cell culture experiments with β-glucan

HT-29 cells (Kindly donated by Professor Tor Lea, Norwegian University of Life Sciences) were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% heat inactivated fetal calf serum, 1% non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. All solutions were obtained from Invitrogen (Carlsbad, CA, USA). The cells were maintained at 37 °C with 5% CO_2 in a humidified incubator. For stimulation experiments freeze dried β-glucan samples (original preparations, enzyme treated samples and fractions obtained by P-SEC) were dissolved (cereal β-glucans) or suspended (yeast β-glucans) in culture medium at a concentration of 1 mg/mL. Cells were plated in 12 well plates at a concentration of 1.5×10^5 cells/mL and incubated until they were approximately 80% confluent (48 h). The medium was then exchanged by fresh culture medium containing the β-glucan samples or enzymes. Medium without β-glucan or enzymes was used as a control. Cells were incubated with the samples for 24 h and cell culture supernatants were stored frozen until assayed for IL-8 concentration by ELISA as previously described (Rieder et al., 2011).

2.8. Control for bacterial contaminants like endotoxins

IL-8 secretion by HT-29 cells stimulated with β-glucan samples in the presence or absence of the LPS inhibitor Polymyxin B sulfate (10 μg/mL, Sigma–Aldrich) was measured to assess the contribution of LPS in the stimulating effect as previously described (Rieder et al., 2011). The ability of very low concentrations (0.05–10 ng/mL) of LPS derived from two serologically different *Escherichia coli* strains (*E. coli* O111:B4 and *E. coli* O55:B5, both from Sigma–Aldrich) to stimulate IL-8 secretion by HT-29 cells was investigated to estimate the LPS sensitivity of this particular test system.

A commercial test kit based on the *limulus amoebocyte* lysate (LAL) test, where the specific recognition protein for β-glucan (factor G) has been removed (ToxinSensor™ Endotoxin Detection System, GenScript, Piscataway, NJ, USA) was used to determine the possible presence of LPS in some β-glucan preparations. Furthermore, TLR-4 and TLR-2 were blocked on HT-29 cells using azide-free anti-TLR-4 (5 μg/mL HTA 125, BD Bioscience, San Diego, CA) and anti-TLR-2 antibodies (5 μg/mL 11G7, BD Bioscience). The pretreatment with anti-TLR-2 and anti-TLR-4 antibodies was done 1 h before the cell culture medium was changed to medium containing β-glucan samples and TLR-antibodies.

2.9. Statistics

Analysis of variance of cell culture data sets was carried out in Minitab (Version 16) using a previously described general linear model (Rieder et al., 2011). Dunnett's test was used to compare the treatment effects with a control, while a Tukey test was used to compare treatment effects pairwise. Significant differences were set at $p < 0.01$.

3. Results

3.1. Enzyme treatment of cereal β-glucan samples

The cereal β-glucan preparations with 40 and 359 kDa M_w have been previously described to markedly (CGB40) or slightly (CBG359) stimulate IL-8 secretion by HT-29 cells (Rieder et al.,

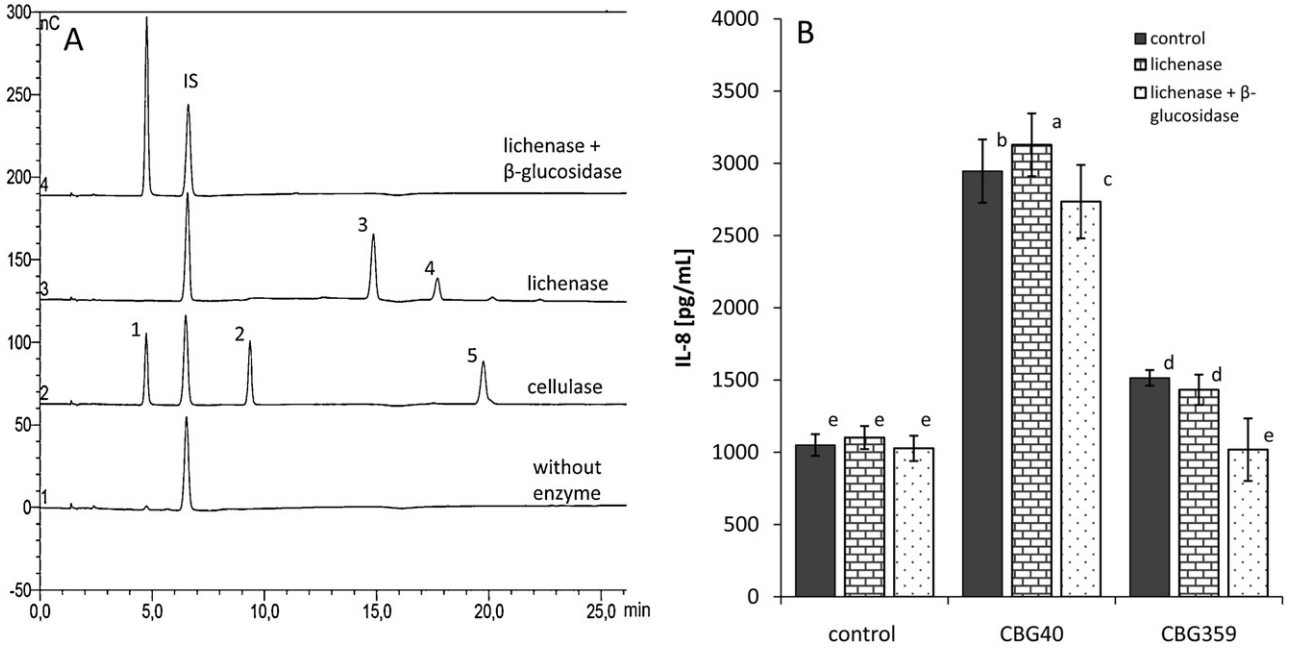

Fig. 1. Enzymatic degradation of cereal β-glucans CBG40 and CBG359. (A) HPAEC-PAD chromatograms of CBG359 treated with different enzymes; IS = internal standard, [1]glucose, [2]cellobiose, [3]G4G3G, [4]G4G4G3G, [5]G4G3G4G. (B) IL-8 concentrations in cell culture supernatants of HT-29 cells in response to treatment with lichenase and lichenase + β-glucosidase treated CBG40 and CBG359 or control medium with or without enzymes. Data shown are the mean ± SEM of 4 independent cell culture experiments each run in duplicate. Columns sharing a common letter are not significantly different (Tukey's pairwise comparison; $p < 0.01$).

2011). In the present study their carbohydrate specific effects were investigated by treatment with the cereal β-glucan specific enzyme lichenase. Lichenase specifically binds to the β-1,3 linkages in the cereal β-glucan backbone and cleaves the adjacent β-(1,4) linkage thereby producing two characteristic oligosaccharides G4G3G and G4G4G3G. These occur as two distinct peaks (at 14.8 and 17.9 min) in the HPAEC-PAD chromatogram of CBG359 (Fig. 1A) and CBG40 (data not shown) treated with lichenase. The lichenase treatment did not abolish the ability of CBG40 to induce IL-8 secretion (Fig. 1B). This indicates that the stimulatory property of the sample is not related to the macromolecular structure of the carbohydrates. Since G4G3G and G4G4G3G released during lichenase treatment may have an effect on IL-8 secretion alone these samples were further degraded to glucose by β-glucosidase. Glucose is a major part of the cell culture medium and the additional glucose released by β-glucosidase (corresponding to 1 mg/mL) will not affect IL-8 secretion. Fig. 1A shows the complete conversion of the two oligosaccharides G4G3G and G4G4G3G to glucose by β-glucosidase. Treatment of the β-glucan samples with both enzymes resulted in a 100% decrease of the small effect of the CBG359 sample on IL-8 secretion (Fig. 1B) but did not affect the ability of the CBG40 sample to stimulate IL-8 secretion to a large extent. Hence, the stimulatory effect of CBG 40 seems to be unrelated to the presence of G4G3G and G4G4G3G alone or as part of the macromolecule.

However, lichenase treatment of CBG40 released a lower amount of oligosaccharides with a lower ratio of G4G3G to G4G4G3G compared to treatment of CBG359 (Table 1). Further treatment by β-glucosidase increased the amount of β-glucan equivalents released as glucose or oligosaccharides for both samples, even though CBG40 still showed lower digestibility (Table 1). The use of cellulase in addition to lichenase and β-glucosidase resulted in a glucose release corresponding to 95% of the original β-glucan weight for the 40 kDa sample. Analysis of the oligosaccharides released by either lichenase or cellulase from the two cereal β-glucan samples by MALDI–TOF revealed the presence of oligosaccharides with higher degree of polymerization (DP) in the lichenase treated CBG40 sample (Fig. 2A). The use of a special oligosaccharide (borohydride reduced xyloglycan heptasaccharide) with MW between DP 6 and DP 7 (M_w = 1064.94) as an internal standard (0.75 mg/mL) in all 4 samples enabled a quantitative comparison of the two differently treated β-glucan samples. The oligosaccharides with higher DP, which were shown in the lichenase treated CBG40 sample, could not be observed after treatment with cellulase. CBG359 treated with either lichenase or cellulase did not contain higher DP oligosaccharides. The disappearance of these higher DP oligosaccharides after cellulase treatment points to a cellulosic structure. This is also coherent with the fact that cereal β-glucans may contain β-(1,4)-linked segments with DP 5–15 accounting for up to 10% of the mixed-linked β-glucan chains (Lazaridou & Biliaderis, 2007; Wood et al., 1994). It should be noted that the β-glucosidase used in the present study was unable to completely degrade these cellulosic fragments in the lichenase treated CBG40 sample as higher DP oligosaccharides were also observed in the lichenase and β-glucosidase treated sample by MALDI–TOF (data not shown). Hence, the ability of CBG40 degraded with both lichenase and β-glucosidase to increase IL-8 secretion may be due to the presence of a certain amount of longer cellulosic oligosaccharides in this particular sample. If these cellulosic oligosaccharides are indeed involved in the observed increased cytokine secretion, this effect should be abolished in cellulase treated samples, which no longer contain these oligosaccharides. The cellulase enzyme preparation, even though it was boiled and freeze dried, showed a destructive effect on the HT-29 cells, which was evident in a remarkable reduced IL-8 secretion. The enzyme preparation was therefore purified as described in materials and methods. Fig. 2B shows the effect of the two cereal β-glucans as such or after treatment with the purified cellulase on IL-8 secretion. It is evident that cellulase treatment did not considerably reduce the stimulatory ability of CBG40, while the small stimulatory effect of CBG359 was further decreased by cellulase treatment. The IL-8 secretion stimulating property of the CBG40 sample both before and after treatment with lichenase and β-glucosidase can therefore not be

Table 1
Degradation of cereal β-glucan preparations by different enzymes.

	Lichenase G4G3G/G4G4G3G (molar ratio)	β-Glucan equivalent (%)	Lichenase + β-glucosidase β-Glucan equivalent (%)	Lichenase + cellulase + β-glucosidase β-Glucan equivalent (%)
CBG40	1.78	63	78	95
CBG40 fraction 4	1.78	62		
CBG359	3.19	80	83	85

related to the presence of longer β-(1,4)-linked oligosaccharides in the enzyme treated samples or corresponding short β-(1,4)-linked structures in the original macromolecules.

3.2. Cereal β-glucan fractionation

Parallel to the use of different enzymes to investigate the carbohydrate specific effect of the cereal β-glucan samples, P-SEC was used in an attempt to isolate molecular populations with the highest effect on IL-8 secretion from CBG40. The presumably more narrow MW distributions of the obtained fractions should in combination with analysis of their stimulating effects and molecular structure reveal possible structure function relationships. Fig. 3A shows a chromatogram of a typical separation. Due to the absence of salts in the eluent, the β-glucan recovery could be determined by the weight of the freeze dried fractions. Recovery was above 90% and fractions 3–5 contained the main part of the β-glucan. Fractions 2 and 6 contained small amounts (but visible) freeze dried material with a weight less than 4 mg/tube, while fractions 1, 7 and 8 did not contain any visible material after freeze drying. Fig. 3B shows the effect of the different fractions on IL-8 secretion by HT-29 cells. It is evident that the IL-8 stimulating effects of the original CBG40 sample was remarkably reduced by P-SEC in all three β-glucan containing fractions 3–5. The composition of the β-glucan in the mid peak fraction (F4), which contained the most β-glucan, was analyzed by lichenase degradation and quantification of the specific released oligosaccharides. No difference between the original CBG40 sample and fraction 4 could be observed with this respect (Table 1). Equally to the enzyme treatment experiments, the P-SEC experiments also showed that the stimulatory effect of CBG40 is unrelated to the macromolecular structure of the main β-glucans in the preparation. Thus it was not possible to establish any structure–functional relationship regarding immune-stimulation by cereal β-glucans from the present data. Surprisingly, the non-β-glucan containing fractions 7 and 8 showed the highest stimulating effect of all the fractions on IL-8 secretion by HT-29 cells, comparable with the effect of the original CBG40 sample.

The original CBG40 sample, fraction 4 and a combination of fractions 7 and 8 were compared by NMR analysis after freeze drying and dissolution in D_2O. The obtained NMR data for fraction 4 confirm that the sample consists of highly pure β-glucan (result not shown). For fractions 7 + 8 the 1D NMR spectrum (Fig. 3C) shows all significant proton resonances in the region between approximately 0.7–5.5 ppm. This can be subdivided into three regions, where signals from region I (0.7–1.6 ppm) are normally associated with saturated protons and region II from 1.6 to 2.6 ppm represents protons attached to the C^{α} of carbonyl containing groups (e.g. in fatty acids). For region III from 3 to 5.5 ppm signals are typically related with protons from carbohydrates. Therefore, it is clear that

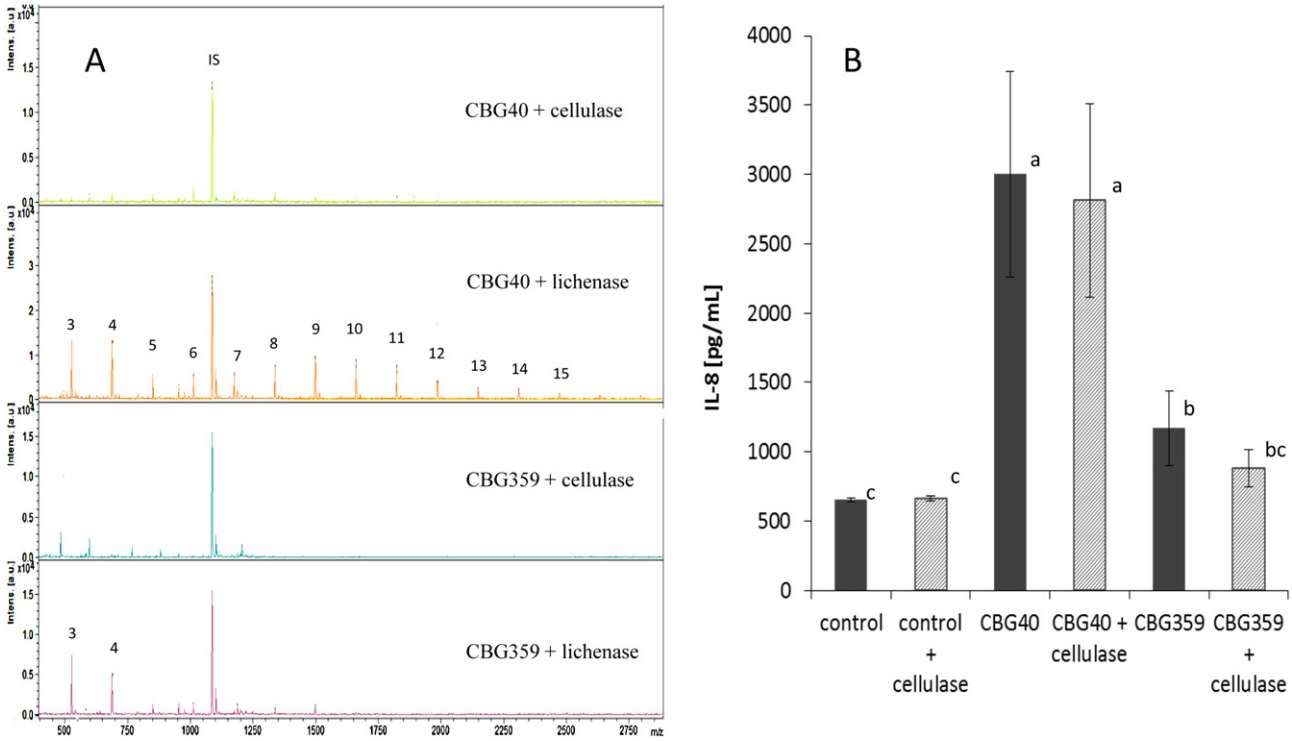

Fig. 2. Degradation of cereal β-glucans CBG40 and CBG359 by cellulase: (A) MALDI–TOF–MS spectra of cellulase and lichenase treated β-glucan samples; IS = internal standard, numbers indicate DP. (B) IL-8 concentrations in cell culture supernatants of HT-29 cells in response to treatment with cellulase degraded cereal β-glucan or control medium with or without enzymes. Data shown are the mean ± SEM of 3 independent cell culture experiments each run in duplicate. Columns sharing a common letter are not significantly different (Tukey's pairwise comparison; $p < 0.01$).

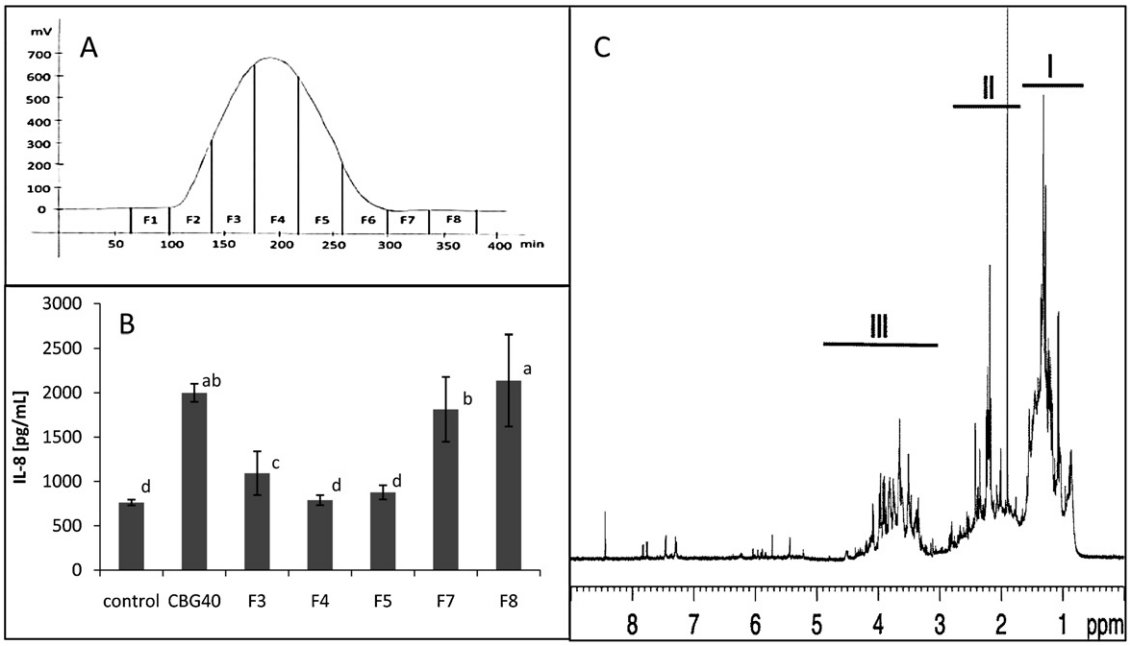

Fig. 3. P-SEC separation of CBG40 (A) P-SEC chromatogram of a typical separation of CBG40. (B) IL-8 concentrations in cell culture supernatants of HT-29 cells in response to treatment with P-SEC fractions 3–5, 7 and 8, original CBG40 cereal β-glucan or control medium. Data shown are the mean ± SEM of 3 independent experiments (each run in duplicate) based on 3 different P-SEC runs. Columns sharing a common letter are not significantly different (Tukey's pairwise comparison; $p < 0.01$). Part (C) 1D NMR spectrum of fractions 7 + 8 in 99.9% D_2O recorded with water suppression at 25 °C. Region I indicate proton signals associated with saturated protons and region II are characteristic of protons attached to the C^{α} of carbonyl containing groups like in fatty acids. Region III ppm are typically related to proton signals from carbohydrates.

the 1D NMR spectrum for fractions 7 + 8 shows the sample contains carbohydrates and fatty acids moieties, which is also in agreement with the obtained 2D ^{1}H, ^{13}C HSQC (result not shown). This strongly indicates that fractions 7 + 8 consist of LPS or LPS like compounds.

3.3. Control for bacterial contaminants like endotoxins

The LPS content of different β-glucan samples was analyzed by the LAL-test. The CBG40 sample clearly contained the highest amount of 4.6 ng LPS per mg β-glucan, followed by the 359 kDa sample with 0.25 ng/mg. Another cereal β-glucan sample of M_w = 123 kDa, which has not shown any effect on IL-8 secretion (Rieder et al., 2011), did not contain LPS amounts above the detection limit of 0.1 ng LPS per mg β-glucan. The P-SEC fraction 4, which likewise did not stimulate IL-8 secretion by HT-29 cells, also contained less than 0.1 ng LPS per mg β-glucan.

The detection of LPS in the CBG40 sample was a bit surprising, since earlier experiments showed no difference in induction of IL-8 secretion by this sample in the presence or absence of Polymyxin B (Rieder et al., 2011). However, following addition of Polymyxin B to the lichenase degraded CBG40 sample a clear reduction in the IL-8 secretion stimulating effect was obtained (Fig. 4). This finding

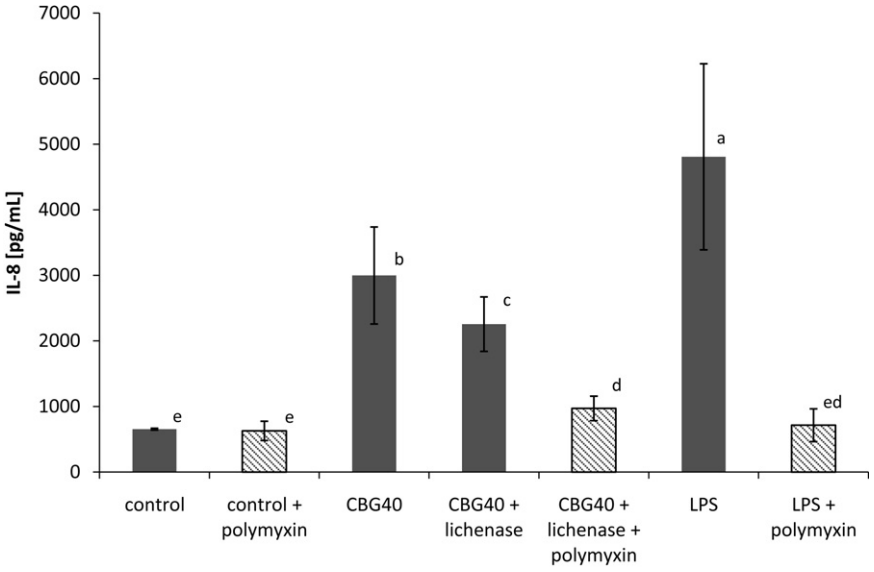

Fig. 4. IL-8 concentrations in cell culture supernatants of HT-29 cells in response to treatment with CBG40, lichenase digested CBG40 and LPS (100 ng/mL) with (striped bars) and without (filled bars) the addition of Polymyxin B (10 μg/mL) to the cell culture medium. Data shown are the mean ± SEM of 3 independent cell culture experiments each run in duplicate. Columns sharing a common letter are not significantly different (Tukey's pairwise comparison; $p < 0.01$).

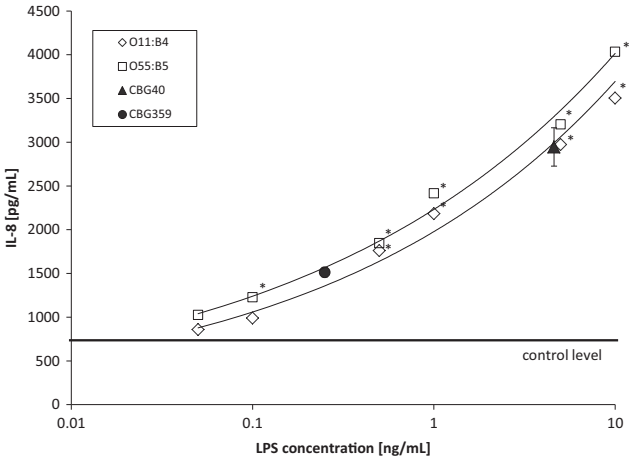

Fig. 5. IL-8 concentrations in cell culture supernatants of HT-29 cells in response to different concentration of LPS from *E. coli* serotypes O11:B4 and O55:B5. Average values for CBG40 and CBG359 (1 mg/mL) are plotted against their endotoxin content as determined by the limulus test and average basal IL-8 secretion is indicated by a black horizontal line. Data shown are the mean ± SEM of 3 independent cell culture experiments each run in duplicate. Significant differences from the control were analyzed with Dunnet's test at $p < 0.01$ and are indicated by an asterisk.

suggests that the LPS in the CBG40 sample may be inaccessible for the binding to Polymyxin B by e.g. forming aggregates with the β-glucan chains. Degradation by lichenase may have increased the accessibility of LPS for Polymyxin B binding e.g. by releasing LPS from β-glucan aggregates. One could hypothesize that the observed cellular effects are due to the LPS content in the CBG40 and CBG359 samples.

In order to estimate the LPS sensitivity of the employed test system, LPS from two serologically different *E. coli* strains (O111:B4 and O55:B5) were added to the cells in a concentration range of 0.05–10 ng/mL. Fig. 5 shows that equal amounts of LPS derived from *E. coli* O55:B5 or *E. coli* O111:B4 generally led to similar stimulation of IL-8 secretion. The stimulations were concentration dependent and followed power functions. Already very low concentrations of LPS of 0.1–0.5 ng/mL increased IL-8 secretion significantly (Fig. 5). Plotting the average values of IL-8 secretion by 1 mg/mL CBG40 and CBG359 against the measured content of LPS in these samples revealed that their effect on IL-8 secretion may be completely explained by their content of LPS.

Blocking of TLR-4 and TLR-2 by specific antibodies was tested as an alternative to Polymyxin B to test for the contribution of bacterial contaminants like LPS (binds to TLR-4) or peptidoglycan (binds to TLR-2) to the observed effects of various test samples. However, antibodies (5 μg/mL) did not inhibit the effect of 10 ng/mL LPS from *E. coli* O111:B4 (data not shown). Furthermore, the addition of TLR-4 and TLR-2 antibodies was unable to reduce the stimulatory effects of CBG40 and CBG359 on IL-8 secretion (data not shown).

3.4. Enzyme treatment of yeast β-glucan samples

Treatment of samples with specific enzymes was also applied to assess the carbohydrate specific effects of three yeast β-glucan preparations (Zymosan, MacroGard and soluble Wellmune). As yeast β-glucans are structurally distinct from cereal β-glucans a different array of enzymes had to be used. First, the effect of lyticase, which breaks down yeast β-glucan and solubilizes it (Danielson et al., 2010), on the ability of yeast β-glucan preparations to increase IL8-secretion was tested. HPAEC-PAD of the lyticase treated yeast β-glucans showed degradation to glucose, laminaribiose and two un-identified peaks for all three samples (data not shown). Due to the un-identified peaks no quantification of the degradation products was attempted. Fig. 6A shows a 57% reduction in the ability of MacroGard to induce IL-8 secretion after lyticase treatment, while Zymosan was less affected by lyticase degradation (11% reduction). In contrast to Zymosan and MacroGard Wellmune showed only a small increase in IL-8 secretion. Lyticase treatment of Wellmune returned the IL-8 secretion to control level. However, the control containing the boiled and freeze dried enzyme alone (control + lyticase) also showed a slight but non-significant reduction in IL-8 secretion when compared to cell culture medium (control). Lyticase is a relatively crude enzyme preparation and lyticase treatment might have resulted in degradation of other yeast cell wall components than β-glucan like e.g. mannans. To verify the results obtained with lyticase, yeast β-glucan samples were therefore also treated with a combination of 1,3-exo-β-D-glucanase and β-glucosidase. This treatment resulted in the release of glucose as the only sugar component observed by HPAEC-PAD (data not shown). For MacroGard and Zymosan the amount of liberated glucose (calculated as polymeric glucose) corresponded to approximately 45 and 42% of the original β-glucan preparation by weight, respectively. For soluble Wellmune the amount of liberated glucose was considerably higher (78%). This points toward a higher β-glucan content in the soluble Wellmune compared to Zymosan and MacroGard samples, respectively. However, it should be noted that the β-glucanase preparation contained low molecular weight components which decreased IL-8 secretion and had to be removed (as described for cellulose above) prior to treatment of samples intended for cell culture. The level of IL-8 secretion stimulation by different yeast β-glucans before and after treatment with β-glucanase and β-glucosidase is illustrated in Fig. 6B. The results obtained with β-glucanase and β-glucosidase correlate with the observations from the lyticase treatment. Again the ability of MacroGard β-glucan sample to increase IL-8 secretion was reduced by 56% after enzymatic treatment while the corresponding Zymosan sample only showed an 8% reduced ability to stimulate IL-8 secretion. Soluble Wellmune β-glucan did not show any ability to increase IL-8 secretion, which was not changed by enzymatic degradation. The reduction in IL-8 secretion by enzymatic degradation of MacroGard (both with lyticase and β-glucananse plus β-glucosidase) points toward an important role of the β-glucan fraction of MacroGard in the observed immune-modulating effect.

4. Discussion

The different approaches aimed at determining the particular structural component responsible for the observed increase in IL-8 secretion by the CBG40 sample all point toward an effect which is unrelated to the β-glucan present in the sample. CBG40 is a highly pure β-glucan preparation with a monosaccharide composition of solely glucose and protein/peptide levels below detection limit of 8 pg/mg (Rieder et al., 2011). Clearly, chemical analysis of the samples was insufficient to determine the active component as this study shows that trace amounts of LPS in the CBG40 sample can explain most of the observed stimulating effects. This was surprising as it was previously shown that Polymyxin B was unable to reduce the increase in IL-8 secretion by CBG40 (Rieder et al., 2011). Treatment of cell cultures with the LPS inhibitor Polymyxin B is commonly used to rule out LPS contamination (Bhattacharyya et al., 2008; Estrada et al., 1997; Han et al., 2003; Schepetkin & Quinn, 2006). Polymyxin B acts by binding to the lipid A part of LPS (Morrison & Jacobs, 1976), which is the bioactive component of the molecule and whose structure is highly conserved among gram negative bacteria (Lerouge & Vanderleyden, 2002) and can therefore block the action of different LPS types. However, our data suggests that Polymyxin B may be unable to inhibit the effect of LPS

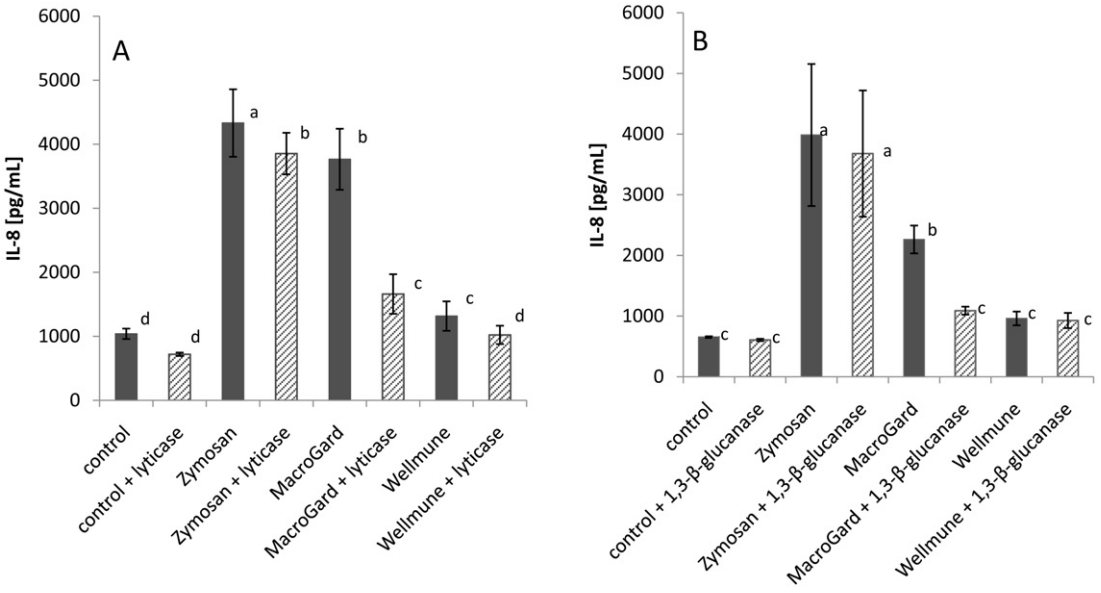

Fig. 6. IL-8 concentrations in cell culture supernatants of HT-29 cells in response to yeast β-glucan preparations Zymosan, MacroGard and soluble Wellmune. (A) Degradation by lyticase; (B) degradation by 1,3-β-glucanase and β-glucosidase. Data shown are the mean ± SEM of 3 independent cell culture experiments each run in duplicate. Columns sharing a common letter are not significantly different (Tukey's pairwise comparison; $p < 0.01$).

in certain polymeric samples like β-glucans. After lichenase degradation of the 40 kDa sample the stimulatory effect on IL-8 secretion could be suppressed by the addition of Polymyxin B. This suggests that LPS may be entrapped in the 40 kDa β-glucan sample making it inaccessible for Polymyxin binding. How this entrapped LPS were able to increase IL-8 secretion is not clear at present. Another possibility is the uptake of the β-glucan/LPS complex into the cells and the activation of cytokine secretion by intra-cellular released LPS. HT-29 cells have been shown to contain considerable amounts of TLR-4 protein in the cytoplasm, while cell surface expression of TLR-4 was low (Suzuki, Hisamatsu, & Podolsky, 2003).

The low level of cell surface expression of TLR-4 on HT-29 cells may also be the reason for the lack of response of these cells to blocking with anti-TLR-4 antibody. The murine anti-human TLR-4 antibody (HTA 125) used in this study has been previously described to reduce the effect of LPS on cytokine secretion in various other cell types (Akashi et al., 2000; Shimazu et al., 1999; Yonekawa et al., 2011). However, a complete reduction to the basal level has not been reported (Akashi et al., 2000; Shimazu et al., 1999; Yonekawa et al., 2011). TLR-4 blocking antibodies are therefore only of limited use for the investigation of the contribution of non-LPS constituents in samples with immune-stimulating properties.

Due to the specific test kit, where the factor G reacting with β-glucan had been removed, the LAL test could be applied to quantify LPS even in β-glucan samples. The samples showing stimulatory effects in the cell culture tests also contained low levels of endotoxin. Comparison of the effect of a sample with the effect of a LPS control of much higher concentration, as can be observed in the literature (e.g. Roubroeks, Skjåk-Bræk, Ryan, & Christensen, 2000), is of limited use as even small amounts of LPS can have a marked effect. Therefore, LPS concentrations corresponding to those found in the test samples have to be used in order to test if an observed effect may be induced by LPS in the sample. Several methods for the removal of LPS from biological samples have been described. The use of immobilized Polymyxin and TritonX-114 phase separation are two examples (Liu et al., 1997). We have now demonstrated that P-SEC with pure water as eluent can also be used for endotoxin removal from polymeric samples. However, all these methods are time consuming and require fully soluble polysaccharides, the complete removal of endotoxin is not always possible and the test samples may contain other active components that can confound the results.

In this study we have used different enzyme preparations to investigate the carbohydrate specific immune modulating effects of different β-glucan samples. The main advantage of this approach is that it specifically explores the components of interest, in this case β-glucans, instead of excluding the potential contribution of other components like LPS or peptidoglycan. Enzymes highly specific for the component of interest are required since degradation of other components by the enzymes can confound the results. The enzyme preparations used in this study were specific for the β-glucosidic linkages in the β-glucan chains. The polysaccharide parts (O-polysaccharide) of LPS consist of unique sugars, which are rarely found elsewhere (Lerouge & Vanderleyden, 2002), and a degradation of LPS by the enzymes used in this study was unlikely. Both 1,3-β-glucanase and cellulase showed no effect on LPS activity (data not shown).

The use of enzymes to degrade samples intended for cell cultures can be challenging as the enzyme preparations may decrease cytokine secretion by themselves even after heat inactivation. We have shown that removal of low MW constituents from the enzyme preparations could reduce this negative effect on the HT-29 cells and thereby improve the usefulness of the enzyme preparations in determining carbohydrate specific effects. In order to demonstrate the specific immune stimulating effect of a certain components (e.g. β-glucan) it is essential that the enzymatic degradation products of this component do not have any immune-stimulating effect by themselves and that the degradation of the components can be quantitatively monitored. Taking these precautions into account, the use of specific enzymes is a powerful tool to investigate specific biological effects of polymeric test samples. Targeted enzymatic degradation as an alternative to the sole use of Polymyxin B, which seems to be insufficient to rule out LPS contamination of polymeric samples, has the additional advantage of not only excluding the effect of contaminants but being able to relate an effect to a defined structural component of interest.

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