Rupturing fungal cell walls for higher yield of polysaccharides: acid treatment of the basidiomycete prior to extraction

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Abstract

The fungal cell wall of *Agaricus bisporus* powder was degraded by ethanol-acid treatment in order to improve the yield of the hot water extractions. Polysaccharides from multiple hot water extractions of treated and untreated mushroom were precipitated with ethanol and characterised separately. The treatment and the sequenced extractions changed the anomeric compositions, the molecular weights, and the sugar contents of the extracted polysaccharides. The total yield of the first extraction of treated *A. bisporus* increased by 46% with over 10 percentage points higher glucan content compared to untreated batch. Bioactivities were decreasing within the extraction batches and after the treatment. This was found to be connected to the amount of polysaccharides and the content of mannitol in the precipitates.

Keywords: Beta-glucans, Fungal cell wall, Pretreatment, Extraction, Polysaccharide, Mannitol

1. Introduction

Polysaccharides fulfill many different roles in living organisms, like structural, cell communication and energy storage. Humans have been always using polysaccharide resources, like plants, for energy and food. Extensive research granted novel purposes and applications to these biopolymers in many different fields, like pharmaceuticals, biomaterials and tissue engineering, food ingredients, viscosity modifiers, and nutraceuticals (Oliveira and Reis, 2011, Liu et al., 2015). For these applications they posses several desirable properties, such as non-toxicity, biodegradability and -compatibility, and low cost. Due to high and versatile demand, commonly utilised sources for polysaccharides are readily abundant biomasses, such as wood (cellulose, cellulose derivatives, hemicelluloses; Gatenholm and Tenkanen (2003)), crops (dietary fibers, cellulose, starch, glucans; Fuentes-Zaragoza et al. (2010), Charlton et al. (2012)), algae and lichens (fucoidans, laminars, carrageenans, ulvans; Wijesekara et al. (2011)). Many of these resources have proven to contain polysaccharides with multiple bioactivities, for example immunostimulatory, anti-tumour, free radical scavenging, antioxidativity, and anti-inflammatory activities (Kim and Li, 2011, Harlev et al., 2012, Jin et al., 2013).

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Traditionally, many of these polysaccharides are extracted simply by hot water. In the past decades, more feasible extraction technologies have been developed in order to harness plants for polysaccharide extraction and to boost the efficiency, such as microwave and ultrasonic-assisted extractions (Normand et al., 2014, Chao et al., 2013). These technologies require minimal sample preparations and lower time and energy consumption, and still increase the yield. However, Cheng et al. (2013) found out in their study that the antioxidant activity of the plant hot water extract was stronger than polysaccharides from other extractions technologies. It is shown that the secondary structure, e.g. the triple helical structure of the polysaccharides, is connected to its bioactivity (Surenjav et al., 2006) and it is possible that these technologies destroy that structure. Nevertheless, many applications are based on the characteristics of the primary structure and the subunits of the polysaccharides (Rees and Welsh, 1977), so their applicability does not depend on the secondary structure.

Also pretreatment methods, such as enzymatic hydrolysis, improve the yield and purity, while reduce the use of solvents (Puri et al., 2012). They open up the cell walls, allowing the diffusion of the desired polysaccharides out from the biomass. The drawbacks of enzymatic treatments are the necessity of tailoring for each biomass separately, very limited work conditions, and high scale-up costs.

Another way to open up the cell walls is to use chemical hydrolysis. Chemical hydrolysis is non-specific for the cell wall components, only guided by the activation energy of the hydrolysing bonds. Acid hydrolysis is usually done with a concentrated acid at low temperature or with a diluted acid at high temperature (Adel et al., 2010). It has been shown, however, that aqueous acid may hydrolyse different polysaccharides unequally (Sundberg et al., 1996). Also, if polysaccharides are hydrolysed to smaller units, the increase in solubility might reduce hydrolysis efficiency. Hydrolysis in alcoholic medium would prevent the solubilisation of degraded polysaccharides before the extraction.

Fungal cell wall is generally accepted to be structured in three layers: an outermost layer of glucans and polysaccharides bounded to glycoproteins; a middle layer of glucans; an inner layer of glucans and covalently bounded chitin (Chen and Cheung, 2014). These layers are usually not extractable only with one single solvent. Sequential extractions with, for example, hot water, cold alkali, and hot alkali have been used for extracting polysaccharides from fungal sources. Especially the third layer is considered difficult to extract, which presents a major challenge since it covers the major part of the cell wall.

Trygg and Fardim (2011) have demonstrated how even short treatment time with acid in alcoholic medium at relatively low temperature can rupture the primary cell wall of cellulosic fibres and change their dissolution mechanism. Disruption made possible for the solvent to penetrate the cell wall and enhance the dissolution of the otherwise resilient fibres. Lin et al. (2009) showed that this treatment punctures small holes in the primary cell wall of the cotton fibres.

In our current work, we applied the ethanol-acid treatment to fungal cell walls of *Agaricus bisporus* (Button mushroom). Our objective was to increase the yield of the polysaccharides with traditional hot water extractions and to study how the treatment influences the structure and the biological activities of the extracted polysaccharides.

2. Materials and methods

2.1. Agaricus bisporus - sample preparation

Ripe batch of commercial fruiting bodies from *Agaricus bisporus* was provided by Mykora Ltd. The batch was cut to **<**1 cm slices, frozen at -40°C, freeze-dried with an industrial freeze-dryer, and stored in sealed vacuum containers at -20°C until further use. Dried slices were ground with a Grindomix GM 300 knife mill (Retsch GmbH, Germany) for 1 min at 1000 rpm. Ground powder was sieved with a mesh 20. Approximately 24 w-% of the powder passed the mesh and the median diameter of these particles was measured with a RODOS dispenser and a HELOS particle size analyser. Median diameter was 150-180 µm. 76 w-% of the particles did not pass the mesh and their average diameter was measured with Fiji image analysis software (Schindelin et al., 2012). Median diameter was 1400-1600 µm with the largest population at 1200 µm. Dry content of the mushroom powder was **>**97%.

2.2. Ethanol-acid treatment

Ground *A. bisporus* powder was refluxed in round-bottom flask with preheated ethanol (Altia PLC, technical grade, 92.5 w-%) and hydrochloric acid (37%, Merck KGaA, Germany) for 1 h at 75 ±1°C. Consistency was ~10% and the ratio of ethanol and hydrochloric acid 20:1. (Trygg and Fardim, 2011)

After the treatment the mixture was cooled down in an ice-water bath and neutralised with sodium bicarbonate. Solid fraction was separated by centrifuging at 14334 g (9000 rpm) for 15 min with Avanti J-26S XP centrifuge and JA-10 rotor. Solids were separated from the supernatant by decantation and filtration. The treated mushroom was stored at 4°C for further use. The supernatant was concentrated with rotavapor and freeze-dried. Its composition and bioactive properties were studied together with other extracted polysaccharide samples.

2.3. Hot water extractions

Approximately 75-80 g (dry mass) of treated and untreated mushroom were washed thrice with 900 cm^3 of technical ethanol for 6 h to remove alcohol soluble substances, such as lipids. Ethanol was removed by centrifugation (see section 2.2). After the ethanol washes, polysaccharides were extracted by refluxing with 750 cm^3 of boiling water for 6 h. Again, both mushrooms were extracted thrice and the supernatants were separated from the solid residues by centrifugation and stored at 4°C until all the extractions were completed, and then polysaccharides were precipitated by adding technical ethanol dropwise into the containers while stirring. Precipitation was allowed to continue overnight at 4°C. Afterwards, polysaccharides were filtered, rinsed with acetone and left to dry at room temperature, stored in a desiccator for a couple of days, and weighed. The precipitates were labelled as AB1-3 (untreated *A.bisporus*) and tAB1-3 (treated *A.bisporus*) according to the extraction cycle. Additionally, the first hot water extract of untreated mushroom precipitated after the supernatant had cooled down and stored at 4°C for two days, before the ethanol precipitation. This precipitate was separated by decantation and labelled as AB1p.

2.4. Analytical methods

2.4.1. Sugar content of the hot water extracts

After the hot water extractions the sugar content of the extraction liquid was monitored by phenol-sulfuric acid method on a 96-well microplate (Dubois et al., 1951, Masuko et al., 2005). Briefly, 400 mm^3 of diluted samples were mixed with 1.2 cm^3 of concentrated sulfuric acid (Sigma-Aldrich, 95-97%) and 240 mm^3 of 5% phenol solution, and incubated for 5 min at 90°C. Absorbance was read at 490 nm and glucose (Merck KGaA, Germany) was used for constructing the standard curve.

2.4.2. Molar mass distribution

Approximately 10 mg of dry precipitates were dissolved in 5 cm^3 of 100 mM $NaNO_3$, sonicated, and filtered with 0.45 µm RC microfilters. 50 mm^3 were injected into Alliance 2690 HPLC separation module (Waters corporation, Massachusetts, USA) with 30_X 7.5 cm GMPW TSKgel-column (Tosoh Bioscience, Tokyo, Japan) connected to RID-20A refractive index detector (Shimadzu, Kyoto, Japan). The temperatures of the samples, column, and RI-detector were set to 40°C and the flow rate of the mobile phase 100 mM $NaNO_3$ was 0.5 cm^3 min^{-1}. Molar masses of the polysaccharides were correlated with the retention times of the commercial pullulan standards from 342 to 708000 Da (PSS Polymer Standards Service GmbH, Mainz, Germany). The peak areas of the pullulans and samples were integrated with time intervals of 0.1 min with Origin (OriginLab, Northampton, MA) and used for molecular weight calculations (M_w and M_n) and relative concentrations of the different molecular weight populations. (Rasmussen and Meyer, 2010)

2.4.3. α- and β-glucan content

Total, α- and β-glucan content was determined with commercial Mushroom and Yeast β-glucan Assay Procedure K-YBGL 02/2016 (Megazyme Int.). Briefly, total glucan content was measured by hydrolysing the polysaccharides with sulphuric acid and enzymatically breaking the remaining oligomers to glucose units. Glucose was then further reacted with glucose determination reagent (GOPOD; glucose oxidase, peroxidase, and 4-aminoantipyrine) for colourimetric analysis. Absorbance values were correlated with glucose standard solutions and the results were translated to mass percentages from the total weights of the precipitates. α-Glucans were measured using the same colourimetric analysis after alkaline extraction and enzyme hydrolysis. The amount of β-glucans were computed by reducing the α-glucans from the total glucans. The method was validated by measuring the mentioned values for yeast β-glucan standard provided in the analysis kit. All measurements were done in duplicate and they fit in the given 5% deviation of the procedure.

2.4.4. Sugar composition of polysaccharides

Approximately 10 mg of precipitates were hydrolysed with 2 M trifluoroacetic acid (≥99%, Merck KGaA, Germany) for 6 h at 100°C. After the hydrolysis, samples were dried under nitrogen flow at 40°C overnight, dissolved in 1 cm^3 of MQ-water, and filtrated with 0.45 µm RC microfilter. Then 100 mm^3 of each sample was mixed with 30 mm^3 of the internal standard solution (myo-inositol) and dried under nitrogen flow at 60°C. Samples were stored in desiccator until silylation with Tri-Sil (ThermoFisher Scientific) for 1 h at 60°C.

1 mm^3 of silylated samples were injected to Shimadzu GC-2010 via a split injector (210°C, split ratio 15) into SPB-1 fuesed silica capillary column(30 m × 0.25 mm × 0.25 µm; Supelco, USA). Column temperature was stabilised for 2 min at 150°C, then heated 4°C min^{-1} to 210°C, followed by 40°C min^{-1} to 275°C where it was kept for 5 min. Carrier gas was helium and the total flow 33.4 cm^3 min^{-1}. The temperature of the flame ionization detector was 290°C.

Chromatogram peaks were identified by comparing the retention times with those of the peaks of the standard sugar solution containing exact amounts of arabinose (Ara), rhamnose (Rha), galactose (Gal), glucose (Glu), mannose (Man), xylose (Xyl), L-fucose (Fuc), and mannitol (ManOH) (all from Merck KGaA). Peak areas were integrated with LabSolutions -software (version 5.71, Shimadzu) and correlation factors were calculated for each sugar. Relative composition was computed for each sample. Samples were prepared and analysed in triplicate.

2.4.5. FTIR

Fourier transform-infrared (FTIR) spectra of the precipitates were collected with a Bruker Vertex 70 FTIR spectrometer equipped with a Harrick VideoMVP™ diamond attenuated total reflectance (ATR) accessory. Spectra were recorded at the range of 5000-450 cm^{-1} with a resolution of 2 cm^{-1}. Samples were subjected to 32 scans and air was excluded as a background reference.

2.4.6. Phenolic content

The total amount of phenolic compounds was measured according to Folin-Ciocalteu method (Magalhães et al., 2010). Briefly, ~10 mg of precipitates were weighed accurately and dissolved in 10 cm^3 of distilled water and sonicated overnight. After filtering with a 0.45 µm RC microfilter, 100 mm^3 of samples were pipetted in triplicate into a microplate followed by 100 mm^3 of commercial Folin-Ciocalteu's phenol reagent (Merck KGaA) and 150 mm^3 of prepared 350 mM NaOH. Absorbance at 760 nm was measured after 20 min at room temperature. Gallic acid (Sigma, ≥98.5%) was used to prepare the standard curve. The phenolic content was calculated as gallic acid equivalents.

2.4.7. Bioactivity of the polysaccharides

Oxygen radical absorbance capacity (ORAC) was measured according to a method applied previously with modifications Prior et al. (2003). ~50 mg of precipitates were dissolved in 5 cm^3 of MQ-water by vigorously vortexing and sonicating them for 30 min. Undissolved fragments were removed by centrifugation and filtering with 0.45 µm Minisart-microfilters. The maximum value was found by preparing a dilution series for each sample. 20 mm^3 of diluted solutions were pipetted into microplate wells with 100 mm^3 of fluorescein solution and 60 mm^3 of phosphate buffer solution. Reaction was started by adding 70 mm^3 of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH, 108 mg cm^{-3}; Aldrich 97%). Excitation wavelength was 485 nm and fluorescence at 535 nm were recorded for 40 min at 37°C. Trolox (Aldrich 97%) was used as a reference and results are expressed as Trolox equivalents, TE µmol g^{-1} of sample, and also relative to the amount of each sample, TE µmol µmol^{-1} of sample, calculated from the number averaged molecular weights and glucan content (see sections 2.4.2 and 2.4.3).

Free radical scavenging abilities for 1,1-diphenyl-2-picrylhydrazyl (DPPH; Aldrich) radicals was measured with 10 mg $cm^{-}3$ crude extract solutions according to micronised version of the method from Kozarski et al. (2011). 200 mm^3 of solution was mixed in microplate with 100 mm^3 of 0.2 mM DPPH reagent in dimethyl sulfoxide (DMSO) and absorbance was measured after 35 min at 517 nm. Absorbance of the reagent blank A_j (extract+DMSO) was reduced from the reading A_i (extract+DPPH) and the difference was related to the blank A_c (DMSO+DPPH), according to the equation

$$\text{DPPH scavenging ability (\%)} = 1 - \frac{A_i - A_j}{A_c} \times 100. \quad (1)$$

Gallic and ascorbic acids were used as a reference.

2.4.8. Statistical analysis

In Figure 1 and Tables 3 and 4, statistical significance ($p<0.05$) between samples was tested with One-way ANOVA with Tukey and LSD post hoc tests, and the difference was indicated by different letters. In Table 1, the statistical difference between samples was tested with t-test and marked with different symbols. Analysis was performed with SPSS 25.0 software (SPSS Inc, Chicago, IL).

3. Results and discussion

3.1. Yield of extract and content of polysaccharides

Dried and powdered *Agaricus bisporus* was extracted thrice with hot water, with and without ethanol-acid treatment. A notable precipitation was observed after the first extraction with hot water in the sample without the treatment, when it cooled to 4°C (see section 2.3).

Total sugar content of the extraction liquids was measured by phenol-sulfuric acid method and results were multiplied by their volume for the total sugar yield (Fig 1: green adjacent bars). AB2 had a similar value as AB1 and AB1p, but the amount of sugars and polysaccharides in AB3 clearly decreased compared to earlier extractions. The amount of sugars and polysaccharides in the extraction liquids of treated mushroom were higher than from untreated mushroom, especially in the first hot water extract of the treated mushroom tAB1. Some of the sugars in extraction liquids probably did not precipitate with ethanol due to their low molecular weight, which explains the higher values of the sugars in the liquid compared to glucans in precipitates of AB3 and tAB1-3. Also, it is a worth of noticing that phenol-sulfuric acid method measures all the sugars, not only glucose (glucans), which should be between 46-77% from all sugars (Table 3).

After the extractions the extracts were precipitated in ethanol and precipitates were dried and stored in a vacuum desiccator. Total yield for AB (AB1+AB1p+AB2+AB3) was 9.6 and for tAB (tAB1+tAB2+tAB3) 12.4 g per 100 g of dry mushroom. However, it is notable that the tAB yielded more with one hot water extraction than AB yielded with three extractions. This can be partially due to the swollen state of the cell wall after the ethanol-acid treatment, whereas mushroom powder was dry prior to the extraction of AB. On the other hand, extraction time was six hours, which should eliminate the effect of the pre-swelling in the ethanol-acid treated mushrooms. More likely, the rupturing of the cell wall improved the solvent access and extractability of the polysaccharides, as we speculated in the Introduction.

3.2. Macromolecular properties of polysaccharides

Anomeric configurations. FTIR spectroscopy was used to analyse the structure of the extracted polysaccharides and the spectra were normalised according to the highest intensity, i.e. ~1020 cm^{-1}, for a comparison. Characteristic strong OH stretching of hydroxyl groups and bound water was observed in the spectra of AB1-3 and tAB1-3 at the range 2500-3500 cm^{-1} (Fig 2). The peak of OH stretching in AB1 was clearly shifted towards lower energy to 3215 cm^{-1} compared to others samples at 3283-3325 cm^{-1} (Fig 3; right axis△). The shift derives from the stronger hydrogen bonding network (Lee et al., 2015), which probably was caused by the homogeneity of the glucans, as AB1 was found out to be virtually free from α-glucans (Table 1). Consequently, when the α-glucan content was even 4% (AB3), the shift was not observed any longer. Two symmetric and one asymmetric stretching of CH_2 group were observed at 2855, 2887, and 2925 cm^{-1}, respectively.

tAB-precipitates had notably weaker vibrations than AB-precipitates in the region of amide and amine vibrations, i.e. at 1639, 1523, 1398, and 1236 cm^{-1} (Mohaček-Grošev et al., 2001). The ethanol-acid treatment could have wash out and hydrolysed some of the glycoproteins and chitin residues. tAB-precipitates were observed to be more pure in their total glucan content containing less phenolic compounds (see Tables 1 and 4).

The stretchings C-O-C and C-O of the glucopyranose ring are visible at the range 1000-1200 cm^{-1}. AB1 has a clearly different profile than other precipitates, probably due to negligible α-glucan content (see Table 1 and Fig 2). Also the peak of the fingerprint area at ~1014 cm^{-1} shifted towards higher energy when α-glucan content increased, so that the main peak was actually at 1033 cm^{-1} in AB1 (supplementary material).

However, better correlation with the anomeric configurations of the glucans was found from the region between 750-900 cm^{-1} (Fig 2: insert). (Šandula et al., 1999) The intensity of the peak at 765 cm^{-1} indicated relatively well the ratio between α- and β-glucans (Fig 3; left axes●). Similar tendencies were found with β/α-ratio and the peak at 889 cm^{-1} but less satisfactory (supplementary material).

Fig. 1. Precipitate masses (white bars), total glucans (red inner bar), and the sugar content of the extracts prior to precipitation (green adjacent bar). Total glucans and sugar content were measured with enzymatic kit and phenol-sulfuric acid method, respectively. Significant differences ($p<0.05$) in One-way ANOVA tests in sugar content are marked as a-d. Statistical comparison between precipitate masses was not performed due to the single measurement of the sample. Statistical comparison between glucan content of the extracts is presented in Table 1.

Due to the low yield of tAB3, it was not possible to measure the glucan composition with Megazyme kit, but the position of OH stretching at 3290 cm^{-1} indicates the presence of α-glucans and the absorbance at 765 cm^{-1} refers to similar ratios of α- and β-glucans as in samples AB3, tAB1, and tAB2 (Fig 3), whereas the peaks at 889 and the shift of 1014 cm^{-1} suggest even slightly lower β-glucan content.

It is thought that α- and β-glucans are not evenly distributed in the fungal cells (Kozarski et al., 2011, Novak and Vetvicka, 2008). α-glucans are said to be intracellular glucans, whereas β-glucans are immersed in the cell wall as structural components. In this light, it is intuitive to speculate that the rupturing of the cell wall by hydrolysis would increase the relative content of the β-glucans. Also, considering the higher α-glucan content of AB1p and AB2 compared to AB3, it indicates that α-glucans were depleted after two hot water extractions. In tAB samples similar conclusions could not be made due to insufficient data, but as far as it can be speculated from the FTIR spectra, both α- and β-glucans were extracted at the same levels in tAB3 as in tAB1 and tAB2. Ruptured cell walls would support this model of more constant ratio of different glucans leaching out during the extractions.

Molecular weight distribution. The precipitate AB1p separated from the supernatant of AB1 when the hot water extract was stored at 4°C prior to ethanol precipitation. Polysaccharides with molecular weight *log* $M_p \geq 5$ were detected in AB1p and AB2-3, but not in AB1 (Fig 4:top). It is well-known that high molecular weight glucans are less soluble than low molecular weight ones, so the early precipitation could be explained by the higher concentration of polysaccharides in AB1p compared to AB2-3 (Fig 1) and the fact that 82% of its polysaccharides were high molecular weight polysaccharides (Table 2), whereas in AB2 and AB3 the percentage was only 47% and 32%. Also, it is known that linear α-glucans in mushrooms (Synytsya and Novak, 2014) are more likely to form aggregates than branched

Fig. 2. FTIR spectra of the precipitates from AB1 and AB1p (untreated *A.bisporus*), and tAB1 (treated *A.bisporus*). In the insert, vertical bars indicate the wavenumbers 889 and 765 cm^{-1}.

glucans (Magee et al., 2015). Since these high molecular weight polysaccharides precipitated out from the extraction liquid to form their own AB1p fraction, they were absent in the precipitate and chromatogram of AB1.

Polysaccharides with *log* $M_p \leq 3.5$ were present only in AB1 and AB1p chromatograms (Fig 4). These polysaccharides are oligomers with $\leq$20 glucose units and they are so soluble that they were mostly leached out during the first extraction. Similar results was measured in tAB-samples (Fig 4:bottom). Polysaccharides with *log* $M_p \leq 4.1$ were diminishing stepwise along the extractions. Relatively, the amount of polysaccharides with *log* $M_p \approx 5.3$ were increasing while smaller were decreasing.

Ethanol-acid treatment had a significant effect on the molecular weight distribution. The largest polysaccharides, i.e. *log* $M_p \approx 5.8$-5.9 and 5.5 in AB-precipitates, were degraded and they formed new populations at *log* $M_p \approx 5.2$-5.5 and 4.6-4.8 in tAB-precipitates. It is also noteworthy that the lack of high molecular weight glucans correlated with the absence and the low amounts of α-glucans in the samples.

3.3. Sugar composition

The relative amount of glucose increased in the hydrolysates from AB1 to AB3 and from tAB1 to tAB3 similarly as the total glucan amounts, although the increment was not statistically significant (Tables 3 and 1). The purity of the glucans tends to increase when the solvent gets access to the inner layers of the cell wall; most of the other sugars are in the outermost layer, which is in direct contact with the capillary water between the cells (Paudel et al., 2016).

The precipitate from the neutralised treatment liquor consisted of 1.4% glucans and its hydrolysate was composed of 93.7% mannitol and 3.9% glucose. The role of the mannitol in fungi is diverse (Patel and Williamson, 2016),

Fig. 3. α/β-Glucan ratios (•, left black axis), as measured with enzymatic kit, FTIR absorbances at 765 cm^{-1} (□, left red axis) of extract precipitates. On the right axis, the FTIR wavenumber peak of the OH stretching at 3150-3350 cm^{-1} (△) in the FTIR spectra. Lines are drawn to highlight changes.

e.g. it protects plants and fungi from reactive oxygen and osmotic stress, so a simple explanation for its existence and location in the cell cannot be stated here. However, we observed that AB1 had a notable amount (11.19%) of mannitol compared to other extracts (Table 3) and the precipitate of neutralised ethanol-acid liquor consisted mainly of mannitol. Lower amount in AB1p indicates that mannitol is not bound to AB1p polysaccharides, but it precipitated together with β-glucans of AB1. Difference between the amounts of mannitol in AB1 and tAB1 as well as in AB1 and AB1p was statistically significant.

The amount of arabinose and rhamnose remained almost constant in each sample, regardless the ethanol-acid treatment or the number of the extraction. This may indicate that these sugars cover the whole cell wall evenly, or at least they are evenly bound to outcoming glucans. On contrary, galactose, mannose, and fucose decreased in each batch along with the extractions, which indicates that they have a decreasing gradient when moving inwards the cell wall layers or the difference may arise from the relative increment of glucose. Then again, only decrease of fucose was statistically significant.

It has been shown that the biosynthesis of the galactomannan takes places before the crosslinking to the cell wall β-glucans (Engel et al., 2012), which may explain their higher amount in the outer cell wall layers. Also, statistically the amounts were higher in untreated extracts than in treated extracts.

The amount of xylose increased notably only in AB3 extract compared to earlier extractions of the untreated sample. On contrary, tAB1 had the highest amount of xylose and after that the amount decrease significantly. It could be speculated that the xylose is embedded in the whole cell wall, and leached out all at once from the ruptured cell

Fig. 4. HPSEC-RID chromatograms of sequential extracts from untreated (top; AB1-3, AB1p) and treated (bottom; tAB1-3) *A. bisporus*. The analytical system was calibrated using pullulan standards from 342 to 708000 Da. Molecular mass values, based on the calibration curve and expressed as $\log(M_p)$, are reported on the top y-axis.

Table 1: α- and β-glucan compositions as w-%[1].

	α	β	Total
AB1	0.3 ±0.0*	7.0 ±0.0*	7.2 ±0.0*
AB1p	12.0 ±0.4˙	6.8 ±0.4*	18.8 ±0.1˙
AB2	14.4 ±0.5°	4.9 ±1.0*	19.3 ±0.5˙
AB3	4.4 ±0.1†	15.8 ±4.4˙	20.2 ±1.2˙
tAB1	7.9 ±0.1ɣ	23.0 ±0.8°	30.9 ±0.8°
tAB2	9.0 ±0.0¤	27.2 ±0.3°	36.2 ±0.3°
EtOH-HCl[2]	0.2 ±0.0*	1.2 ±0.1†	1.4 ±0.3†

[1]Means ± standard deviation; significant differences between samples in the t-test ($p < 0.05$) are marked with different symbols.
[2]Precipitate from the neutralised ethanol-acid solution.

Table 2: Weight averaged-molecular weights (M_w) of polysaccharides, as computed from the integrated areas at certain time range and the peak values (time t_p, molecular weight M_p). Division expresses the relative amount of population from the total population.

	time range / min	*log* M_w	Division / %	t_p / min	*log* M_p
AB1	14.1-16.7	4.2	100	15.8	4.1
AB1p	10.0-14.2	5.9	82	11.9	5.9
				13.1	5.5
	14.2-16.7	4.3	18	15.7	4.2
AB2	10.1-14.0	5.9	47	12.3	5.8
	13.9-17.3	4.4	53	15.9	4.1
AB3	10.2-13.9	5.8	32	13.1	5.5
	13.9-17.3	4.4	68	15.7	4.2
tAB1	11.2-14.0	5.4	22	13.3	5.4
	14.0-17.6	4.4	78	15.9	4.1
tAB2	10.5-17.9	5.3	100	13.3	5.3
			100	14.9	4.6
tAB3	10.5-17.2	5.2	100	13.5	5.3
			100	14.5	4.8

walls of tAB1 but the relative amount of xylose in untreated samples increased when other components, mainly glucose, started to decrease. Also, the glycosidic bond between the xylose and the backbone could have been hydrolysed during the ethanol-acid treatment, which would explain the high amount of xylose in tAB1.

3.4. Antioxidative activity of crude polysaccharides

The maximum ORAC values were found by diluting sample solutions to different concentrations and choosing one dilution factor for parallel measurements (for all samples, n=10-12). Clearly, the highest ORAC value within the samples was found with AB1, and the values were decreasing rapidly within the AB-batch along the extractions (Table 4). In the tAB-batch the values remained practically the same throughout the samples, except the precipitate from the neutralised ethanol-acid liquor, i.e. mannitol (see section 3.3), which had the highest measured ORAC value. The amount of mannitol in one gram of this precipitate is very high, which explains the high ORAC values. When compering the ORAC values relative to the quantity of substance, assuming that the polysaccharides in the precipitate are consisting purely of mannitol, its ORAC values are similar to tAB-samples. The ORAC value of mannitol given in the literature is 51% higher than the one of glucose, which correlates well with our results (Nakajima et al., 2013).

When the ORAC values were related to the total glucans (Table 1) and the number averaged molecular weights, ORAC values became more comparable (Table 4). AB1 still has the highest value but the difference with AB1p is smaller. Other samples, however, demonstrated much lower capacity to absorb oxygen radicals.

Table 3: Relative composition (%) of major sugars in hydrolysates[1].

	AB1	AB1p	AB2	AB3	tAB1	tAB2	tAB3
Total sugars[2] / g	1.33±0.03^{a,b}	1.33±0.00^{a,b}	1.45±0.20^{a}	0.99±0.03^{b}	10.22±0.25^{c}	2.58±0.04^{d}	1.43±0.05^{a}
Arabinose	2.36±0.29^{a}	1.87±0.36^{a}	2.52±0.50^{a}	4.35±2.07^{a}	2.44±0.30^{a}	2.28±0.05^{a}	2.85±0.02^{a}
Galactose	21.28±1.22^{a}	16.94±2.24^{b}	18.56±1.06^{a,b}	19.52±1.00^{a,b}	12.51±1.46^{c}	12.34±0.24^{c}	9.29±0.05^{c}
Glucose	52.12±3.46^{a}	64.41±1.67^{b}	63.67±0.30^{b,c}	56.15±6.78^{a,c}	74.10±1.25^{d}	76.97±0.61^{d}	79.96±0.56^{d}
Mannose	8.50±0.99^{a}	8.34±1.14^{a,b}	8.18±0.97^{a,c}	9.25±2.28^{a}	3.61±0.51^{d}	6.81±0.74^{a}	5.30±0.57^{b,c,d}
Rhamnose	n.d.	n.d.	1.11±0.05^{a}	0.69±0.39^{b}	0.20±0.07^{c}	0.08±0.01^{c}	0.18±0.01^{c}
Xylose	1.98±0.92^{a}	3.00±0.53^{a}	3.31±0.19^{a}	8.03±3.91^{b}	5.82±0.18^{a,b,c}	0.91±0.03^{a,d}	2.00±0.03^{a}
Mannitol	11.19±5.38^{a}	3.33±1.45^{b}	1.21±0.08^{b}	0.63±0.46^{b}	0.34±0.03^{b}	0.12±0.01^{b}	0.11±0.01^{b}
Fucose	2.58±0.51^{a}	2.11±0.24^{a}	1.46±0.11^{b}	1.38±0.12^{b}	0.99±0.10^{b,c}	0.49±0.03^{c}	0.32±0.01^{c}

[1]Means ± standard deviation; significant differences ($p<0.05$) in One-way ANOVA between samples are marked as a-d.
[2]Measured with the phenol-sulfuric acid method (Section 3.1 and Figure 1)

The value of tAB3 could not be computed due to missing total glucan amount. However, if we speculate that the amount is similar to tAB2, as indicated by FTIR (see section 3.2), value would be 3-4 1±µmol TE/µmol of sample.

ORAC values of diluted samples were plotted against their concentrations and curves were fitted to the polynomial functions with QtiPlot software (version 0.9.8.9). The dilution factors of maximum ORAC values followed inversely the total glucan content (see Tables 1 and 4), meaning that the total glucan content positively correlates with the oxygen radical absorbance capacity of the samples, and not only β-glucans or phenolic compounds.

DPPH radical scavenging capacity increased within the batches, being the highest on the AB3 and tAB3. The scavenging capacity of the tAB1 and the precipitate from the neutralised ethanol-acid liquor was so low that they could not be measured reliably. Since the DPPH values were inversely related to the phenolic content of the precipitates (Table 4), excluding AB1p, the reason for these interpretations could be explained with an inhibition of polysaccharide radical scavenging activity by phenolic compounds. Refining the results with total glucan content and the number averaged molecular weights did not provide any new insights to the results.

Table 4: ORAC-values, DPPH and phenolic content of the AB- and tAB-samples[1].

	ORAC / TE[2]	ORAC / mol[3]	Dilution factor	DPPH / %	Phenolics / mg g^{-1}
AB1	150±9^{a}	24±2	14.7	12.8±8.2^{a}	5.3
AB1p	89±7^{b}	21±2	7.2	15.7±3.2^{a}	12.9
AB2	78±4^{c}	8±1	5.9	39.9±3.5^{b}	4.7
AB3	42±5^{d}	4±1	5.0	60.5±1.4^{c}	1.8
tAB1	39±2^{d}	2±1	5.6	n.d.[5]	4.8
tAB2	37±4^{d}	3±1	5.3	10.8±1.4^{d}	2.9
tAB3	35±4^{e}	-	4.4	65.3±1.5^{c}	0.8
EtOH-HCl[4]	276±14^{e}	4±1	4.4	n.d.[5]	5.6

[1]Means ± standard deviation; significant differences ($p<0.05$) in One-way ANOVA test between samples are marked as a-e.
[2]µmol Trolox equivalents per gram of sample [3]µmol Trolox equivalent per µmol of sample
[4]Precipitate from the neutralised ethanol-acid solution [5]No detectable activity

3.5. Discussion

According to the review from Kalač (2013) the carbohydrate content of *Agaricus bisporus* is between 50.9-74.0%. Besides glucans, this includes also insoluble fibres, heterosaccharides, and chitin. Tian et al. (2012) extracted polysaccharides from *A.bisporus* with hot water (HW), microwave-assisted (MA) and ultrasonic-assisted (UA) extractions, and after purification the yield of polysaccharides was 2.36%, 4.71%, and 6.02%, respectively. Alzorqi et al. (2017)

extracted *Ganoderma lucidum* with HW, soxhlet (SE) and UA, with the yield of the latter being 8.09%, similar then to *A.bisporus*. From three different extraction techniques, SE gave the highest yield (after 16 h of extraction) and HW the highest carbohydrate content, but the glucans from UA were the most active biologically. The difference in biological activity was explained by the higher β-glucan content of the UA extracted fraction and the higher branching degree of such polysaccharides. This was speculated to originate from so-called micro-jets and hot-spots, that are created by the ultrasound treatment. Cavitation effects assist the diffusion of the dissolved substances through the cell walls and the cell wall destruction due to thermal and mechanical effects. (Vinatoru, 2001)

In our study, the yields of crude extracts AB and tAB from the first extractions were 3.2% and 10.1%, respectively. Out of these crude extracts, 7.2% and 30.9% were glucans (Table 1), which computes to 0.23 and 3.12 g (as seen in Fig 1). All together, the three sequential extractions of treated and untreated *A. Bisporus* produced nearly 1.5 g and 3.7 g of glucans, respectively. The increment in the yield of pure glucans by acidic ethanol treatment in our study was approximately 246%, which is quite similar as the increment 255% between HW and UA reported by Tian. Also, in the latter work, the average molecular weight of the polysaccharides was five times lower than ours, 158 kDa compared to 790 kDa. Even though the purification passages adopted by those authors are different from ours, it is clear that the rupturing of the cell wall plays an important role when aiming to increase the yield of polysaccharide extraction from mushrooms. All these techniques are possible to implement in industrial scale.

High yield of polysaccharide extraction is desired when we aim at new glucan-based applications and products. In our opinion, by combination of different extraction techniques and possibly pretreatments and processings, it could be possible to gain the maximum yield.

4. Conclusions

Agaricus bisporus was treated with ethanol-acid solution at elevated temperature for one hour. The treatment ruptured the fungal cell wall which increased the total yield by 29% and the yield of the first hot water extraction by 46%. The yield of first hot water extraction of the treated mushroom was higher than the total yield of untreated mushroom. Purely considering the energy and time consumption of the extractions from industrial perspective, the treatment could be used to improve the efficiency of the extraction processes. The rupturing of the cell walls makes the inner and less accessible cell wall layers more accessible for the extractive solvents.

The ethanol-acid treatment hydrolysed glucans so that the largest populations of the polysaccharides were diminished and the molecular weight of the polysaccharides shifted towards lower values. However, the crude extracts of the treated mushroom were richer in glucans compared to extracts from untreated mushroom and their β-glucan anomers were more abundant. On the downside, bioactivities, mainly the radical-scavenging capacity of tAB fractions, decreased when compared with the extracts from the untreated mushrooms as well as within the extraction batches. Our results indicate, however, that this might be connected to the relative concentrations of mannitol, rather than molecular weight or anomeric compositions of the glucans. AB1 had the highest proportion of mannitol and also oligomeric polysaccharides. Mannitol, common multifunctional compound in mushrooms, was mainly removed during the treatment and was found in precipitate from the neutralised ethanol-acid solution.

Acknowledgements

The research was part of the project "Innovative Technologies and Concepts for Business Growth Based on Finnish Mushrooms" (3135/31/2015), funded by Tekes (the Finnish Funding Agency for Innovation) and companies from Finland and Germany. We would like to acknowledge Nature Lyotech ltd for freeze-drying the mushroom and M.Sc. Anna Puganen for ORAC and DPPH measurements. We are also thankful for PhD Pia Damlin and the department of Materials Chemistry, University of Turku, for the use of FTIR spectrometer and for the laboratory of Pharmaceutical Sciences, Åbo Akademi, for the use of HELOS particle size analyser.

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