SUPPLEMENTARY MATERIAL

Anti-bacterial and anti-oxidant properties of Mixed-Linkage Beta-Oligosaccharides from extracted β-glucan hydrolyzed by *Penicillium occitanis* EG_L lichenase

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Abstract

The aim of the present study was first to ascertain the chemical composition and the physico-chemical properties of cereal extracted β-glucan from barley flour. Secondly, to assess the antioxidant properties and the antibacterial properties of extracted β-glucan hydrolysates. The proximate composition, FT-IR and Scanning electron microscopic of extracted β-Glucan were studied. Hydrolysates from extracted β-glucan, obtained by lichenase EG_L from *Penicillium occitanis,* were a mixed linkage beta-oligosaccharides (MLBO) of trisaccharides and tetrasaccharides. MLBO showed a DPPH radical-scavenger with IC50 about 1.8±0.01 mg/ml whereas the IC50 of extracted β-glucan was about 5±0.01 mg/ml. MLBO showed a high antioxidative capacity 175 µmol/ml α-tocopherol equivalents at 5 mg/ml. The antimicrobial activity was confirmed against all tested bacteria especially at 20 mg/ml of MLBO while no inhibition was observed for all the strains used after the addition of either EG_L or extracted β-glucan.

Key words: extracted β-glucan, β-glucan hydrolysates, DPPH radical-scavenging, total antioxidant capacity, antibacterial activity

Experimental

Samples, Reagents and Enzymes

Barley flour (125 µm) was used for the extraction of β-glucan fraction. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich, Inc (St. Louis, MO, USA).

Alcalase, Amyloglucosidase (300L), Termamyl (120L) were purchased from Novozymes (Danemark). Lichenase EG_L from *P. occitanis* was purified as described by Chaari et al. (2014).

Extraction of β-glucan fraction from Barley flour

β-glucan fraction was sequentially extracted using the method of Papagergiou et al (2005) with slight modifications: Barley flour refluxed with 80% (v/v) ethanol at a ratio of flour/ethanol solution of 1:10, for 2 h at 90 °C. The supernatant was removed by vacuum filtration and the residue was dried at 40 °C overnight. The pretreated barley was suspended in distilled water (1:10 w/w flour to water ratio), and heated at 50 °C for 2 h with continuous stirring. The insoluble residue was removed by centrifugation (3000×g, 10 min). 0.04 % (v/v) thermostable alpha-amylase (Termamyl 120L) was added to the supernatant (at 90 °C, 3 h, pH 4.5). Following hydrolysis, the suspension was cooled and sodium azide was added (0.02% w/v); the pH was adjusted to 4.0 (2M HCl) and held at 25°C overnight. The suspension was centrifuged and the pH of the clear viscous supernatant raised to 7.0 with 2 M NaOH. Two volumes of 80% (v/v) ethanol were added to the supernatant holding it overnight at 4 °C. The fibrous precipitate was collected by filtration under vacuum. The residue was washed exhaustively with isopropanol and acetone several times. The residues obtained was dried in oven at 40 °C and stored at room temperature for subsequent physicochemical analyses.

Chemical analysis

Moisture, ash and fat contents were analyzed in triplicate according to AACC methods (1990). The mineral constituents were analyzed separately, using an atomic absorption spectrophotometer (Hitachi Z6100, Tokyo, Japan). Protein content was determined by the Kjeldahl method as described by Pearson (1970). Protein was calculated using the general factor (5.7). Cellulose content was determined as described by Updegraff (1969). Starch was determined according to the enzymatic method using α-amylase (Termamyl 120L) and amyloglucosidase (AMG 300L). Starch percentage was calculated as the amount of glucose released after hydrolysis (Ellouze-Ghorbel, 1996). Insoluble and soluble dietary fibres (DF) were determined according to the AOAC enzymatic-gravimetric method of Prosky et al. (1988). Briefly, the samples were gelatinized with heat-stable alpha amylase (Termamyl 120L) (100 °C, pH 4.5,15 min) and then enzymatically digested with protease (Alcalase) (60°C, pH 7.5, 30 min), followed by incubation with amyloglucosidase (AMG 300L) (60 °C, pH 4.5, 30 min) to remove protein and starch. Then, the samples were filtered, washed (with water, 95% ethanol and acetone), dried and weighed to determine insoluble fibre. Four volumes of 95% ethanol were added to the filtrate and to the water washings. Then, the precipitates were filtered and washed with 78% ethanol, 95% ethanol and acetone. After that, the residues (soluble DF) were dried and

weighed. The obtained values were corrected for ash and protein. Total DF was determined by summing insoluble DF and soluble DF.

Infrared spectroscopy

The Fourier-transform infrared (FT-IR) spectroscopy was used for extracted β-glucan from barley flour. The spectra were recorded with a Perkin Elmer Spectrum One FT-IR instrument (Perkin Elmer Ltd, Bucos, England) in the range of 400 $cm^{-1}$to 4000 cm^{-1} using the KBr pellets method.

Scanning electron microscopy (SEM)

The surface morphology of extracted β-glucan was observed using SEM. The dried samples were ground and then coated with gold under by Ion Sputter (Bio-Rad SC-500, Hercules, CA). The specimens obtained were viewed under an accelerating voltage of 20 kV in a scanning electron microscope (SEM) Philips XL 30 (Philips, Leimeil-Brevannes, France).

Production of β-glucan oligosaccharides using P. occitanis lichenase

The extracted β-glucan was soaked in citrate-phosphate buffer pH 3 at 50°C for 30 min to yield a suspension with 1% concentration (w/v). Lichenase EG_L of *P. occitanis* at 20 U / g substrate was added into a reactor containing β-glucan suspension, and the reactor was maintained in a thermostatic water bath at 50°C for 16 h. The resulting hydrolysate was then incubated in a boiling water bath for 10 min and then centrifuged. The supernatant containing the sugars was concentrated by evaporation.

Thin Layer Chromatography analysis

The sugars in the hydrolysis were analyzed by Thin layer chromatography analysis (TLC) which was carried out with a solvent system of Chloroform/acetic acid/water (6:7:1 by volume) using percolated silica gel plates (Merck). After developing the products, the sugar spots were visualized by spraying ethanol (95%) and H_2SO_4 (5%) and then drying the plates in an oven at 105 °C for 10 min.

Antioxidant activity

DPPH radical-scavenging assay

DPPH radical-scavenging activity of the hydrolysates was determined as described by Bersuder et al., (1998). A volume of 500 µl of each sample at different concentrations was mixed with 500 µl of 99.5% ethanol and 125 µl of 0.02% DPPH in 99.5% ethanol. The mixture was then kept at room temperature in the dark for 60 min, and the reduction of DPPH radical was measured at 517 nm using a UV–Visible spectrophotometer. The DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical} - \text{scavenging activity } (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} * 100$$

The control was conducted in the same manner, except that distilled water was used instead of sample. A lower absorbance of the reaction mixture indicated a higher DPPH radical scavenging activity. Butylated hydroxyanisole (BHA) was used as a standard. The test was carried out in triplicate.

Total antioxidant capacity

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH (Prieto et al., 1999). An aliquot of 0.1 ml of sample solution at different concentrations was combined in an Eppendorf tube with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated at 90 °C for 90 min. After that, each sample was allowed to cool to room temperature and the absorbance was measured at 695 nm against a control. The control solution consists of 1 ml of reagent solution and 0.1 ml distilled water. The activity of the samples was expressed as α-tocopherol equivalent using the following linear equation:

$A = 0.011C + 0.0049$ $R^2 = 0.987$;

where A is the absorbance at 695 nm and C the concentration as α-tocopherol equivalent (μmol/ml). The values are presented as the means of triplicate analyses.

Antibacterial activity

The antibacterial activity of the barley flour extracted β-glucan hydrolysates at different concentrations against Gram positive bacteria including *B. thuringiensis (*ATCC 2663), *B. subtilis* (ATCC6633) *and Actinomycete sp*. and Gram negative bacteria involving *E. coli* (ATCC 8739), *E.faecalis* (ATCC 51299), *K. pneumoniae* (ATCC 13883), *P. aeruginosa (*ATCC49189*)* and *S. typhimurium* (ATCC14028) was determined using the disc diffusion method. Cell suspension (10^7 cfu/ml) was added onto agar plates before paper discs (diameter of 5.0 mm) containing test solutions were placed on plates. The bacterial resistance of samples was examined for a zone of inhibition of the disk after incubation at 37 °C for 24 h. The total diameter of the inhibition zone was measured.

Figure captions

Figure S1: FT-IR spectrum of extracted and commercial β-glucan

Figure S2: Scanning electron microscopy of extracted β-glucan (A) and commercial β-glucan (B)

Figure S3: TLC analysis of extracted β-glucan hydrolyzed by EG_L (1) and standard mixture of sugars ranging from DP 1 to DP 4 (G1 to G4) (2).

Figure S4: DPPH radical-scavenging activity (A) and total antioxidant activity (B) of BHA, MLBO, extracted β-glucan and EGL at different concentrations.

Figure S5: Some examples of anti-bacterial activity of Ampicillin: **1**, EG_L (10 mg/ml): **2**, EG_L (20 mg/ml): **3**, extracted β-glucan (10 mg/ml): **4**, extracted β-glucan (20 mg/ml): **5**, MLBO (10 mg/ml): **6**, MLBO (20 mg/ml) : **7**.

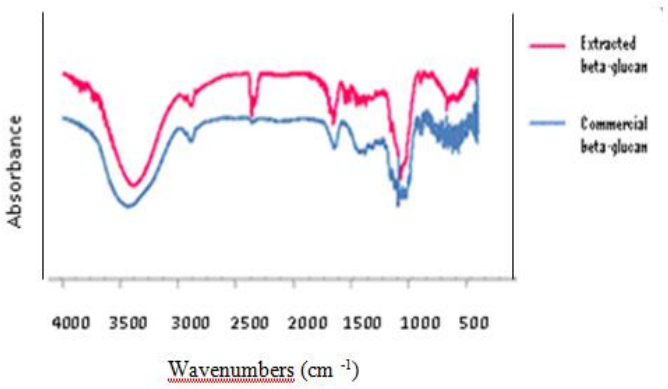
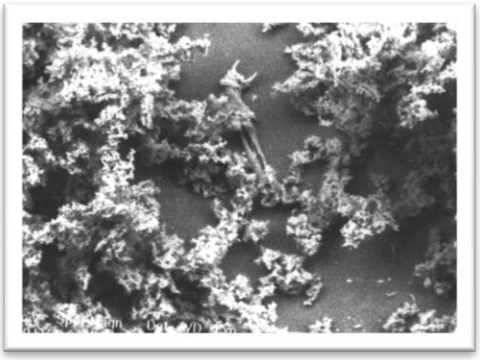
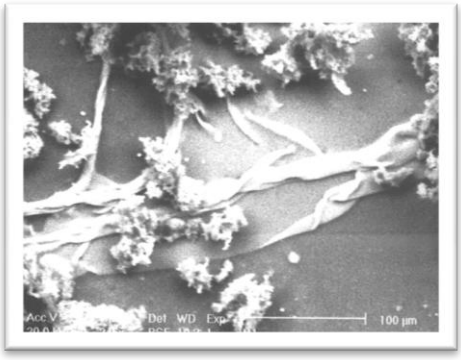


Figure S1: FT-IR spectrum of extracted and commercial β-glucan

A  B 

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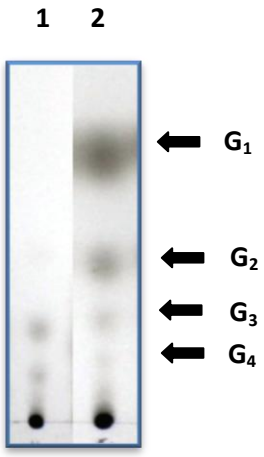
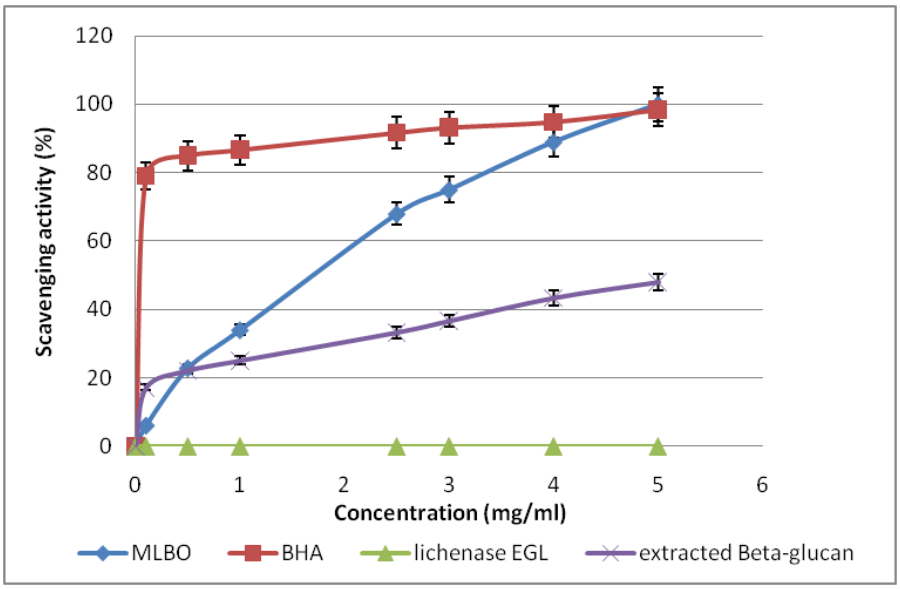


Figure S 3: TLC analysis of extracted β-glucan hydrolyzed by EG_L (1) and standard mixture of sugars ranging from DP 1 to DP 4 (G1 to G4) (2).

A



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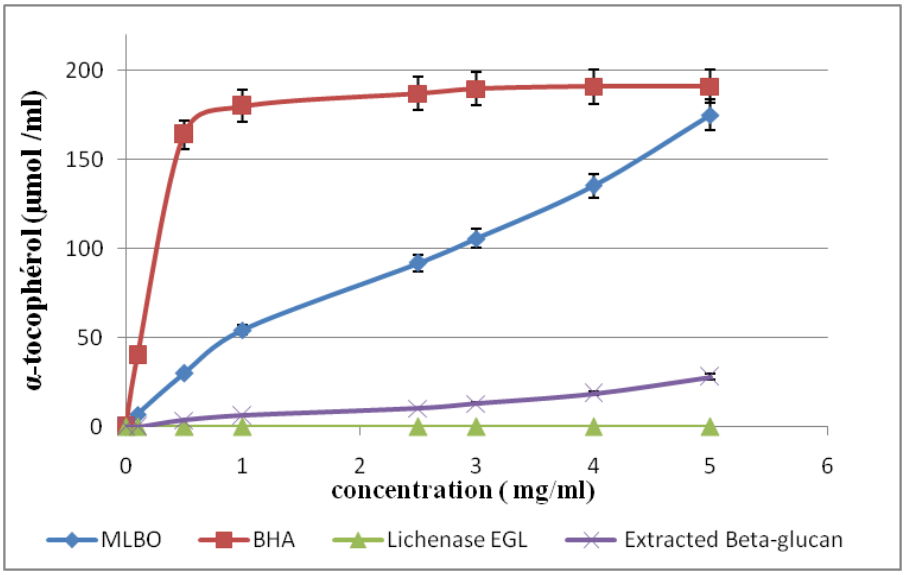

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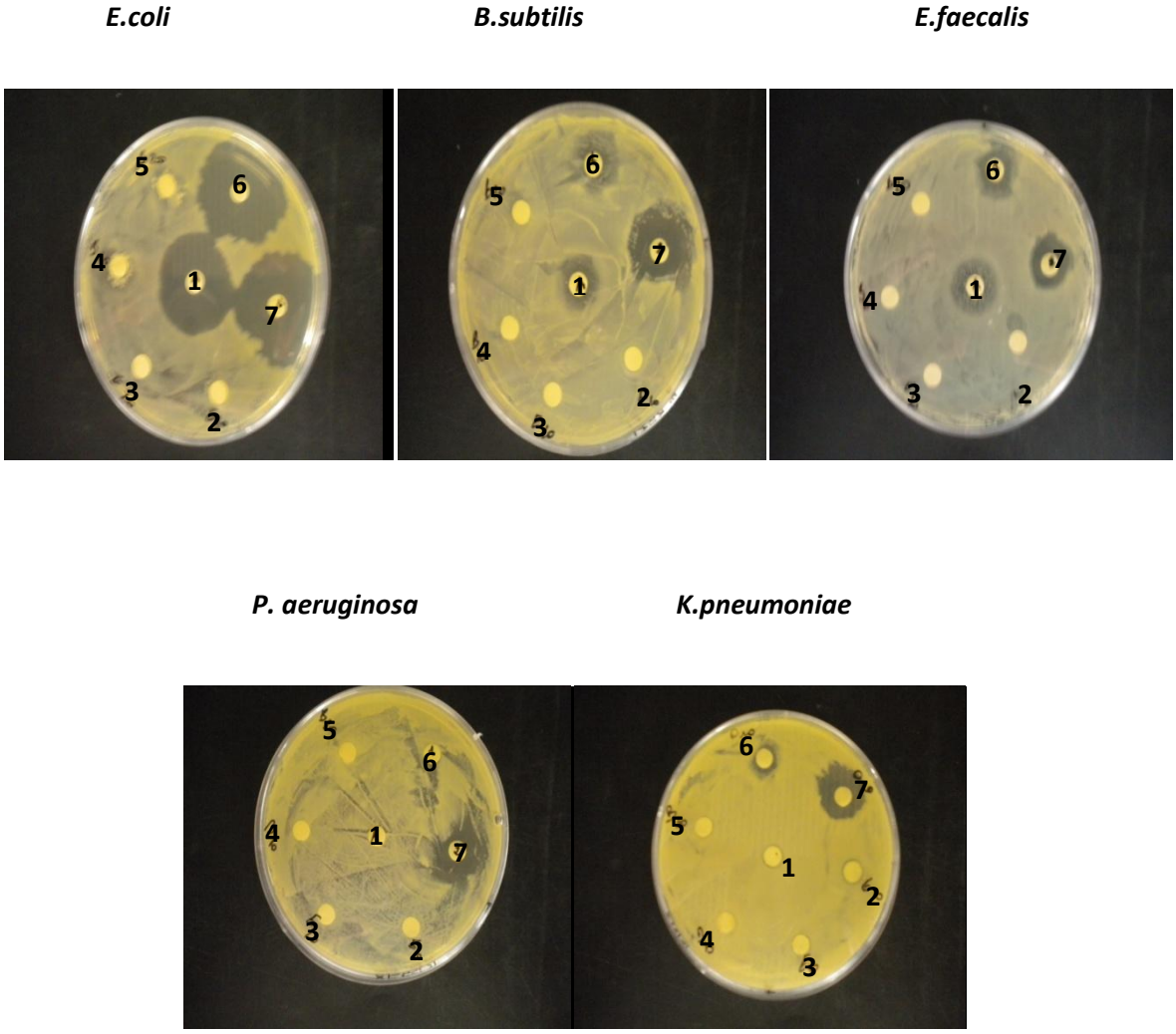

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Table S1: Chemical composition of barley flour and extracted β-glucan (g/100g of dry weight).

	Barley flour	Extracted β-glucan
Moisture	10.26±0.08	6.55±0.016
Ash	1.23±0.05	10.41±0.04
Protein	8.73±0.1	5.04±0.13
Fat	1.59±0.013	0.036±0.008
Starch	51.46±1.8	15.19±0.33
Cellulose	0.4±0.01	0
Total DF	33.75±0.7	72
Insoluble DF	30.84±1.1	0
Soluble DF	2.91± 0.3	72±3.1

Table S2: Diameters (mm) of inhibition zones determined after 24 h incubation at 37 ◦C.

	Strains	Ampicillin	MLBO		Lichenase EGL	Extracted β-glucan
			10mg/ml	20mg/ml		
Gram+	***B. thuringiensis***	20	Nd	nd	Nd	Nd
	B. subtilis	12	16	24	Nd	Nd
	Actinomycete sp.	13	Nd	10	Nd	Nd
Gram-	***E.faecalis***	14	12	16	Nd	Nd
	E.coli	26	34	40	Nd	Nd
	K. pneumoniae	Nd	6	14	Nd	Nd
	P. aeruginosa	Nd	Nd	10	Nd	Nd
	S. typhimirium	10	Nd	27,5	Nd	Nd