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Structural characterization of polysaccharides isolated from grape stalks of *Vitis vinifera* L.

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ABSTRACT

The main structural polysaccharides of grape stalks are cellulose, heteroxylan, and glucan. Cellulose contributes 30.3% of grape stalk matter and has an unusually high degree of the crystallinity (75.4%). Among hemicelluloses, xylan was the most abundant one, contributing ~12% to the weight. The heteroxylan was isolated from the corresponding peracetic holocellulose by DMSO extraction followed by precipitation in ethanol. The M_w of heteroxylan (19.0 kDa) was assessed by size exclusion chromatography (SEC) and the structure was inferred by methanolysis and methylation linkage analysis, as well as 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. The heteroxylan is a partially acetylated (DS = 0.49) glucuronoxylan possessing the main backbone composed by β-(1→4)-linked D-xylopyranosyl units ramified with α-(1→2)-linked 4-*O*-methyl-α-D-glucuronosyl residues (MeGlc*p*A) at a molar ratio 25:1. The isolated heteroxylan contained concomitant β-glucan (ca. 15%), whose structure was elucidated by methylation linkage analysis and by NMR spectroscopy. The results obtained revealed mixed β-(1→3; 1→4)-D-glucan with a molar ratio of β-(1→3)- to β-(1→4)-linked glucopyranosyl units of 1:2.

1. Introduction

Grape stalks are a large byproduct of the wine sector and are mainly composed of cellulose, hemicelluloses, tannins, and lignin.[1–3] Previous studies have shown that polysaccharides constitute more than 50% of grape stalk components representing an economically attractive source of fiber material for energy crops (renewable fuels) or papermaking/biocomposites.[1–3] However, only limited knowledge is available concerning structural features of polysaccharides from grape stalks.

Hemicelluloses are non-cellulosic polysaccharides surrounding the cellulose fibers and can be divided into four groups of structurally different polysaccharide types: the xylans, the mannans, the xyloglucans, and the mixed linkage β-glucans. The hemicelluloses together with cellulose have a support function in the cell wall.[4] The hemicelluloses are mainly heteropolysaccharides that are composed by repeating units of pentoses (D-xylose and L-arabinose), hexoses (mainly D-galactose, D-glucose and D-mannose), and uronic acids.[5] In particular, glucuronoxylans are the major hemicelluloses class in angiosperms, representing 15–30% of total dry weight and they are widely found in plant species, especially in woods.[5,6] *O*-Acetyl-4-*O*-methylglucurono-β-D-xylan, usually designated as glucuronoxylan, consists of the main backbone composed by β-(1→4)-linked β-D-xylopyranose units (β-D-Xyl*p*) partially substituted at *O*-2 by the 4-*O*-methyl-α-D-glucuronic acid (MeGlc*p*A). The glucuronoxylans are also substituted at *O*-2 and/or at *O*-3 by acetyl groups. The degree of acetylation ranges from 10% to 20%, corresponding to approximately from 3 to 7 acetyl groups per 10 units of D-xylose.[5,6] In some plants, the glucuronoxylans can be structurally associated with other cell wall polysaccharides. Thus, in *Eucalyptus globulus*, the glucuronoxylan backbone is substituted at *O*-2 with MeGlc*p*A substituted in turn with galactosyl and glucosyl structural units.[7–9]

Among structural glucans, xyloglucans and the mixed β-(1→3;1→4)-D-glucans are the most abundant. The xyloglucans have a β-(1→4)-glucopyranose backbone branched with α-D-Xyl*p* residues at position 6 of the Glc*p* residues, when 30–65% of Glc*p* units are xylosylated.[5,9] The mixed β-(1→3;1→4)-D-glucans possess a usually unbranched backbone composed of β-D-Glc*p* units with a diverse number of molar ratios between β-(1→3) and β-(1→4)-linked units.[5,9] As a rule, the structural blocks constituted by several β-(1→4)-linked D-Glc*p* units (cellobiose, cellotriose, cellotetraose, etc.) are irregularly spaced by β-(1→3)-linked D-Glc*p* residues.

Further research and development trials on the conversion of grape stalks into value-added products (fuels, chemicals and materials) run into the difficulties related to the lack of knowledge

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about the structure of its carbohydrates. Hence this study aims to fill this gap by clarifying the structural features of the carbohydrate polymers in grape stalks.

The present work deals primarily with the study on the xylan isolated from peracetic holocellulose of grape stalks by extraction with dimethyl sulfoxide (DMSO). The xylans and concomitant β-glucan were characterized by methanolysis and methylation linkage analysis, SEC, 1D, and 2D NMR spectroscopy and mass spectrometry techniques.

2. Experimental

2.1. Materials

The grape stalks sample of the variety *Vitis vinifera* L. (Touriga Nacional) was supplied by Tavfer Group (Quinta do Serrado in Penalva do Castelo in Dão Region of Portugal). The grape stalks were separated from the grape clusters by a mechanical destemmer operation. The material was dried at room temperature, milled on a Retsch cross-beater mill SKl, and sieved to 1–2 mm particles. The grape stalks were characterized regarding the ash content (Tappi T 211 om-91), extractives content in acetone (Tappi T 204 om-88), cellulose *Kürschner* and *Höffer* content,[10] proteins and tannins content,[1] and lignin content according to Tappi T 222 om-88.

2.2. Preparation of holocellulose and isolation of xylan

Holocellulose in∼41% yield was obtained by delignification of grape stalks with peracetic acid (14% AcOOH) at 85 °C during 30 min.[8] The holocellulose (ca. 2 g) was milled in a vibratory zirconium ball mill for 40 min and extracted twice by dimethylsulfoxide (DMSO) at 60 °C for 24 h under a nitrogen atmosphere while being stirred (solid-to-liquid ratio 50). The resulting filtrates were precipitated in 800 mL of ethanol, acidified with formic acid to pH 2–3 and kept for two days in the fridge at +4 °C to coagulate the precipitated xylan. After centrifugation, the residue was washed five times in absolute methanol and dried under vacuum. A part of crude xylan was re-dissolved in DMSO (ca. 1:50 w/w) and purified by graded precipitation with ethanol thus obtaining the purified xylan.

2.3. Neutral sugars analysis

The holocellulose and xylan were submitted to the neutral sugar analysis after Saeman hydrolysis.[11] The neutral sugars were analyzed by GC as alditol acetates (Varian 3350 gas chromatograph equipped with a FID detector (260 °C) and with a DB-225J&W column), under the following conditions: initial temperature −220 °C (5 min); temperature gradient of 10 °C/min; final temperature −240 °C (6 min).

2.4. Acid methanolysis for analysis of sugars and uronic acids

The xylan was subjected to acid methanolysis and subsequent silylation according to methodology described previously.[12] The samples were analyzed by GC (Trace Gas Chromatograph 2000 series), equipped with mass detector (Thermo Scientific DSQII), using helium as carrier gas (35 cm/s). The chromatographic conditions were as follows: column capillary DB-1J&W (30 m × 0.32 mm i.d. 0.25 μm); initial temperature of the column −100 to 4 °C/min −175 °C following by 175–12 °C/min; final temperature −290 °C; detector temperature −290 °C.

2.5. Methylation linkage analysis

The xylan was activated with powdered NaOH and methylated with CH_3I[13,14] followed by a remethylation to ensure complete methylation of the polysaccharides.[15] The methylated xylan was hydrolyzed by treatment with 2 M trifluoroacetic acid for 1.5 h at 120 °C, and the partially methylated sugars were reduced with sodium borohydrate and acetylated.[15,16] Partially methylated alditol acetates (PMAA) were dissolved in dichloromethane and analyzed by GC/MS (Trace GC 2000 series coupled with Thermo Scientific DSQII mass spectrometer) using a column capillary DB-1J&W (30 m × 0.32 mm i.d. 0.25 μm). The chromatographic conditions were as follows: injector and detector operating at 220 and 280 °C, respectively. Temperature program used was as follows: 10 min at 45 °C with a linear increase of 10 °C/min until 140 °C and standby 5 min at 140 °C; a linear increase from 140 to 170 °C by 0.5 °C/min and standby 1 min at 170 °C; a linear increase from 170 to 280 °C by of 15 °C/min and standby 5 min at 280 °C.

2.6. Size exclusion chromatography (SEC)

The xylan solution was prepared immediately before the analysis by dissolution of ca 5 mg of xylan in 50 μL of 10% LiCl solution in *N*,*N*-dimethylacetamide (DMAC) at 100 °C for 15 min and further dilution with DMAC to a xylan concentration of about 1% (w/w). The xylan was analyzed by GPC in a PL-GPC 110 system, equipped with a 10 μm Plgel pre-column and two 10 μm Plgel columns MIXED D 300 × 7.5 mm in a series, and a refraction index detector. The pre-column, SEC columns, and the injection system were maintained at temperature of 70 °C during the analysis. The eluent flow (0.1 M LiCl in DMAC) was 0.9 mL/min. The SEC columns were calibrated using pullulan reference materials (Polymer Laboratories, UK) in a range of 1–100 kDa.

2.7. NMR spectroscopy

Holocellulose was analyzed by Cross Polarization/Magic Angle Spinning (CP/MAS) ^{13}C NMR. Solid state ^{13}C NMR spectra were recorded on a NMR BRUKER AVANCE 400, with a magnetic field of 9.4 T. The sample was spun in a zirconium rotor sealed with Kel-FTM caps at 9 kHz. The acquisition parameters used were as follows: proton pulse of 4 μs, contact time of 2 ms, recovery delay of 4 s and 7000 scans were accumulated.

The isolated and extracted xylan with DMSO was analyzed by ^1H NMR and ^{13}C NMR on a Bruker AVANCE 300 spectrometer operating at 300.13 MHz for proton and at 75.2 MHz for carbon, respectively, at 303 or 323 K. The xylan was dissolved in D_2O (ca. 2% w/w) and the sodium 3-(trimethylsilyl)propionate-d_4 (TMSP, δ 0.00) was used as the internal standard. The acquisition parameters for the proton spectra were as follows: 12.2 μs pulse width (90°), 18 s relaxation delay, and 300 scans were collected. The acquisition parameters for the carbon spectra were as follows: 60° pulse, 8 s relaxation delay, and 5000 scans were collected. All 2D NMR spectra were recorded on a Bruker AVANCE 300 spectrometer. 2D ^1H–^1H (absolute-mode COSY spectrum) spectrum was recorded at 323 K using a standard COSY sequence (relaxation delay 2 s) acquiring 2 K × 512 increments transformed to a 2 K × 1 K data matrix after zero-filling, FT, and squared sine-bell apodization applied to both dimensions. COSY spectrum was acquired over a 7.0 ppm window in both F2 and F1 directions. For each t1 value 600 scans were accumulated. 2D ^1H–^1H (TOCSY) spectroscopy was recorded at 323 K and the spectra were acquired at a spectral width of 2185 Hz in both dimensions (τ_{mix} = 0.050 s).The relaxation delay was 2.0 s. For each FID, 128 transients were acquired; the data size was 1024 in t_1 × 512 in t_2. The phase sensitive ^1H–^{13}C(HSQC) spectrum was acquired at 50 °C over a F1 spectral weight of 12,000 Hz and a F2 width of 2000 Hz with a 2048 × 1024 matrix and 128 transients per increment. The delay between scans was 2 s and the delay for polarization transfer was optimized for $^1J_{CH}$ = 148 Hz.