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Microbial (1→3)-β-D-glucans from Libyan figs (*Ficus carica*)

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Abstract

The cell walls from Libyan natural fig fruits is obtained from the fresh weight of the mycelium, the material released by successive extractions with hot water and sodium hydroxide. The alkali extract was fractionated by ion-exchange and gel-filtration chromatography. Analysis of the first fraction showed that D-glucans was the unique component.

Keywords: Polysaccharides; *Ficus carica*; D-glucan

1. Introduction

The common fig is well known for its tasty fruits, which are enjoyed around the world. These fruits can also be dried, roasted and used as a coffee substitute. The fig, to top its wonderful taste, has many medicinal properties as well. Taken internally, the fig has mildly laxative properties, which are often used in conjunction with senna and carminative herbs. Vitamins B1 and B2 are also found in figs. Much work has been carried out on the compositon of figs of the maturity stage (Burks, Dowell, & Xie, 2000; Ramulu & Vdayasekhara, 2003). D-glucans have been found as common polymers of the fungal cell wall, they consist of a mixture of linear (1→3)-β-D-glucans with various (1→6)-branched oligsaccharides chains, the length of those chains being modulated by culture conditions. We wish to study the possibility to produce polysaccharide by microbial contaminants in natural figs, which contain a high proportion of sugars, creating a suitable environment for microbial growth.

2. Experimental

2.1. General

IR spectra were recorded by an Acculab 10 Beckman instrument with KBr pellets. NMR spectra were recorded on a Bruker 500-instrument. For the ^{1}H NMR spectroscopy at 70 °C, the sample (10 mg) was repeatedly dissolved in D_2O (5×5 ml), and the solution was lyophilized. The final freeze-dried sample was dissolved in 1 ml of 99.99% D_2O. GLC was analyzed on a Packard Model 419 and Hewlett-Packard Model 5713 gas chromatographs each equipped with flame-ionisation detector and columns of 1.3% of ECNSS-M on Gas Chrom Q (100–200 mesh); and 2.3% of OV-225 on GC-Q (100–200 mesh). GLC-MS was conducted with a Hewlett Packard 5895 instrument, using a fused-silica capillary column (30×25 mm) coated with a 0.2 μm film of OV-1. The ionisation potential was 7 eV and the temperature of the ion source was 200 °C.

2.2. Plant material

Natural figs were used in this study (500 g) (*Ficus carica*). The dry fruits were uniform in shape, size, and color and were obtained from Libya at weekly intervals

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from July 15 to August 15, 2003. Dry figs were cut to small pieces after removing all the seed and calyx. The maturity of the fig fruits selected for this study was in the stage of full ripeness, the stage at which figs contains larger amounts of polysaccharides (Ishurd et al., 2002).

2.3. Microbiological techniques

Natural figs after harvesting were cultured in a Czapek Dox liquid medium containing 5% (w/v) of D-glucose. A fermenter vat (8 l) was inoculated with a spore suspension from a 10 day culture grown on a Czapek Dox agar medium. The vat was incubated at 25 °C, mechanically stirred (200 rpm), and aerated (2 l/min). After 21 days, the mycelium was removed by centrifugation and the supernatant solution was filtered through glass wool.

2.4. Extraction of glucans

Glucans were isolated from mycelial walls, and extracted with hot water (5 l) for 3 h. The process of extraction was repeated and the extracts were combined. The insoluble residue was suspended in 1 M NaOH for 6 h at 70 °C and then centrifuged for 20 min at 5000 rpm. The supernatant solution was neutralized with acetic acid and dialyzed against distilled water for 2 days, and was lyophilized. The resulting material was dissolved in 0.01 M sodium phosphate buffer, pH 7.0, and fractionated by column chromatography column (2.8×25 cm) on DEAE-cellulose (Pharmacia) anion-exchange resin with the same buffer (500 ml) an initial element and then with a linear gradient of NaCl (0–1 M) in the same buffer. Each fraction was dialyzed and subjected to chromatography on a column of Sephadex G-25 (1×30 cm) and eluted with water.

2.5. Determination of molecular weight

A solution of glucans (3 mg) in distilled water (0.5 ml) was applied to a column (1.6×80 cm) of Sepharose CL-4B. The column was equilibrated and eluted with distilled water at a flow rate 10 ml/h and the effluent was collected in 4 ml fraction.The carbohydrate content of each fraction was determined with anthrone reagent (Shields & Burneu, 1960). The column was calibrated with standard dextrans.

2.6. Methylation analysis

The glucan (5 mg) was methylated by the method of Hakomori (1964) and the product showed no IR absorption for hydroxyl group. After methylation, the product was purified by elution from a column Sephadex LH-20 with ethanol–chloroform 2:1, dried, and hydrolyzed by heating in 85% aq. formic acid (2 ml) at 100 °C for 6 h. After evaporation of the formic acid, the residue was hydrolysed with 1 M trifluoroacetic acid (2 ml) under the same conditions. After evaporation to dryness, the methylated sugars were reduced with $NaBD_4$, acetylated with acetic anhydride, and analyzed as the alditol acetates by GLC. The identification of the methylated sugars was analysed by GLC-MS (methylated glucitol acetates). GLC of methylated derivatives of D-glucose enables estimation of the molar of tetra-*O*- and tri-*O*-methylglucosides, but not quantification of di-*O*-methylglucosides, which have a lower mass response. The molar ratio of tri-and di-*O*-methyl derivatives was obtained by GLC of the acetylated, methylated glucosides, but most of the tetra-*O*-methyl-D-glucoside was lost by this method (Bjorndal, Lindberg, & Svenndon, 1967; Perret et al., 1992).

2.7. Smith degradation

A sample (10 mg) was oxidized with 0.05 M $NaIO_4$ (10 ml) at 4 °C in the dark for 1 week. The oxidation was stopped by addition of 1,2-ethanediol and the solution was dialyzed against distilled water for 48 h. The dialyzed material was reduced with $NaBH_4$ for 17 h, neutralized with 50% acetic acid, dialyzed, and partially hydrolyzed in 0.5 M trifluoracetic acid for 16 h at 20 °C. The supernatant was neutralized by evaporation of the excess acid and fractionated by chromatography on Sephadex G-15.

2.8. Acetolysis

Acetolysis of glucans (10 mg) was performed according to Dubourdieu et al. (1981) in 10:10:1 acetic anhydride–acetic acid–H_2SO_4 (10 ml) were stored in sealed tubs at 20 °C for 18, 24, or 36 h. Each mixture was poured on to ice (5 ml) and neutralized to pH 4–6 with sodium hydrogen carbonate. Acetylated sugars were extracted with chloroform (5×4 ml), and the combined extracts were washed with conc. aqueous sodium hydrogen carbonate, dried ($CaCl_2$), and concentrated. Each residue was dissolved in acetone (4 ml), and 0.2 M sodium hydroxide (4 ml) was added. After 30 min at 4 °C, the reaction was stopped by adding Dowex 50-X8 (H^+) resin to pH 5.The resin was removed and filtrate was concentrated at 40 °C under diminished pressure. The carbohydrate present in the residue were eluted from a column (64×1.6 cm) of Sephadex G-15 with water. Appropriate fractions were combined and freeze-dried. Polysaccharides excluded from the gel were methylated, and mono- or oligo-saccharides were characterized by GLC of the *O*-trimethylsilyl derivatives (Bayard & Montreuil, 1972).

3. Results and discussion

The cell walls glucans of figs were prepared as previously reported (Dubourdieu, Ribereau, & Fournet, 1981), and obtained in yields of 2–4% of fresh weight of the mycelium. The material was released by successive hot water and sodium hydroxide extractions accounting for 23% of

the walls. The alkali extract was fractionated by column chromatography on DEAE-cellulose. Analysis of the first fraction, eluted with 10 mM potassium phosphate buffer, showed that D-glucose was the sole compound. Gel filtration of these glucans on Sepharose CL-4B indicated a range of molecular weight from 2×10^6 to 2×10^4 with a preponderance of material of higher molecular weight (yield, 50%). The glucans were twice methylated by the Hakomori method (Hakomori, 1964). After hydrolysis, reduction and acetylation, GLC of the alditol acetates from fully methylated glucans showed three peaks corresponding to 2,3,4,6-tetra-*O*-methyl, 2,4,6,-tri-*O*-methyl and 2,4-di-*O*-methyl derivatives in molar ratio of 1.0:4.0:0.8 (Table 1). These results indicated a (1→3)-linked backbone with (1→6)-linked branches.

During acetolysis, the glucan rapidly dissolved in the acetolysis mixture. Gel filtration on Sephadex G-25 gave only two fractions (A and B). The higher molecular weight fraction (A) was eluted with the void volume, and methylation, hydrolysis, reduction, acetylation, and GLC of derivatives analyzed its structure. The identification of 2,3,4,6-tetra-*O*-methyl derivatives agrees with a (1→3)-linked oligsaccharides resulting from complete cleavage of the branched chains and probably from some cleavage of (1→3) bonds of the backbone. The second peak (B) was composed of only glucose monomer, suggesting the presence of single D-glucosyl groups as side chains attached at O-6 of some of the main chain units.

The insoluble glucans were submitted to periodate oxidation, borohydride reduction, and hydrolysis under mild conditions by heating with 0.5 M trifluoroacetic acid at 20 °C for 17 h (Smith degradation) (Aspinall & Ferrier, 1957; Dlixon & Lipkin, 1954). The Smith-degraded polysaccharide was obtained as an insoluble product that was separated by centrifugation. Methylation analysis of this polymer gave 2,4,6-tri-*O*-methyl and 2,3,4,6-tetra-*O*-methyl derivatives in a molar ratio of 17:1 (Table 1). This result was in agreement with a (1→3)-linked backbone chain. Detection of only D-glycerol but not of D-glucosyl-glycerol in the soluble fraction confirm the presence of single glucosyl groups as side chains. The anomeric proton single at δ 5.17 (d, J 7.4 Hz) in the ^{1}H NMR spectrum and (δ 111.7 for C-1) confirmed that the sugar residues were linked β-glycosidically, which agrees with presence of an IR band 890 cm^{-1} (Ishurd, Sun, Xiao, & Ashour, 2002; Ishurd, Zahid, Ahmad, & Pan, 2001). The β configuration of the D-glucosyl groups was clearly evidenced by the presence of two anomeric peaks in the region δ 102.09 and branchings of C-6 were shown by signals of *O*-substituted C-6 at δ 70.3 and of unsubstituted C-6 at δ 61.8. The predominance of the latter, together with the typical signal of O-substitued C-3 at δ 82.6 supported the high proportion of β-D-glycosyl (1→3)-linkages in linear arrangement that was previously demonstrated by chemical analysis (Ishurd, Yousef, Wanxing, & Ashour, 2003; Ishurd, Zahid, Zhou, & Pan, 2001; Zahid, Ishurd, & Pan, 2002). The multiplicity of the signals and the broad C-3 at δ 82.6 could be ascribed to the presence, in the glucans, of linear (1→3), branched (1→3,1→6), and terminal β-D-glucopyranosyl residues, which was similar to that of lentinan (Saito, Okhi, Takasuka, & Sasaki, 1977). The results of methylation analysis, acetolysis and Simth degradation suggested structure **1** for the D-glucan of the Libyan fig (*Ficus carica*).

β-D-Glcp
1
↓
6
→β-D-Glc*p*-((1→3)-[β-D-Glc*p*-(1→3)-]$_3$-β-D-Glc*p*

Figs produce β-D-glucans of the same type as the β-D-glucan from Libyan dates (*Phoenix dactylifera* L) with a straight chain having (1→3)-linked β-D-glucose residues and substituted with branched chains. Some differences were found in the structure of the branched chains. The D-glucans of Libyan fig have a more complex structure, branches having either one D-glucosyl group or di-, tri-, or tetra-D-glucose side chains, whereas only monosaccharide chains were found in the Libyan. The D-glucans of the Libyan fig is similar to the D-glucans obtained from basidiomycetes (Bartnicki, 1968) and ascomycetes which have a moderate degree of branching of one out of five D-glucose residues.

Table 1
Molar ratio of the hydrolysis products of methylated native glucans and Smith degraded glucans

O-Methyl-D-glucose	Linkage indicated	Molar ratios of glucans	
		Native	Smith
degraded			
2,3,4,6-Tetra-	Glc(→	1.0	1.0
2,4,6-Tri	→3)-Glc(1→	3.0	16.0
2,4-Di	→3)-Glc(1→	0.7	–
	6		
	↑		

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