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A GALACTOMANNAN IN CARBONATED-BEVERAGE FLOC FROM RAW CANE SUGAR

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ABSTRACT

A galactomannan that contributes to the formation of acid floc in carbonated beverages, isolated from Australian raw cane sugar, consists of D-mannose and D-galactose in 2.3:1.0 molar ratio, has $[\alpha]_D$ +97.3° in water, and has molecular weight $\sim 3.5 \times 10^6$ by gel filtration. The galactomannan has a main chain of α-(1→6)-linked D-mannopyranose residues, 86% of which are branched through O-2. All D-galactopyranose and some of the D-mannopyranose residues are present as non-reducing terminals in side chains that may be attached by α-(1→2)-links to the main chain. Two possible structures for the galactomannan are proposed, depending upon whether α-(1→2)-linked D-mannopyranose residues are present in the main chain or the side chains.

INTRODUCTION

The formation of carbonated-beverage floc is a matter of great concern to manufacturers of bottled beverages and to sugar refiners. Several studies aimed at determining floc constituents and the cause of floc formation have been undertaken[1–7]. These indicated that proteins, neutral and acidic polysaccharides, lipids, and silicates are the major constituents, and that floc formation most probably results from protein–polysaccharide aggregation[7].

This paper deals with the isolation of a galactomannan from a polysaccharide designated CP, reported previously[8], and the results of its structural analysis.

RESULTS AND DISCUSSION

Purification. — As the polysaccharide CP was found to contain a small proportion of starch and dextran, it was treated with alpha amylase and dextranase. The resulting polysaccharide was further purified by precipitation with 75% ethanol and successive dialysis against water.

The purified polysaccharide was then fractionated on a column of Sephadex G-200 to yield two main fractions designated F-I and F-II. The F-I polysaccharide was preponderant and found to be composed of D-mannose and D-galactose in the

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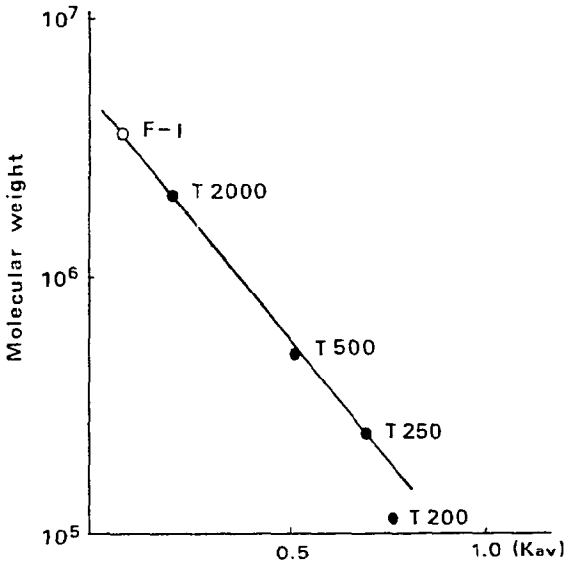

Fig. 1. Estimation of the molecular weight of F-I polysaccharide by gel filtration on Sepharose 2B.

molar ratio of 2.3:1.0, and had $[\alpha]_D$ +97.3° in water. The polysaccharide eluted in the F-II fraction contained mainly glucose plus traces of galactose, arabinose, and xylose.

As the F-I polysaccharide showed a single, symmetrical elution-curve on columns of Sepharose 2B and DEAE-cellulose, it was concluded that it may be a homogeneous galactomannan. Its molecular weight was estimated by gel filtration on Sepharose 2B to be ~3.5×10^6 (Fig. 1).

Methylation analysis. — The galactomannan was methylated by the Hakomori method[9], and the alditol acetates obtained from the hydrolyzate of the methylated galactomannan were subjected to g.l.c.–m.s. analysis (Table I). As shown in Table II, 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3,4,6-tetra-*O*-methyl-D-

TABLE I

MASS SPECTRA[a] OF ALDITOL ACETATES DERIVED FROM THE HYDROLYZATE OF METHYLATED GALACTOMANNAN

Alditol acetates	m/z
2,3,4,6-Me_4-Man	71, 87, 101, 117, 129, 145, 161, 205
2,3,4,6-Me_4-Gal	71, 87, 101, 117, 129, 145, 161, 205
3,4,6-Me_3-Man	87, 99, 101, 129, 161, 189
2,3,4-Me_3-Man	87, 99, 101, 117, 129, 161, 189
3,4-Me_2-Man	87, 99, 129, 189

[a]RM-50 GC Hitachi GC-mass spectrometer, carrier gas; He, 1 mL/min, split ratio; 30:1, 70 eV, chamber temp; 200°; and 063 Hitachi gas chromatograph, glass WCOT column (PEG 20M on 0.25 mm × 30 m), oven temp; 180°.

TABLE II

MOLAR RATIOS AND RETENTION TIMES OF ALDITOL ACETATES DERIVED FROM THE HYDROLYZATE OF METHYLATED GALACTOMANNAN

Molar ratio	*Alditol acetates*	*Retention times*[a]	
		Column B	*Column* C
1.0	2,3,4,6-Me_4-Man	0.98	0.96
2.7	2,3,4,6-Me_4-Gal	1.20	1.13
1.0	3,4,6-Me_3-Man	1.92	1.71
0.7	2,3,4-Me_3-Man	2.42	2.03
3.6	3,4-Me_2-Man	5.22	3.81

[a]Retention times of the corresponding alditol acetates relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol. Column B: 3 mm × 2 m glass column packed with 3% ECNNS-M on Gas Chrom Q (80–100 mesh). Column C: 3 mm × 2 m glass column packed with 3% OV-225 on Gas Chrom Q (80–100 mesh).

galactose, 3,4,6-tri-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-mannose, and 3,4-di-*O*-methyl-D-mannose were identified in the molar ratio of 1.0:2.7:1.0:0.7:3.6.

As D-galactopyranose residues were detected only as 2,3,4,6-tetra-*O*-methyl-D-galactose, all such residues in the galactomannan constitute the non-reducing ends of side chains. D-mannopyranose residues were present as non-reducing ends, as (1→6)- and as (1→2)-linkages. As the molar ratio of 3,4-di-*O*-methyl-D-mannose was greater than those of the other methylated sugars, the galactomannan was considered to be highly branched, at either O-2 of (1→6)-linked D-mannopyranose residues or O-6 of (1→2)-linked D-mannopyranose residues. Of the side chains, 75% seemed to be terminated by D-galactopyranose residues, and 25% by D-mannopyranose residues.

Periodate oxidation and Smith degradation[10]. — The consumption of periodate and the production of formic acid in the periodate oxidation of the galactomannan (Fig. 2) were 1.46 and 0.44 mol/sugar residue, respectively. The periodate-oxidized galactomannan was reduced and subjected to complete Smith degradation. The degraded products were acetylated and then analyzed by g.l.c. Mannose, galactose, and erythritol were not detected, whereas 0.83 mol/sugar residue of glycerol was formed. These results suggest that (1→3)- and (1→4)-linkages are not present in the galactomannan. The theoretical values for periodate consumption, formic acid production, and glycerol production calculated from the molar ratio of methylated sugars obtained in the foregoing methylation analysis are 1.49, 0.49, and 1.0 mol/sugar residue, respectively. These values are in fair agreement with the experimental values in this work and support the linkages of component sugar residues in the galactomannan deduced by methylation analysis.

Enzymic analysis. — Hydrolysis of the galactomannan with coffee-bean α-D-galactosidase yielded D-galactose (t.l.c.), whereas the hydrolysis with *Escherichia coli* β-D-galactosidase gave no free sugars. Coffee-bean α-D-galactosidase has been extensively studied with respect to its specificity for various galactomannans[11]; the

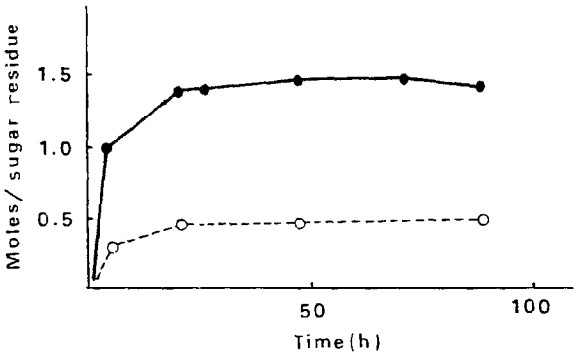

Fig. 2. Consumption of periodate and production of formic acid during periodate oxidation ●, consumption of periodate; ○, production of formic acid.

enzyme is unable to liberate all of the galactosyl groups. In this work, the enzymic release of D-galactopyranose residues from the galactomannan was ~50%.

Methylation analysis of the hydrolyzate of the galactomannan with α-D-galactosidase gave 2,3,4,6-tetra-*O*-methyl-D-mannose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 3,4,6-tri-*O*-methyl-D-mannose, 2,3,4-tri-*O*-methyl-D-mannose, and 3,4-di-*O*-methyl-D-mannose in the molar ratio of 1.0:1.4:1.0:2.0:2.2.

Hydrolysis of the galactomannan with jack-bean α-D-mannosidase yielded D-mannose.

The galactomannan was hydrolyzed with α-D-galactosidase and then treated with α-D-mannosidase. Methylation analysis of the resulting hydrolyzate gave the foregoing five methylated sugars in the molar ratios of 1.0:4.7:3.3:9.0:5.0. These results suggest that the galactomannan has a main chain of α-(1→6)-linked D-mannopyranose residues branched at O-2.

The higher specific rotation of the galactomannan after being treated with both enzymes (+58°) and the lower rotation after acid hydrolysis (+25°) also suggested that the mannosidic linkages have α-D configuration.

The results of this work suggest two possible structures for the galactomannan: *(a)* The galactomannan has a main chain consisting of α-(1→6)- and α-(1→2)-linked D-mannopyranose residues. Single α-D-galactopyranose residues and α-D-mannopyranose residues are attached in the ratio of 3.0:1.0 to 86% of the α-(1→6)-linked D-mannopyranose residues in the main chain through α-(1→2)-links.

(b) The main chain of the galactomannan consists of only α-(1→6)-linked D-mannopyranose residues, 86% of which are branched at O-2. D-mannopyranose residues are α-(1→2)-linked in side chains that are terminated by non-reducing α-D-mannopyranosyl and/or α-D-galactopyranosyl groups. In this possibility, most or all of the α-D-galactopyranose and some non-reducing α-D-mannopyranose residues are considered to be attached through O-2 to α-D-mannopyranose residues of the main chain.

EXPERIMENTAL

Purification. — Polysaccharide (500 mg) dissolved in 50 mL of 0.02M phosphate buffer (pH 7.0) was incubated under a few drops of toluene for 20 h at 38° with 0.3 mL of dextranase (*Chaetomium gracile*, 1,300 U/mL, Sankyo Co., Ltd.) and 1.0 mg of alpha amylase (*Bacillus subtilis*, 1,800 U/mg, Boehringer Mannheim GmbH). After the incubation, ethanol (150 mL) was added and the resulting precipitate collected by centrifugation. The precipitate was dissolved in water and dialyzed against deionized water for 72 h. The dialyzate was precipitated with 5 volumes of ethanol and freeze-dried. The freeze-dried sample (10–20 mg) was fractionated by gel filtration on a column (2.6 × 40 cm) of Sephadex G-200 eluted with water. The carbohydrate content of each fraction was monitored by the anthrone–sulfuric acid method[12]. After repeating the foregoing procedures, the polysaccharides designated F-I (365 mg) and F-II (40 mg) were finally obtained from 500 mg of the original polysaccharide. The F-I polysaccharide (20 mg) was applied to a column[13] of DEAE-cellulose (phosphate form, 2.6 × 30 cm) that was eluted with a gradient of phosphate buffer (pH 6.1). The F-I polysaccharide (4 mg) was also subjected to gel filtration on a column (1.5 × 95 cm) of Sepharose 2B that was eluted with water.

Estimation of molecular weight. — The F-I polysaccharide (3 mg in 0.5 mL of water) was applied to a column of Sepharose 2B that was eluted with water. The carbohydrate content of each fraction was monitored by the anthrone–sulfuric acid method, and the molecular weight estimated from a calibration curve prepared by using a dextran T-series (Pharmacia Fine Chemicals AB).

Analytical methods. — T.l.c. was performed on plates of silica gel (10 × 10 cm h.p.t.l.c. plates, silica gel 60) by multiple ascents with 6:4:3 (v/v) 1-butanol–pyridine–water or 3:3.5:0.5 (v/v) chloroform–acetic acid–water[14]. Component sugars in the acid hydrolyzate of the F-I polysaccharide were detected by spraying the chromatogram with diphenylamine–aniline reagent.

G.l.c. was performed with a Hitachi gas chromatograph Model 163, equipped with a hydrogen flame-ionization detector and a Hewlett–Packard integrator Model 3390A, using glass columns packed with 3% PEGS on Gas Chrom Q (0.3 × 100 cm, column *A*), 3% ECNNS-M on Gas Chrom Q (0.3 × 200 cm, column *B*), and 3% OV-225 on Chromosorb W (0.3 × 200 cm, column *C*), respectively.

Component sugars of polysaccharide. — The F-I polysaccharide (5 mg) was hydrolyzed in a sealed glass tube with 0.6M sulfuric acid for 4 h at 100°. After cooling, the contents were made neutral with Dowex-1 X4 (HCO_3^- form) resin. The hydrolyzate was freeze-dried and converted into the corresponding aldononitrile acetates[15], which were analyzed by g.l.c. on column *A*, which was maintained isothermally at 190° at a nitrogen flow-rate of 65 mL/min.

Methylation of polysaccharide. — The F-I polysaccharide (2–10 mg) was methylated by the Hakomori method[9]. The methylated polysaccharide (2–10 mg) was dissolved in 2 mL of 1.5M hydrochloric acid in dry methanol and the mixture

in a sealed glass tube was kept for 22 h at 85°. The mixture was made neutral with silver carbonate, filtered, and the filtrate was deionized with Amberlite IR-120 (H^+ form) resin and evaporated to dryness under diminished pressure at 40°. The resulting methyl glycosides were then hydrolyzed with 2 mL of 0.6M sulfuric acid for 4 h at 100°. The hydrolyzate was reduced with sodium borohydride and then acetylated, and the alditol acetates were analyzed by g.l.c. on columns *B* or *C*, and also by g.l.c.–m.s. with a 063 Hitachi gas chromatograph connected to a RM-50 GC Hitachi GC-mass spectrometer. A glass WCOT column (0.25 mm × 30 m) coated with PEG 20M was used isothermally at 180°. The helium flow-rate was regulated at 1.0 mL/min, with a split ratio of 30:1. The temperature of the ion source was 200°, and the spectra were recorded at 70 eV.

Periodate oxidation and Smith degradation. — The F-I polysaccharide (22 mg) was oxidized with 0.02M sodium periodate (100 mL) at 5° in the dark. Aliquots (0.5–1.0 mL) of the solution were withdrawn at intervals and the consumption of periodate monitored by the Fleury–Lange method[16]; the amount of formic acid liberated was determined by titration with 10mM sodium hydroxide. After complete oxidation, an aliquot (2.0 mL) of the solution was made neutral with sodium hydroxide, and the oxidized polysaccharide was reduced with sodium borohydride for 20 h at room temperature. The excess of borohydride was decomposed with Amberlite IR-120 (H^+ form) resin. The solution was concentrated under diminished pressure at 40°, diluted with a small amount of methanol, and evaporated to dryness. The resulting polysaccharide-polyalcohol was hydrolyzed with 0.6M sulfuric acid for 1 h at 100°. The hydrolyzate was made neutral with Dowex-1 X4 (HCO_3^- form) and evaporated. The components in the hydrolyzate were reduced with sodium borohydride as already described and then converted into the corresponding acetates, which were analyzed by g.l.c. on column *B*.

Enzymic hydrolysis. — The F-I polysaccharide (15 mg), dissolved in 5 mL of 0.02M phosphate buffer, was incubated for 72 h at 35° with 0.1 mL of α-D-galactosidase (coffee bean, 10 U/mg, Boehringer Mannheim GmbH) under a covering of a few drops of toluene. After incubation, 3 volumes of ethanol were added to the solution. The alcoholic supernatant solution obtained by centrifugation was deionized with ion-exchange resins, evaporated, and then analyzed by t.l.c. for liberated sugars. In addition, the resulting precipitate was dissolved in a small volume of water and dialyzed against deionized water for 3 days. The freeze-dried dialyzate was analyzed for component sugars and also subjected to methylation analysis. Incubation of the F-I polysaccharide (15 mg) with 0.1 mL of α-D-mannosidase (jack bean, 50 U/mL, Boehringer Mannheim GmbH) and the subsequent analysis were performed in the same manner as with α-D-galactosidase. Furthermore, the F-I polysaccharide (45 mg) was treated with both α-D-galactosidase and α-D-mannosidase in the aforementioned manner. The residual polysaccharide that had not been hydrolyzed with the enzymes was recovered by dialysis against water and precipitated with alcohol. The change in specific rotation of the residual polysaccharide during acid hydrolysis with 0.1M hydrochloric acid was measured.

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