

Chemical Characterization of the Immunomodulating Polysaccharide of *Aloe Vera* L.

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ABSTRACT^a

Carbohydrate compositional analysis, linkage analysis, and one- and two-dimensional NMR were used to investigate the structure of a bulk water soluble polysaccharide (BSW) isolated from *Aloe vera* gel by alcohol precipitation. Hydrolysis of BSW with strong organic or inorganic acids at elevated temperatures produced a mixture of oligosaccharides and an acid resistant fraction, accounting for nearly 37% of the bulk material. BSW was found to be composed primarily of 84% mannose, 6% glucose and 4% galactose. In contrast, the acid resistant fraction contained arabinose (18%), galactose (18%), xylose (9%), glucose (9%), and galacturonic acid (5%). Oligosaccharides purified from the endo- β -mannanase degradation of BSW were shown by linkage analysis and NMR to contain β Glc1,4 β Man1,4Man and β Man1,4[α Gal1,6]Man, as well as di-, tri-, and tetrasaccharides of β -1,4-linked mannose. Comparison of ¹³C NMR of β -mannanase treated BSW, before and after treatment with exo- α -galactosides, suggests the presence of single galactose residues α -1,6-linked to a mannose backbone. Furthermore, α -1,6-galactose substituted mannoses are the only branched mannoses detected by the ¹³C NMR experiment. The cumulative results suggest that the majority structure of BSW is a β 1,4-linked mannose backbone, substituted with β 1,4-linked glucose residues, and having mannose-substituted branches of single α 1,6-linked galactose residues. However, the presence of an acid resistant fraction with a carbohydrate composition inconsistent with this majority structure suggests that substructures other than those determined may exist in BSW.

^a Keywords: ¹³C NMR, acemannan, *Aloe vera*, polysaccharide, immunostimulant, immunomodulator

INTRODUCTION

Aloe vera L. is a tropical or sub-tropical plant with turgid lance-shaped green leaves with jagged edges and sharp points. The plant is a member of the lily family (*Liliaceae*) not the cactus family as many would believe from the rosette-like arrangement of the long spiked leaves on the central stem [1]. There are over 300 species of *Aloe* known, but *Aloe vera* L. is recognized as the “true *Aloe vera*” for its widespread use and purported healing powers [1-3]. The plant contains two separate juice materials, a yellow latex (exudate), extracted from the vascular bundles at the junction between the rind and the fillets, and a transparent mucilaginous gel, extruded from the inner pulp. While the dried exudate has been used as a cathartic, the gel has been widely accepted since the 4th century B.C. as a traditional medicine for alleviating pain and treating a variety of ailments ranging from burns and lacerations to peptic ulcers, dermatitis, high blood pressure, hair loss and leprosy [1,4]. In more recent times, clinical use of the gel remains controversial, with some workers verifying its positive effects in treating radiation and skin burns [1,5-8] while others have reported its use associated with a significant delay in wound healing [9].

Acemannan, an acetylated β -1,4-linked glucomannan which makes up the majority of the mucilaginous *Aloe vera* gel carbohydrate that is isolated by alcohol precipitation [10-17]. Acemannan HydrogelTM has been incorporated in commercial proprietary wound care products and has been reported to effect wound closure in chronic wounds [18], aphthous ulcers [19-20] and reduction of dry socket associated with 3rd molar extraction sites [21]. Acemannan Hydrogel, further refined, has been shown to act as an immunostimulant, displaying adjuvant activity on specific antibody production [15] and enhancing the release of interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α) and interferon- γ (INF- γ) [22]. Release of these cytokines stimulates an increase of up to 300% in the replication of fibroblasts in tissue culture and enhances macrophage phagocytosis [12, 23]. Proliferation

of fibroblasts is known to be responsible for healing burns, ulcers and other wounds of the skin and gastrointestinal lining. In addition, this product has been shown to have an antiviral effect and be of significant benefit in FIV-infected cats and inhibit AIDS virus replication [24, 25].

There is considerable discrepancy in the literature as to the structure of the polysaccharide isolated from *Aloe vera* mucilaginous gel. Gowda et al. [13], the first workers to isolate a product by the alcohol precipitation method, found a polysaccharide with a glucose:mannose ratio of 1:19 and a degree of acetylation of 0.78/residue. Linkage analysis showed the presence of β -1,4-linked glucose and mannose, suggesting a linear mannan structure, where mannose residues are randomly substituted with glucose residues [16]. Later work by others suggests the presence rhamnose, fucose, arabinose, xylose, galactose and uronic acids in minor abundance [11, 15-16]. It is not known if these minor sugars occur as integral parts of the polysaccharide structure or are present as minor carbohydrate contaminants. In one preparation in which acemannan was chromatographically purified, the polysaccharide was found to contain 93% mannose, 3% glucose, 3% galactose and less than 1% arabinose [11]. On the basis of carbohydrate compositional and linkage analysis, three different structures have been proposed, all having as a backbone β -1,4-linked glucomannan backbone. In the first, β -1,4-linked galactose branches are 2,6-linked to the glucomannan backbone [11]. In the second, extended galactose branches are α -1,6-linked to the backbone [17] and in the third, extended β -1,4-linked mannans are 1,6-linked to the glucomannan backbone [16].

An approach which has proven quite successful in the past for determining the fine structures of galactomannan, glucomannan and galactoglucomannans is one in which structures of oligosaccharides produced by enzymatic or chemical hydrolysis are identified using a variety of chemical and spectroscopic methods [26-37]. In the present report the fine structure of the *Aloe vera* BSW component is further defined using a similar combined approach.

EXPERIMENTAL

BSW polysaccharides. Acemannan Hydrogel™ was obtained from Carrington Laboratories (Irving, TX) and was isolated by clarification and ethanol precipitation of the inner leaf gel of *Aloe vera* L. The product consists on average 60% soluble polysaccharides, 30% fibrous insoluble material, 8.5% organic salts (oxalate, uronate, and malate) and 1-2 % protein. Acemannan Hydrogel was dissolved in distilled water by gentle shaking overnight and vacuum filtered through a 0.45 µm nitrocellulose membrane to yield BSW. BSW was found to contain 90% soluble carbohydrate, 1-2% protein, < 1% insoluble materials, and the remainder organic salts.

Enzymes and other reagents. β-mannanase (*Aspergillus niger*, 500 U, E.C. 3.2.1.25) and α-galactosidase (*Guar seed*, 270 U, E.C. 3.2.1.22) were obtained from Megazyme, Ltd. (County Wicklow, Ireland) and β-glucanase (*Trichoderma Longibrachiatum*, 2250 BGLU/ml, E.C. 3.2.1.4) was obtained from Genencor, Inc. (Rochester, NY). The β-mannanase and the α-galactosidase were used without further purification. 100 units (50 µL) of β-glucanase dissolved in 0.2 mL of 10 mM sodium acetate, pH 4.5 and this solution was dialyzed against two 1 L changes of the same sodium acetate buffer. All other chemical reagents were purchased from Sigma-Aldrich Company Co. (St. Louis, MO) and were used without further purification.

Acid hydrolysis of BSW. A solution of BSW was prepared by dissolving 10-25 mg of the polysaccharide material in 5 mL of distilled water. The solution was made 2 M in trifluoroacetic acid (TFA) and was then heated and stirred in a sealed vial at 85 °C for twenty-two hours. These conditions yielded a mixture of oligosaccharides having a degree of polymerization (Dp) of 1 (monosaccharides) to greater than 10. A similar distribution of oligosaccharides was obtained when the hydrolysis was performed with 2 M TFA at 120 °C for three hours or with 0.5M sulfuric acid at 90 °C for two hours. In

the later case a purified oligosaccharide fraction was obtained following hydrolysis by the addition of barium carbonate to the reaction mixture. The solution was centrifuged and the barium sulfate precipitate was discarded. Acid traces were removed from the supernatant solution under a stream of nitrogen and the aqueous solution was lyophilized.

Chromatographic purification of BSW oligosaccharides. The lyophilized mixture of oligosaccharides obtained from the TFA catalyzed hydrolysis of BSW (2 M TFA, 85 °C, 22 hrs) were dissolved in distilled water, filtered through a 0.45 µm Millipore cellulose acetate membrane and injected into a Dionex liquid chromatograph equipped with a Phenomenex Rezec column (200 x 10 mm), Rezec guard column (60 x 10 mm), and a column oven (85 °C). The oligosaccharide fractions were eluted at 0.4 ml/min with filtered water and detected with a BioRad refractive index detector (Model 1770) connected to a Hitachi D-2500 integrator. The oligosaccharide fractions were collected with a BioRad fraction collector (Model 2110) and freeze dried.

The Phenomenex Rezec column was calibrated with a solution (3 mg/ml) of dextran standard (MWCO 1000, Polymer Service-USA). A correlation between retention time and degree of polymerization was made using this standard where the retention time corresponding to the 1080 MW component (Dp 6) was set to the most abundant saccharide, as was specified by the manufacturer.

Some fractions of shorter oligosaccharides (Dp < 3) were further purified on a Dionex liquid chromatograph equipped with a Biorad Aminex HPX-87C column (300 x 7.8 mm), and a column oven temperature of 85 °C. Slightly longer oligosaccharide fractions (Dp 4-5) were chromatographed on a Rainin Microsorb-MV (C18) column (250 x 5 mm). The oligosaccharide components were eluted at 0.2 mL/min with filtered water.

Isolation of acid resistant oligosaccharides. A solution (3 mg/ml) obtained from 2 M TFA hydrolysis at 120 °C for three hours was freeze dried. The mixture of saccharides (120 mg) was

resuspended in a total volume of 25 ml distilled water and put in 1000 daltons nominal molecular weight dialysis tubing (Spectra/Por® CE, Cellulose Ester) and dialyzed for thirty hours against distilled water (2 L, pH 7). The aqueous sample (Fraction 1B) was removed from the dialysis bag, freeze dried, and further purified using a Biorad Aminex HPX-87C column, as previously described. Oligosaccharides fractions were collected for further analysis.

Enzymatic hydrolysis of BSW with β -D-mannanase. Solutions of BSW were prepared by dissolving 24 mg of polysaccharide material in distilled water (8 ml) and deacetylation was initiated by the addition of 100 μ l of 5 M NaOH. After allowing the reaction to proceed for one hour at room temperature, the polysaccharide solutions were titrated with 5 M acetic acid to pH 4.5 and an aliquot (4 μ l, 1U) of β -D-mannanase in 2 M ammonium sulfate was added immediately. Solutions were incubated for one day at 45 °C. Reactions were terminated by heating to 100 °C for 5 minutes. The quenched reaction mixtures were filtered through a 0.45 μ m filter and freeze dried.

Enzymatic hydrolysis of oligosaccharide mixture by α -galactosidase 24 mg of freeze dried deacetylated oligosaccharide previously treated with β -mannanase was dissolved in distilled water (8 ml) and the solution was adjusted to pH 4.5 with 5 M acetic acid. To this solution was added an aliquot (13 μ l, 2U) of α -galactosidase in 2 M ammonium sulfate. The solution was incubated for one day at 45 °C. The reaction was terminated by heating at 100 °C for 5 minutes and the quenched mixture was filtered through a 0.45 μ m filter and freeze dried.

Enzymatic hydrolysis of oligosaccharide mixture by β -glucanase. The freeze dried deacetylated oligosaccharides, previously treated with β -mannanase and α -galactosidase, were dissolved in distilled water (8 ml). The solutions (3mg/ml) were adjusted to pH 4.5 with 5 M acetic acid followed by the addition of 34 units of freshly dialyzed β -glucanase in 10 mM sodium acetate. The solution was incubated for one

day at 45 °C. The reaction was terminated by heating at 100 °C for 5 minutes and the quenched mixture was filtered through a 0.45 µm filter and freeze dried.

Monosaccharide Composition by GC-MS of their Trimethylsilyl (TMS) Methyl Glycosides. An aqueous solution containing 20-50 µg of inositol was added as an internal standard to 50-500 µg of a deacetylated oligosaccharide sample previously treated with β-mannanase or to 50-500 µg of a monosaccharide standard in a 1 mL vial. The mixture was lyophilized to dryness and 500 µl of dry 1 M methanolic HCl (Supelco) solution was then added. The vial was sealed and the solution was heated with stirring at 85 °C for sixteen hours . The vial was then cooled and the methanolic HCl was evaporated under a stream of nitrogen. An additional 500 µl of dry methanol was added to the dried methyl glycosides and evaporated once again. 200 µl of Sil-A reagent (Sigma Chemical Co.) was added and the reaction mixture was heated to 80 °C for twenty minutes in a sealed vial with stirring. The sample was cooled and the Sil-A reagent was evaporated under a stream of nitrogen. The dried sample was extracted with 200 µl of hexanes, filtered and was ready for GC-MS analysis.

The TMS-methyl glycosides were analyzed by GC-MS on a Hewlett Packard 5970 MSD instrument using a DB-1 column (J&W Scientific). Glycosides were eluded by first holding the temperature constant 2 min at 80 °C, then increasing the temperature to 170 °C at 30 °C/min, increasing the temperature to 240 °C at 40 °C/min, and finally, holding the temperature at 240 °C for 15 minutes. Identification of TMS methyl glycosides from *Aloe* samples was made by comparing retention times of peaks occurring in the MS total ion chromatograph (TIC) to retention times of known standards. Concentrations with respect to inositol (added as an internal standard) were determined by comparing the integrated TIC peak area to that for the TMS-inositol derivative, taking into account appropriate response factors. Response factors were determined from four standard mixtures, where the concentration ratio of inositol to monosaccharide standard was varied from 0.5 to 5.0. The ratio of the peak areas (A_{inos}/A_{MeGly}) were measured in the TIC

and fit to the equation $A_{\text{inos}}/A_{\text{MeGly}} = (C_{\text{inos}}/C_{\text{MeGI}})k_1 + k_0$ using a linear least-squares routine. Values determined for (k_1, k_0) were: arabinose, (0.98, 1.40); xylose, (0.41, 0.51); fucose, (0.68, 0.57); rhamnose, (0.69, 0.89); galacturonic acid, (0.66, 0.50); glucuronic acid, (0.60, 0.35); mannose, (0.42, 0.33); glucose, (0.39, 0.16); galactose, (0.47, 0.44).

Glycosidic Linkages by GLC of Partially Methylated Alditol Acetates. To a solution of 1-2 mg of carbohydrate sample in 0.3-0.5 mL of dried Me_2SO was added 25 - 30 mg of finely powdered NaOH and 0.3 mL of methyl iodide [34,35]. The mixture was stirred in a sealed vial overnight at room temperature. Water (1 mL) and chloroform (1 mL) were then added, and the chloroform layer was washed with water (3 x 10 mL), dried with sodium sulfate and evaporated under a stream of nitrogen. The permethylated material was dissolved in acetonitrile and cleaned by passing the organic layer through a Sep-Pak C18 Reverse Phase Cartridge, preconditioned with acetonitrile. The eluent (pale yellow solution) was evaporated under a stream of nitrogen at room temperature.

To the dry permethylated carbohydrate, 500 μl of 2 M trifluoroacetic acid and 50 μg of inositol were added. The mixture was stirred and heated at 120 $^\circ\text{C}$ in a sealed vial for two hours. The acid was removed under a flow of nitrogen and the aqueous sample was lyophilized to dryness.

The hydrolyzed sample was dissolved in 300 μl of 1 M ammonium hydroxide solution containing 10 mg/mL of sodium borodeuteride and kept at room temperature for three hours in a sealed vial. The reaction was quenched by adding glacial acetic acid, dropwise until bubbling ceased. An amount of 500 μl methanol-acetic acid mixture (9:1 (v/v) %) was added to the reaction mixture and evaporated. This last step was repeated for 3 more times. Similarly, 500 μl of methanol was added to the reaction mixture and evaporated under a stream of nitrogen. This step was repeated for two more times.

To the dry residue, 200 μl of acetic anhydride and 20 μl of 1-methyl imidazole were added. The mixture was stirred in a sealed vial at room temperature for twelve hours. The reaction was quenched by

adding 500 μ l of water and waiting 10 minutes, then the partially methylated alditol acetates were extracted into 500 μ l of dichloromethane. The organic layer was transferred to a clean vial where the solvent was dried with sodium sulfate and evaporated under a stream of nitrogen to dryness. The final residue was dissolved with 100 μ l of methanol and filtered through a 0.45 μ m filter. The clear solution was ready for GC-MS analysis [37, 38].

The partially methylated alditol acetates were analyzed by GLC-MS on a Hewlett Packard 5970 MSD instrument using a SP-2330 column (SUPELCO). A temperature gradient program identical to that described for elution of methyl glycosides was used for the chromatography of partially methylated alditol acetates.

NMR of Aloe vera oligosaccharides. Dried oligosaccharide samples were dissolved in 0.5 ml D₂O (Sigma, 100 atom % D). The ¹H-NMR spectra were recorded on a 500 MHz Varian spectrometer, with a single 90° pulse at a temperature of 45 °C. All ¹H-NMR spectra were taken with a sweep width of 8000 Hz. The residual HOD resonance was presaturated during the second delay period. All ¹³C-NMR spectra were recorded on a 500 MHz Varian spectrometer at a temperature of 45 °C and a sweep width of 28996 Hz for at least two hours. Dioxane was used as an external standard (67.5 ppm).

COSY spectra were acquired using a sweep width of 1607 Hz, and a matrix of 1024 x 1024 data points. When Fourier transformed, the absolute-value data was processed using sine bell weighting to diminish peak tailing effects. TOCSY spectra were acquired using a sweep width of 1500 Hz, a mixing time of 70 ms and a delay time of 2 s. Data was processed (2048 x 2048 data matrix) with Gaussian weighting.

RESULTS

Characterization of acid hydrolyzed and enzyme degraded BSW. Fig. 1A shows the chromatographic profile observed for the separation of oligosaccharides arising from the TFA catalyzed hydrolysis of BSW. Nine well resolved fractions (F1-F9) appear in the chromatogram. Peaks appearing in the chromatogram were assigned an apparent degree of polymerization (Dp) based on retention times of a calibration standard mixture of oligosaccharides. Table 1 summarizes assigned Dp's, retention times and fractional areas of the peaks. As estimated from peak area, the acid resistant fraction, F1, makes up 37% of BSW. When the acid hydrolyzed mixture of oligosaccharides was further reacted with TFA under more severe conditions such as higher acid concentration or longer times, F2 through F8 were degraded to monosaccharides, while F1 remained intact. An acid resistant fraction was also observed when BSW was hydrolyzed with 0.5 M sulfuric acid (2-6 hours at 90 °C). F1 can, however, be further degraded to smaller oligosaccharides when treated with acidic methanol (1 M methanolic HCl, 85 °C, 22 hrs) following TFA hydrolysis. Under these conditions, F1 was substantially decreased and made up no more than 3% of the total integrated peak intensity.

In order to obtain a greater diversity of hydrolysis products which would complement those obtained by TFA hydrolysis, enzymatic methods of degrading BSW were explored. Mannans are relatively resistant to attack by exo-mannosidases but can be degraded by endo-mannanases [26-30, 43-45]. The enzyme endo- β -D-mannanase hydrolyses mannans (including galactomannans, glucomannans and galactoglucomannans) having β -1,4-linkages. Because a minimum four or five contiguous mannose residues are required for binding, mannobiose and mannotriose are not degraded by the enzyme [26].

Enzymatic hydrolysis of BSW was initially attempted with both endo-mannanase and exo- β -D-mannosidase. Neither enzyme was found to hydrolyze the complex saccharide. However, BSW could be hydrolyzed by endo- β -D-mannanase if acetyl groups were first removed. Although deacetylation of BSW

is known to decrease its solubility in water [14], the relatively fast hydrolysis by endo- β -D-mannanase prevented precipitation.

Fig. 1B shows the chromatographic profile of BSW following treatment of the carbohydrate by endo- β -D-mannanase for one day. By comparing the intensity of the peak eluting at the void volume with the corresponding peak in the chromatograph of a reaction blank containing an equivalent amount of enzyme and sodium acetate, it was estimated that 90% of this peak was β -D-mannanase. Thus the majority of the polysaccharide was degraded into oligosaccharides having Dp 2-4 which eluted at 36.51 min, 32.32 min and 28.64 min. The observed hydrolysis of the *Aloe* polysaccharide by β -mannanase to shorter oligosaccharides is consistent with action of the enzyme previously observed on galactomannans [24-30].

Carbohydrate composition of BSW and BSW oligosaccharides obtained by acid hydrolysis.

The monosaccharide composition of intact BSW and BSW previously treated with β -mannanase is summarized in Table 2. The greater fraction of mannose found relative to glucose, galactose and other minor monosaccharide constituents in BSW agrees with results previously found for the major alcohol precipitable polysaccharide component isolated from *Aloe vera* gel [10-16]. The β -mannanase hydrolyzed BSW had a lower xylose content (0.7 vs 3.3%), a higher mannose (84% vs 75%) and fucose (1.5% vs 0.2 %) content than intact BSW. These differences may be accounted for by the greater recovery of material in the enzyme treated samples (84 wt% vs 30 wt%).

The carbohydrate composition of fractions chromatographically purified from the TFA hydrolyzate of BSW are summarized in Table 3. Mannose, glucose, galactose and xylose were found to be present in all fractions isolated from the TFA hydrolysis of BSW, while both uronic acids, arabinose, rhamnose and fucose were not. With the exception of the monosaccharides (Dp 1), mannose was relatively more abundant in smaller oligosaccharides (Dp 2-4) compared to larger ones (Dp 5-10), while

the relative abundance of glucose decreases in the smaller oligosaccharides. Galacturonic acid appears in larger oligosaccharides but is absent in the smaller ones.

Acid resistance has been observed for pectins, where the majority of glycosidic linkage arise from α -1,4-linked galacturonic residues [40, 41]. With the exception of Dp 1, monosaccharides usually associated with pectins and hemicelluloses, such as glucuronic and galacturonic acid, arabinose, rhamnose and xylose, are more concentrated in the higher molecular weight fractions, F1-F2. On the other hand, the higher relative abundance of arabinose, xylose and rhamnose in Dp 1 may be explained by the finding that pentoses are more acid labile than the corresponding hexoses by a factor of 10 to 1000 [40]. Similarly, the fact that deoxyhexoses or methylsugars hydrolyze 5 times faster than regular hexoses [42] may explain why fucose was relatively more abundant in smaller oligosaccharide fractions. The observation that the relative fraction of mannose is greater in the smaller oligosaccharides (Dp 2-5) is probably the result of the relative decrease of galactose, glucose and other minor sugars in these fractions. No other clear trends appear for any of the other component sugars as a function of degree of polymerization.

Because of the limited quantities of F1 which could be obtained by chromatographic purification, a new method was developed for isolation of the acid resistant fraction in order to facilitate its further characterization by NMR and linkage analysis. Fraction 1b (F1b) was isolated after dialysis of a mixture of oligosaccharides obtained from acid hydrolysis of BSW at 120 °C for three hours. The carbohydrate composition of F1b is compared to the composition of BSW and β -mannanase degraded BSW in Table 2. Compared with the BSW, F1b is much more abundant in arabinose (19% vs. 1%), xylose (9% vs. 1-3%) and galactose (18% vs. 4-6%). As a result of the enrichment in these components, F1b has a much lower abundance of mannose than BSW (40% vs. 75-83%). Compared to F1 isolated by chromatographic

methods (Table 3), F1b is slightly less abundant in glucose (9% vs. 12%) and more abundant in arabinose (18% vs. 8%).

Linkage analysis of BSW oligosaccharides obtained by enzyme hydrolysis. We used GC-MS of partially methylated alditol acetates derivatives to determine the type of linkages present in β -mannanase treated BSW and in the acid resistant fraction of BSW hydrolyzed with TFA (F1b). Results are summarized in Table 4. The most abundant derivatives found in the BSW sample correspond to 1,4-linked mannose as the major component and minor amounts of 1,4-linked glucose, 1,2,4-linked mannose, 1,3,4-linked mannose, 1,4,6-linked mannose, and 1,4,6- and 1,3,6-branched galactose. In addition to these derivatives, the acid resistant fraction was found to contain derivatives corresponding to terminal arabinose, galactose and xylose, and 1,4-linked xylose. For both of the samples the overall composition of glucose, galactose and mannose determined from linkage analysis (summarized at the bottom of Table 4) are in reasonable agreement with the saccharide composition determined from the GC-MS of TMS-methyl glycosides (Table 2).

^{13}C NMR of β -mannanase hydrolyzed BSW. The ^{13}C NMR spectrum of BSW treated with β -mannanase is shown in Fig. 2A. The more intense resonances in the spectrum could be assigned to the carbons of internally β -1,4-linked mannose (M_I) and β -1,4-linked mannose at the reducing ($M_{R\alpha}$, $M_{R\beta}$) and non-reducing (M_{NR}) terminal mannose residues by comparing measured chemical shifts to the corresponding assigned resonances of $\text{Man}\beta(1\rightarrow4)[\text{Gal}\alpha(1\rightarrow6)]\text{Man}\beta(1\rightarrow4)[\text{Gal}\alpha(1\rightarrow6)]\text{Man}\beta(1\rightarrow4)\text{Man}\beta(1\rightarrow4)\text{Man}$ oligosaccharide ($\text{Gal}_2^{3,4}\text{Man}_5$) [17]. The two closely spaced resonances at 95.19 and 95.04 ppm could be readily assigned to C1 of the α - and β -anomeric forms of a reducing terminal mannose while the resonance at 101.56 ppm may be assigned to C1 of either internally β -1,4-linked mannose or mannose at the non-reducing end of the oligosaccharides present in the mixture. The presence in the reaction mixture of oligosaccharides

having a Dp of at least 3 is confirmed by resonances which are unique to internally β -1,4-linked mannose (M_1). These include the resonances for C2 (71.37 ppm), C3 (72.86 ppm) and C5 (76.41 ppm). The presence of internally linked mannose in the β -mannanase hydrolysis reaction mixture also is in agreement with chromatographic results, which show the presence tri- and tetrasaccharides (Fig. 1B).

Less intense resonances in the ^{13}C NMR spectrum of the β -mannanase hydrolyzed BSW sample at 70.96 ppm, 74.59 ppm and 77.40 ppm are unique to C4, C2 and C5 of a non-reducing terminal β -glucose while a minor resonance just above the noise level at 79.95 ppm may be assigned to C4 of internally β -1,4-linked glucose [46]. Resonances arising from either non-reducing terminal or internally β -1,4-linked glucose can be assigned to C1 (104.0 ppm) and to C3 (76.93 ppm). From peak integration of the anomeric carbon signals it may be determined that mannose and glucose account for 89% and 11% of the composition. These values compare with 83% mannose and 6% glucose determined from compositional analysis (Table 2).

Compositional and linkage analysis suggest that galactose is a component of β -mannanase hydrolyzed BSW equal in abundance to glucose. However, no resonances which can uniquely be assigned to galactose appear in the ^{13}C NMR spectrum. In other galactomannans galactose is commonly found α -1,6-linked to the β -1,4-linked mannose backbone [26-31, 31, 33, 34]. In the ^{13}C NMR spectrum, the C6 resonance of 1,4,6-branched mannose is shifted downfield by roughly 6 ppm compared to unbranched mannose. If present in the spectrum of the BSW oligosaccharide mixture, the C6 resonance of branched mannose would likely overlap with the much more intense resonance arising from C4 of non-reducing terminal mannose (68.10 ppm). Much smaller but significant shifts are also observed in the C4 and C5 resonances of 1,4,6-branched mannose relative to internally β -1,4-linked mannose [31, 34-36, 47]. Furthermore, shifts of these resonances, as well as that of C6, also appear to be sensitive to galactose branching at a near-neighbor residue [31-36, 46]. There is a resonance in the spectrum of hydrolyzed

BSW at 77.94 ppm which lies slightly upfield of the resonance assigned to C4 of unbranched internally β -1,4-linked mannose (78.15 ppm) and slightly downfield of the resonance assigned to C4 of the β -form of reducing terminal mannose and C5 of non-reducing terminal mannose (77.77 ppm). This resonance can be assigned to C4 of 1,6-branched mannose on the basis of the similarity of its chemical shift with the C4 resonance of branched mannose in Gal₂^{3,4}Man₅ oligosaccharide [27, 31]. The C5 resonance of 1,4,6-branched mannose is observed at 74.64 ppm in the ¹³C NMR of Gal₂^{3,4}Man₅ oligosaccharide. The fact that it is not observed in the spectrum of the hydrolyzed BSW sample may be due to the overlap of the C5 resonance with the much more intense C3 resonance of the non-reducing terminal mannose. A summary of all of the assigned resonances is presented in Table 5.

It is quite unusual that resonances from the structurally more abundant monosaccharide, mannose, suggest branching to galactose while the resonances of galactose are not observable in the ¹³C NMR spectrum of BSW hydrolyzate. An alternative explanation is that mannose 1,6-branching occurs to glucose rather than galactose. In order to test this possibility the β -mannanase hydrolyzed BSW sample was treated with α -galactosidase, an exo-glycosidase which cleaves only non-reducing terminal α -linked galactose. The spectrum of the reaction mixture is shown in Fig. 2B. The C4 resonance of 1,6- branched mannose at 77.94 ppm is absent from the spectrum and a new resonance appears at 98.45 ppm, which can be assigned to C1 of β -galactose [48]. On the other hand, the C1 of non-reducing terminal and internally β -linked glucose at 104.0 ppm does not disappear from the spectrum. The reaction mixture was then treated with the endo-glycosidase, β -glucanase. In the spectrum of the glucanase hydrolyzate (Fig. 2C) the resonance at 104.0 ppm has disappeared and C1 resonances of the α - and β -forms of glucose appear at 93.5 and 97.0 ppm. Resonances previously assigned to C2, C3 and C5 of non-reducing terminal β -glucose also disappear from the spectrum and new resonances appear which can be assigned to C2, C3 and C5 of β -glucose monosaccharide.

Identification of oligosaccharides isolated from the acid hydrolysis of BSW and from β -mannanase treatment of the acid resistant fraction (F1b). Table 3 shows that fractions F4-F8 (Dp 6 through Dp 2), isolated from the acid hydrolysis of BSW, were composed of a number of monosaccharide components higher than their expected degree of polymerization, suggesting that these fractions were impure mixtures of oligosaccharides. For example, F7 (Dp 3) should contain three saccharides instead of four found. Therefore, for structural analysis purposes, further purification with other types of chromatography was required. Mixtures contained in F7 and F8 (Dp 3 and Dp 2) were further purified via ion exclusion chromatography, while F5 and F6 (Dp 4 and Dp 5) were purified using reverse phase chromatography. The β -mannanase treated acid resistant fraction, F1b, was also purified by ion exclusion chromatography. Chromatograms for these purifications are shown in Fig. 3. Chromatograms of F6 and F7 (Dp 3 and Dp 2) show a major peak (Dp3.2 and Dp2.2) with three smaller components (i.e., Dp2.1, Dp2.3 and Dp2.4 for chromatography of F7). The chromatogram for the fractionation of the enzyme treated acid resistant fraction, F1b, shows seven subfractions. The most abundant subfractions were collected and further characterized using a combination of carbohydrate compositional analysis, ^1H NMR and two-dimensional ^1H NMR and ^{13}C NMR, when adequate sample was available.

The carbohydrate compositions of Dp2.2, Dp2.3, Dp3.2, Dp4.2 and Dp5.2 are summarized in Table 6. The data show that fractions Dp2.2 and Dp2.3 contain greater than 80% mannose with minor constituents of galactose and glucose, respectively. Fraction Dp4.2 contains greater than 90% mannose. In contrast, fractions Dp3.2 and Dp5.2 contain 20-30% glucose, in addition to the more abundant mannose component.

The anomeric proton region of the ^1H NMR spectra of Dp2.2, Dp3.2, Dp4.2 and Dp5.2 are shown in Fig. 4. The spectrum of Dp2.2 (Fig. 4A) shows resonances which can be assigned to the anomeric protons of the reducing terminal mannose residue at 5.27 and 5.00 ppm ($M_{R\alpha}$ and $M_{R\beta}$) and to the non-

reducing terminal mannose at 4.81 ppm ($M_{NR\beta}$) of β Man1,4Man disaccharide (Man_2) [35, 36, 53, 54]. The ratio of the integrals for the anomeric protons assigned to the non-reducing residue to those assigned to the reducing residue is about 1, as would be expected for a disaccharide. The anomeric carbon region of the ^{13}C NMR of Dp2.2 shows resonances of nearly equal intensity which can be assigned to the anomeric carbon on the non-reducing mannose residue (101.56 ppm) and on the reducing terminal residue (95.3 ppm) of Man_2 [54]. These results are consistent with the assignment of the Man_2 structure made using 1H NMR. Additional resonances are present in the 1H NMR spectra of Dp3.2, Dp4.2 and Dp5.2 near 4.84 ppm, and likely arise from anomeric protons on non-reducing mannose residues at the terminal and in the center (1,4-linked) of longer oligosaccharides present in these samples. The ratio of integrals of non-reducing to reducing anomeric protons in samples Dp4.2 and Dp5.2 are close to 2 and 3, suggesting that these fractions contain β Man1,4 β Man1,4Man trisaccharide (Man_3) and β Man1,4 β Man1,4 β Man1,4Man tetrasaccharide (Man_4). The near absence of minor monosaccharides other than mannose in the Dp4.2 sample suggests that this sample is nearly pure trisaccharide. The relative intensities of resonances in the ^{13}C NMR spectrum of this fraction (Fig. 5B) confirm the assignment of Dp4.2 to Man_3 . However, since Dp5.2 was shown by carbohydrate compositional analysis to contain ~30% glucose, other oligosaccharides may be present in this sample which are not observed in the 1H NMR spectrum. The ratio of integrals for the non-reducing anomeric protons to reducing proton resonances is about 1.5 in the spectrum of Dp3.2. This result suggests that there is more reducing terminal mannose present than would be expected for Man_3 but more non-reducing mannose present than would be expected from Man_2 . The COSY NMR of this fraction shows a resonance at 4.52 ppm which can be assigned to the anomeric proton of a non-reducing β -1,4-linked glucose. The complete assignment of glucose resonances from the COSY NMR of this fraction suggest the presence of β Glc1,4 Man_2 [36, 55]. Of the remaining subfractions obtained from the acid hydrolysis of BSW which were characterized

by NMR, Dp2.3 was found to be a mixture of Man₂ and glucose. Other subfractions contained non-carbohydrate components.

The ¹H NMR spectra of oligosaccharides isolated from the enzyme treated acid resistant fraction, AR.3 and AR.5, are also shown in Fig. 4. The ratio of integrals of the non-reducing anomeric proton resonances to the reducing anomeric proton resonances is 0.7 for AR.3. This means that there are more reducing mannose terminal mannose residues present in the sample than would be expected if Man₂ were the only component present. Complete proton assignments of glucose and mannose residues of βGlc1,4Man were made from the COSY NMR of AR.3 (Fig. 6) and the presence of a resonance at 104.0 ppm in the ¹³C NMR spectrum confirms the presence of this disaccharide [54]. From integration of proton resonances in the ¹H NMR and the COSY spectrum, we estimate that this fraction contains about 30% βGlc1,4Man and 70% Man₂. These results are consistent with the compositional analysis of AR.3. The ¹H NMR of AR.5 shows the presence of an anomeric proton resonances which can be assigned to a non-reducing α-galactose [31]. The ratio of integrals of non-reducing anomeric proton resonances to reducing proton resonances is exactly 1.0, consistent with the presence of Man₂ or a substructure containing Man₂. Furthermore, since H1 of galactose couples only to H2, one would expect a doublet in the ¹H NMR spectrum rather than the triplet that is observed for this resonance. The apparent “triplet” likely arises from a partial overlap of doublets, each arising from a single anomer at the reducing terminal of the structure. That is effect is observed suggests that the galactose is linked to a reducing mannose in the structure βMan1,4[αGal1,6]Man. Integration of the anomeric galactose proton is about 0.5, suggesting that more than a single component is present in the AR.5 subfraction. From this value and the confirmation of the presence of both βMan1,4[αGal1,6]Man and Man₂ in the COSY spectrum, we estimate that AR.5 consists of an equal mixture of these two oligosaccharides. AR.5 also contains

glucose monosaccharide and anomeric proton resonances for this component are observed in the ^1H NMR at 5.30 and 4.78 ppm.

The NMR of AR.1 and AR.2 were not of sufficient quality to carry out structural determination. A summary of assigned ^1H and ^{13}C shifts for the oligosaccharides identified in the fractions is given in Table 8. A summary of the fractions analyzed and their structures is given in Table 9.

DISCUSSION

Acemannan, the most abundant polysaccharide to be isolated from *Aloe vera*, is not unique in its ability to elicit pharmacological activity. A variety of glycans have been reported to have antiviral and antitumor activity, believed to be a result of their ability to activate macrophages and T cells [17, 23, 50] or to stimulate complement [15]. In some cases, minor changes in structure, molecular weight or conformation of the polysaccharide may have dramatic effects on potency. For example, different antiviral activity of the xylo-mannans from *Nothogenia fastigiata* was explained on the basis of a flexible backbone, molecular size, content and distribution of sulfate groups and of the single stubs of β -1,2-linked xylose [50]. Mouse macrophages were stimulated by an immobilized glycan but not by soluble glycan of the same structure, leading to the postulate that the spatial arrangement of the glycan was decisive for the activity [51].

The carbohydrate composition of the mucilaginous polysaccharide from *Aloe vera* gel has long been disputed [10-17, 52]. Recent work in which the polysaccharide was either purified chromatographically or alcohol precipitated in a manner similar to the isolation method for BSW have shown that mannose comprises greater than 75 % of the carbohydrate fraction with traces of xylose, arabinose, fucose, rhamnose and uronic acids [10,11,15]. Except for the recent study by Fememia et al. [10], most studies

are also in agreement that glucose comprises between 3 and 6% of the *Aloe vera* polysaccharide [11-16]. There appear to be more striking discrepancies in the fractions of galactose found. While some suggest that galactose comprises less than 1.5% [10,13,16], others suggest that the composition with respect to galactose is roughly equal to that of glucose [11,15]. It has been suggested that discrepancies in carbohydrate composition may be traced to differences in the methods used to isolate the polysaccharide [10,11]. It is also quite possible that some of the discrepancies arise from the methods used to hydrolyze the polysaccharide for compositional analysis. Results from the present study suggest that only 30% of BSW is hydrolyzed under conditions more robust (2 M TFA, 120 °C, 48 hrs.) than are commonly used for analysis by GC-MS of alditol acetates [10]. Differences in overall composition occurring as a result of incomplete hydrolysis are reflected in results showing that a BSW sample more completely hydrolyzed by β -mannanase is found to have a greater fraction of mannose than a TFA hydrolyzed sample (Table 3, 84% vs 75%).

Results of the present study also suggest that minor monosaccharides (arabinose, rhamnose, fucose, xylose and uronic acid) found in BSW and Aloe polysaccharide isolated by others are components of separate contaminating pectins and hemicelluloses. If the BSW sample were homogeneous then these minor monosaccharides would be equally distributed among the fractions released from the polysaccharide upon TFA hydrolysis. The fact that they are instead concentrated in the acid resistant fraction suggests that the BSW sample is non-homogeneous, with respect to structure and reactivity. Although acid resistance has been previously observed for pectins, where backbone arises from α -1,4-linked galacturonic acid residues [41], there is not evidence for such a structure in BSW, given the relatively low uronic acid composition.

Linkage analysis results for deacetylated β -mannanase treated BSW are consistent with a mannan with the majority of mannose residues 1,4-linked (76%). Because the majority of glucose is also 1,4-linked

(7%), the data are consistent with previously suggested structures having lengthy 1,4-linked mannose substructures with 1,4-linked glucose substituted randomly along the chain [10-16]. Previous workers have found mannose residues in the polymer to be acetylated at O2/O3 and O6 in a ratio of approximately 1:1, with an overall degree of acetylation of 0.78/residue [11-14]. Because the BSW sample was deacetylated prior to analysis, 1,2,4-linked mannose (6%), 1,3,4-linked mannose (2%) and 1,4,6-linked mannose (2%), must arise from branching. Since residues other than mannose and 1,4-linked glucose account for only 13% of the remaining composition and branched mannose residues collectively account for less than 10% of the composition, an extensively branched structure, if it exists, must have long unbranched 1,4-linked glucomannan sidechains. This type of structure was initially suggested by Mandal and Das [16].

^{13}C NMR results for β -mannanase hydrolyzed BSW show the presence of terminal reducing and non-reducing mannose and glucose and internally β -1,4-linked mannose and β -1,4-linked glucose. The intensity ratio of the C1 resonance of β -1,4-linked mannose and non-reducing terminal mannose to that of reducing mannose is about 1:1 and suggests a mixture of short oligosaccharides, consistent with the results obtained by size exclusion chromatography and linkage analysis. Although no resonances for galactose appear in the spectrum, resonances appear in the spectrum of β -mannanase hydrolyzed BSW for the C4 and C5 of 1,4,6-linked mannose. These resonances disappear upon further treatment of the sample with α -galactosidase, an exo-enzyme able to remove only terminal nonreducing galactose residues. Hence, the ^{13}C NMR data of β -mannanase and α -galactosidase treated BSW suggests that there is a fraction of mannose residues substituted at C6 with single α -galactose residues. Furthermore, α -1,6-galactose substituted mannoses are the only branched mannoses detected by the ^{13}C NMR experiment. This proposed sidechain structure is consistent with linkage analysis results which show that non-reducing

terminal galactose is the most abundant form of galactose in alcohol insoluble Aloe polysaccharide [10] and in our deacetylated β -mannanase hydrolyzed BSW samples (Table 2).

Further information on the majority structure present in BSW is provided by NMR structural analysis of oligosaccharides of fractions purified from acid hydrolysis of BSW. These fractions were found to contain Man_2 , Man_3 , and Man_4 , $\beta\text{Glc1,4Man}$, and $\beta\text{Glc1,4Man}$ and $\alpha\text{Gal1,6Man}_2$. A structure for BSW may be proposed based on the results for chromatography, carbohydrate compositional analysis, linkage analysis and NMR studies. That structure has a linear β -1,4-linked mannose backbone with β -1,4-linked glucose substituting for mannose approximately one in every 30 residues (Scheme 1). Mannose residues are acetylated at O2, O3 or O6, in agreement with previous findings [11-14]. Sidechains are single galactose residues α -1,6-linked to backbone mannoses. The proposed structure for BSW shares both similarities and differences with structures proposed for galactomannans, glucomannans galactoglucomannans. Galactomannans are β -1,4-linked linear mannans with single galactose sidechains but glucose is absent from the structure. Galactoglucomannans have a β -1,4-linked acetylated mannan backbone with substituted β -1,4-linked glucose [26-31]. However, most galactoglucomannans have ratios of mannose:glucose:galactose of approximately 1:1:1 and have sidechains more complex in structure than single galactose α -1,6-linked to mannose [30]. It is possible that the unique structure of BSW is responsible for its potent pharmacological activity.

Finally it should be emphasized that while the majority structure proposed for BSW is based on the most abundant fractions, those most readily available for NMR analysis. The acid resistant fraction makes up 37% of BSW and 60% of the carbohydrate contained in this fraction are non-mannose residues. Unfortunately, the low yield of subfractions containing these non-mannose residues following purification eliminated the possibility of their further structural analysis. The fact that an acid resistant fraction of

BSW exists and comprises over 37% of the bulk polysaccharide is remarkable and emphasizes the heterogeneity present in the sample.

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Table 1. Chromatographic data for BSW oligosaccharides obtained from acid hydrolysis^a

Fraction number	Dp ^b	Retention time (min)	Area ^c
F1	10	12.17	0.366±0.03
F2	8	17.03	0.015±0.00
F3	7	19.73	0.042±0.01
F4	6	21.66	0.064±0.02
F5	5	24.07	0.079±0.01
F6	4	27.14	0.108±0.01
F7	3	31.13	0.141±0.03
F8	2	35.64	0.144±0.07
F9	1	38.11	0.052±0.02

^aOligosaccharides were eluted from a Phenomenex Resec column (200 x 10 mm) using water as eluant.

^bA correlation between retention time and degree of polymerization was made using a dextran standard (see experimental).

^cReported as fraction of total area. Average and standard deviation were calculated from an average of two separate runs.

Table 2. Monosaccharide composition (mole%) of BSW and the acid resistant fraction of BSW^a

Glycoside	BSW ^b (Intact)	BSW ^c (β -mannanase)	F1b ^d
Ara	1.8 (1.1) ^e	1.4 \pm 0.2	18.8 \pm 1.6
Rha	0.2 (0.3)	0.1 \pm 0.0	nd
Fuc	0.2 (0.2)	1.5 \pm 0.1	nd
Xyl	3.3 (0.9)	0.7 \pm 0.1	9.1 \pm 0.1
Man	75.2 (76.8)	83.9 \pm 3.8	40.6 \pm 0.2
Glc	8.8 (12.1)	6.2 \pm 1.2	9.0 \pm 0.6
Gal	6.2 (1.4)	4.4 \pm 1.2	17.9 \pm 0.5
Gal UA	3.5 (7.2) ^f	2.4 \pm 0.4	4.2 \pm 0.4
Glc UA	0.8	0.1 \pm 0.0	0.4 \pm 0.4
Recovery ^g (μ g/mg)	30.3	83.3	

^aComposition was determined by GC-MS of TMS methyl glycosides as described in the Experimental Section. Standard deviations are reported in cases where three analysis were carried out.

^bAnalysis was carried out on intact BSW without prior deacetylation.

^cAnalysis was carried out on deacetylated BSW following treatment with endo- β -mannanase (1U of enzyme at 45 °C for 24 hrs.).

^dF1b was prepared by dialysis of TFA hydrolyzed BSW (120 °C for three hours).

^eNumbers in parenthesis were taken from reference 10 for the alcohol insoluble fraction of the gel extracted with water.

^fUronic acid in reference 10 was analyzed colorimetrically and reflects total Gal UA plus Glc UA.

^gRecovery is estimated by comparing peak areas to those of myo-inositol, added as an internal standard.

A number of factors including the yield of TMS-methyl glycosides from monosaccharides may influence this value.

Table 3: Monosaccharide composition (mole %) of acid hydrolyzed BSW fractions^a

Glycoside	F1 Dp10	F3 Dp7	F4 Dp6	F5 Dp5	F6 Dp4	F7 Dp3	F8 Dp2	F9 Dp1
Ara	8	0.26	nd	0.12	nd	nd	n.d.	17.15
Rha	3.09	0.63	nd	0.09	0.11	nd	n.d.	0.33
Fuc	nd	nd	nd	0.22	0.43	nd	0.33	2.67
Xyl	8.15	3.85	1.24	1.24	1.21	1.42	1.1	2.58
Man	43.86	58.85	68.37	63.48	75.16	80.07	82.8	59.65
Glc	12.41	18.88	20.87	13.41	11.15	7.68	6.07	4.22
Gal	21.06	13.4	4.81	21.44	11.95	10.83	9.69	13.4
Gal UA	1.82	4.13	4.32	n.d.	n.d.	n.d.	n.d.	n.d.
Glc UA	1.61	n.d.	0.39	n.d.	n.d.	n.d.	n.d.	n.d.

^aFractions F1-F10 were obtained following hydrolysis in 2 M TFA at 85 °C for 22 hours and partially purified as described in the caption to Fig. 1. Results are those of a single analysis.

Table 4: Glycosidic linkage analysis of BSW and F1b^a

Methylate Alditol acetate	BSW	F1b ^b
T-Ara	0.4±0.0	9.2±1.6
T-Fuc	nd	nd
T-Xyl	nd	3.6±2.7
T-Glc	nd	nd
T-Gal	0.8±0.3	3.8±0.1
1,4-Xyl	1.5±1.5	12.0±0.3
1,4-Man	76.5±4.0	44.4±1.0
T-Glc UA	2.0±0.8	4.0±0.2
1,4-Glc	7.0±0.7	8.0±1.3
1,3,4-Man	1.8±0.4	4.3±0.6
1,2,4-Man	6.1±0.3	7.5±0.7
1,4,6-Man	2.0±0.3	1.1±0.1
1,4,6-Gal	1.0±0.2	1.3±0.5
1,3,6-Gal	1.0±0.1	1.0±0.2
Total Glc	7.0	8.0
Total Gal	2.8	6.1
Total Man	86.4	57.2
Total Ara		9.2
Total Xyl		15.6

^aBSW and F1b were enzymatically hydrolyzed prior to linkage analysis. Analysis was carried out using GC-MS of partially methylated alditol acetates.

^bF1b was prepared by dialysis of TFA hydrolyzed BSW (120 °C for three hours).

Table 5. ^{13}C chemical shift assignments for oligosaccharides produced from hydrolysis of acemannan by β -mannanase^a.

Residue type ^b	C1	C2	C3	C4	C5	C6
β -Glc _R	104.03 (104.0)	74.21 (74.0)	76.93 (76.8)	79.95 (80.4)	71.37 (71.3)	61.91 (61.8)
β -Glc _{NR}	104.03 (103.9)	74.59 (74.7)	76.93 (77.1)	70.96 (71.2)	77.40 (77.4)	61.91 (62.1)
β -Man _{NR}	101.56 (101.38)	71.68 (71.82)	74.21 (74.19)	68.10 (68.05)	77.77 (77.79)	62.37 (62.34)
α -Man _R	95.19 (95.15)	72.07 (71.67)	70.32 (70.28)	78.15 (78.18)	72.34 (72.31)	61.91 (61.93)
β -Man _{NR}	95.04 (95.01)	72.07 (72.03)	73.04 (73.00)	77.77 (77.86)	76.17 (76.12)	61.91 (61.93)
β -Man _I	101.56 (101.46)	71.37 (71.29)	72.86 (72.85)	78.15 (78.17)	76.41 (76.28)	61.91 (61.93)
α -Gal(1→6)Man _I	101.56 (101.31)	71.37 (71.24)	72.86 (72.74)	77.94 (78.08)	n.d. (74.64)	68.10 (67.93)

^aChemical shifts are with respect to externally referenced dioxane (67.4 ppm). Numbers in parenthesis were taken from reference 42 for the reducing and non-reducing terminal β -1,4-linked glucose (β -Glc_R and β -Glc_{NR}) and from reference 27 for the remaining entries in the table.

^bAbbreviations in the table are β -1,4-linked mannose (β -Man_I), 4-linked α - and β -reducing terminal mannose (α - and β -Man_R), non-reducing terminal β -mannose (β -Man_{NR}) and 6-O- α -galactosyl β -1,4-linked mannose (α -Gal(1→6)Man_I).

Table 6: Composition (mole%) of soluble fractions obtained from TFA hydrolysis of BSW^a

Glycoside	Dp2.2	Dp2.3	Dp3.2	Dp4.2	Dp5.2
Ara	0.1	0.3	nd	nd	nd
Rha	nd	0.1	nd	nd	nd
Fuc	nd	0.21	nd	0.1	nd
Xyl	0.5	0.5	1.2	0.1	1.3
Man	87.6	82.7	73.1	92.6	61.6
Glc	0.9	12.9	21.1	3.64	31.3
Gal	9.86	3.4	4.6	3.7	5.8
Gal UA	1.3	0.2	nd	nd	nd
Glc UA	nd	nd	nd	nd	nd

^aDetermined by GC/MS of TMS-methyl glycosides. Due to small amount of sample purified,

compositional analysis was carried out for only one sample of each fraction.

Table 7: Composition (mole%) of fractions purified from the enzymatic digestion of the acid resistant fraction (F1b^a) of BSW^b

Residue	AR.1	AR.2	AR.3	AR.4	AR.5	AR.6
Ara	9.9±0.5	3.8±0.2	1.3±0.2	0.2±0.1	nd	0.6±0.2
Rha	nd	nd	nd	nd	nd	nd
Fuc	2.6±0.7	0.9±0.01	nd	nd	nd	nd
Xyl	30.6±3.5	11.6±1.8	2.3±0.1	0.4±0.1	nd	1.0±0.1
Man	27.2±1.2	14.0±0.4	60.6±0.5	84.5±2.1	56.5±0.5	72.6±0.3
Glc	9.0±0.5	22.0±1.1	26.1±0.6	9.2±0.7	23.8±0.2	9.8±0.1
Gal	1.7±0.2	39.2±1.1	6.2±0.1	4.2±0.9	18.2±0.3	13.4±0.1
Gal UA	19.3±1.6	8.5±1.6	2.9±0.2	1.2±0.2	nd	1.8±0.1
Glc UA	nd	0.3±0.1	0.8±0.2	0.3±0.1	1.6±0.1	1.0±0.1

^aF1b was prepared by TFA hydrolysis of BSW at 120 °C for three hours and was isolated after dialysis of the oligosaccharide mixture. Fractions were purified as described in the caption to Fig. 3.

^bDetermined by GC-MS of TMS-methyl glycosides. Standard deviations are estimated from three separate sample preparations

Table 8: Summary of assigned ^{13}C and ^1H NMR shifts^a

assignment	Man_2^{b}		Man_3^{c}		$\beta\text{GlcMan}^{\text{d}}$		$\beta\text{Man}1,4[\alpha\text{Gal}1,6]\text{Man}^{\text{e}}$
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1 α	5.27 (5.27)	95.25 (95.3)	5.27 (5.27)	95.26 (95.3)	5.27 (5.26)	95.26 (95.3)	5.27 (5.28)
1 β	5.00 (5.00)	95.12(95.3)	5.00 (5.00)	95.13 (95.3)	5.00 (5.00)	95.14 (95.3)	5.00 (5.01)
2 α	4.08 (4.09)	71.70 (71.7)	4.08 (4.09)	72.43 (71.7)	4.08 (4.08)	71.87 (71.9)	4.08 (4.10)
2 β	4.09 (4.09)	71.97 (72.0)	4.09 (4.09)	71.96 (72.0)	4.09 (4.08)		4.08 (4.10)
3 α	4.05 (4.08)	70.38 (70.4)	4.05 (4.08)	70.39 (70.4)	4.05 (4.08)	70.37 (70.6)	nd (4.09)
3 β	3.90 (3.89)	72.94 (73.1)	3.91 (3.89)	72.93 (73.1)	3.90 (3.89)	70.37 (73.4)*	3.95 (3.90)
4 α	3.98 (3.99)	78.00 (77.8)	3.98 (3.99)	78.05 (77.8)	3.98 (3.99)	77.92 (78.5)	nd
4 β	3.92 (3.95)	78.24 (78.2)	3.92 (3.95)	78.32 (78.2)	3.92 (3.95)	78.26 (78.5)	nd
5 α	3.96 (3.99)	72.38 (72.3)	3.96 (3.99)	72.4 (72.3)	3.96 (3.99)	72.38 (72.6)	nd
5 β	3.71 (3.66)	72.22 (76.2)*	3.71 (3.66)	76.17 (76.2)	3.71 (3.66)	72.38 (76.5)*	nd
6a α	3.92 (3.94)		3.92 (3.94)		3.92 (3.94)		nd
6a β	3.95 (3.99)	62.45 (62.0)	3.97 (3.99)	62.98 (62.0)	3.95 (3.99)		nd
6b α	3.82 (3.85)		3.82 (3.85)		3.82 (3.85)	62.46 62.0)	nd
6b β	3.83 (3.85)	61.93 (62.0)	3.83 (3.85)	62.98 (62.0)	3.83 (3.85)		nd
1' α			4.84 (4.85)			103.96 (104.2)	4.84
1' β	4.82 (4.83)	101.60 (101.6)	4.81 (4.85)	101.61 (101.6)	4.52 (4.60)		4.81
2' α			4.22 (4.22)			74.58 (74.7)	4.22
2' β	4.15 (4.16)	71.96 (72.0)	4.16	71.96 (71.9)	3.35 (3.41)		4.17
3' α			3.90 (3.89)			77.46 (77.6)	3.92
3'b	3.75 (3.75)	74.29 (74.3)	3.75	76.47 (74.3)*	3.54 (3.61)		3.78
4' α			nd (3.94)			70.99 (71.2)	3.88
4' β	3.69 (3.74)	68.16 (68.2)	3.67	68.16 (68.2)	3.47 (3.51)		3.7
5 α			nd (3.65)			76.98 (77.1)	nd
5' β	3.53 (3.52)	77.82 (77.8)	3.57	77.85 (77.9)	3.52 (3.59)		nd
6'a α	3.84 (3.83)	62.44 (62.0)	nd (3.89)	62.01 (62.0)	nd (4.01)	61.99 (62.0)	nd

6'aβ			3.85				nd
6'bα	4.04 (3.04)	61.98 (62.0)	nd	62.01 (62.0)	nd (3.82)		
6'bβ			4.06 (4.01)				
1"α/β			4.84 (4.86)	101.61 (101.6)			5.06 (5.13)
2"α/β			4.22 (4.23)	71.96 (71.4)			3.92 (3.94)
3"α/β			3.91 (3.91)	73.15 (73.0)			3.86 (3.94)
4"α/β			3.95 (3.99)	77.85 (77.9)			nd (4.11)
5"α/β			3.83 (3.86)	76.48 (76.5)			nd (4.01)
6"αα/β			3.86 (3.89)	62.01 (62.0)			nd (3.86)
6"βα/β			4.04 (4.08)				nd

^aTaken from COSY and TOCSY ¹H NMR and ¹³C NMR spectra.

^bNMR shifts in parenthesis were taken from references 35, 36, 53 and 54. Man₂ was found to be the only component of fractions Dp2.2 and AR.6 and was a major component of fraction Dp2.3, AR.3, and AR.4.

^cNMR shifts in parenthesis were taken from references 35, 36, 53 and 54. Man₃ was found to be the only component of fraction Dp4.2 and a major component of fraction Dp3.2.

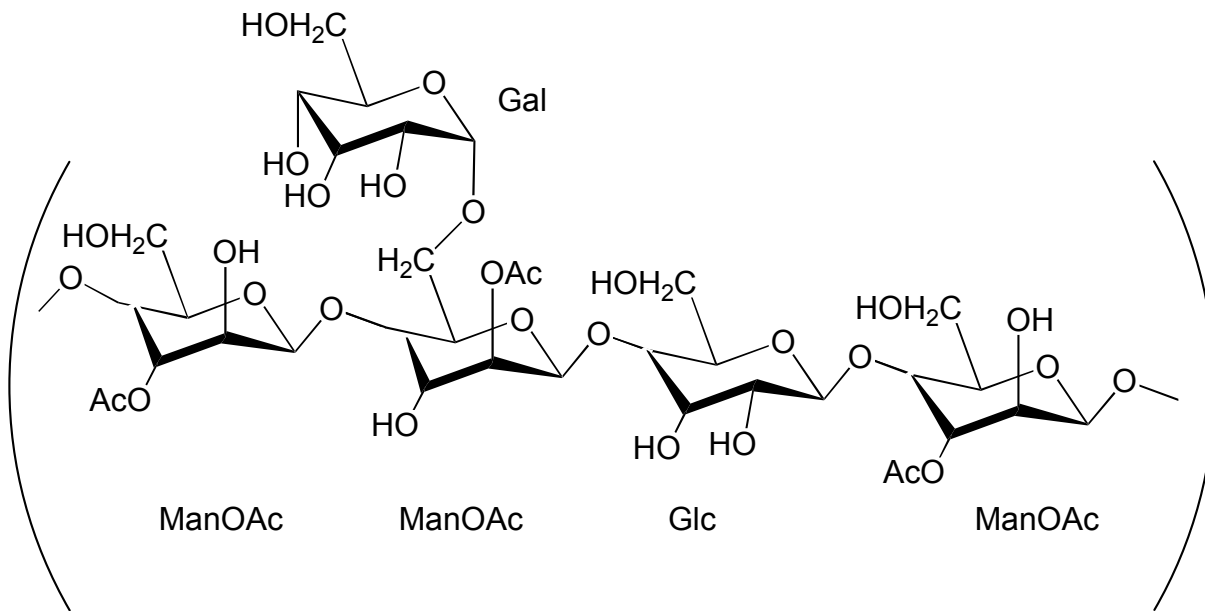
^dNMR shifts in parenthesis were taken from references 36 and 55. βGlc1,4Man occurs as a component of fractions AR.3 and AR.4.

^eNMR shifts in parenthesis were taken from reference 31.

*Reported shift assignments differ substantially from those previously determined by others.

Table 9: Summary of structures determined by NMR for purified oligosaccharide fractions

Fraction	Structures
Dp2.2	Man ₂
Dp2.3	Man ₂ + glucose
Dp3.2	Man ₃ + βGlc1,4Man ₂
Dp4.2	Man ₃
Dp5.2	Man ₄
AR.3	70% Man ₂ + 30 % βGlc1,4Man
AR.4	85% Man ₂ + 15% βGlc1,4Man ₂
AR.5	50% Man ₂ + 50% βMan1,4[αGal1,6]Man



Scheme 1: Proposed structure for the major component of *Aloe vera* polysaccharide

FIGURE CAPTIONS

Fig 1: Chromatograph of *Aloe vera* BSW following (A) acid hydrolysis (2 M TFA at 85 °C for 22 hrs.).

Retention times and percent of total integrated areas for each of the fractions were F1 (12.17 min, 36.6±3.0%), F2 (17.03 min, 1.5±0.0%), F3 (19.73 min, 4.2±1.0%), F4 (21.66 min, 6.4±2.0%), F5 (24.07 min, 7.9±1.0 %), F6 (27.14 min, 10.8±1.0 %), F7 (31.13 min, 14.1±3.0%), F8 (35.64 min, 14.4±7.0%), F9 (38.11 min, 5.2±2.0%). The abundance of all fraction remained nearly constant from run to run, as reflected in the relatively low standard deviations for the values cited. F1-F10 correspond to degrees of polymerization (Dp) 10 through Dp 1. (B) Chromatograph following treatment of deacetylated BSW with β -mannanase (1U of enzyme at 45 °C for 24 hrs.). Chromatography was carried out on a 200 x 10 mm Phenomenex Rezec column (85 °C) using water as eluent (0.4 mL/min). Peaks were detected using a refractive index detector.

Fig. 2: ^{13}C NMR of deacetylated *Aloe vera* BSW (A) treated with β -mannanase (1U of enzyme at 45 °C for 24 hrs.) (B) treated with β -mannanase followed by α -galactosidase (2U of enzyme at 45 °C for 24 hrs.) and (C) treated with β -mannanase and α -galactosidase followed by β -glucanase (34U of enzyme at 45 °C for 24 hrs.). Peak “X” is an unassigned resonance.

Fig. 3: (A) Ion exclusion chromatogram of fraction F8 (Dp2) isolated from the TFA hydrolysis of BSW. (B) Ion exclusion chromatogram of fraction F7 (Dp3) isolated from the TFA hydrolysis of BSW. (C) Reverse phase chromatogram of fraction F6 (Dp4) isolated from the TFA hydrolysis of BSW. (D) Reverse phase chromatogram of fraction F5 (Dp5) isolated from the TFA hydrolysis of BSW. (E) Ion exclusion chromatogram of the β -mannanase treated acid resistant fraction, F1b.

Fig. 4: ^1H NMR showing the anomeric proton resonances of fractions (A) Dp 2.2, (b) Dp3.2, (C) Dp4.2, (D) Dp5.2, (E) AR.3, and (F) AR.5. Fractions were isolated as shown in Fig. 3.

Fig. 5: ^{13}C NMR showing the anomeric carbon resonances of fractions (A) Dp2.2, (B) Dp4.2, (C) AR.3, and (D) AR.4.

Fig. 6: ^1H COSY spectrum of AR.3. As estimated from integration, this fraction is composed of approximately 70% Man_2 and 30% $\beta\text{Glc1,4Man}$. Coupling networks (H2-H4) are shown for the nonreducing terminal β -mannose residue of Man_2 and β -glucose of $\beta\text{Glc1,4Man}$ (nonreducing terminal = primed nomenclature, G = Glc, M = Man)..