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Polysaccharides from *Cannabis sativa* Active in Lowering Intraocular Pressure

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SUMMARY

Aqueous extraction of air dried Cannabis sativa *(marijuana) yields, after dialysis, a mixture of high molecular weight carbohydrate-containing components. This mixture has very potent intraocular pressure-lowering activity (antiglaucoma) when tested by intravenous injection into rabbits. Partial purification by DEAE-cellulose and gel filtration chromatography has yielded very active material (lowers intraocular pressure maximally at 1 μg/animal) with an estimated molecular weight of about 500 000. The active material contains mostly carbohydrate with a small amount of protein. Rhamnose, galactose and uronic acid are the major sugar constituents. The composition of components suggests that the active material is a pectic polysaccharide possibly derived from the cell wall.*

INTRODUCTION

We have discovered a novel activity for polysaccharides solubilized from dried leaves of *Cannabis sativa* by extraction with hot water. These

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carbohydrates, which have certain chemical and physical characteristics of pectic polysaccharides, lower intraocular pressure (IOP) when injected intravenously into rabbits. We have shown that these water-soluble materials lower IOP by a different mechanism (Green *et al.*, 1981, 1982) from certain cannabinoids (Green, 1982). Since the elevated IOP caused by glaucoma is a major cause of blindness, this polysaccharide material may have pharmaceutical applications. Further chemical investigations of these carbohydrates were therefore undertaken, and the results are reported in this paper.

EXPERIMENTAL

Extraction of *Cannabis sativa*

Samples of dried marijuana (primarily leaves and small stems of Czechoslovakian variety) were obtained from the University of Mississippi Research Institute for Pharmaceutical Sciences under the auspices of the National Institute of Drug Abuse. Portions were macerated in a Waring blender (3·8 litre capacity) with warm (55–60°C) water (eight times the weight of the sample). Optimum activity was obtained from samples blended at low speed (15 500 rpm) for 0·5–1·0 min. The macerated samples were filtered through cheesecloth under vacuum, and the residue was then re-extracted with warm water without further maceration. Extracts were centrifuged at 1340 *g* for 60 min; the supernatants were dialyzed against distilled water, lyophilized and tested for IOP-lowering activity.

Ion exchange chromatography

Crude, dialyzed extracts were lyophilized and then applied to a column (2·5 cm × 45 cm) of DEAE-cellulose equilibrated in 0·05 M Tris-HCl, 0·25 M NaCl, pH 6·7. The first 250 ml eluting under these conditions were pooled, dialyzed against water and lyophilized. Highly-coloured material was retained on the column.

Chemical analysis

The carbohydrate compositions of various samples were determined by gas chromatography of the alditol acetates using the method of Albersheim *et al.* (1967). Gas chromatography using flame ionization detection (FID) was performed with a 2 mm × 1·8 m column of SP 2340 and

a temperature programme beginning at 190°C for 5 min and then 5°C per min to 275°C. Helium was used as the carrier gas at a flow rate of 30 ml/min.

Total neutral sugar was determined by the phenol–sulphuric acid method of DuBois *et al.* (1956), using D-glucose as standard.

Partial amino acid analyses were performed on acid hydrolysates (6 M HCl, 18 h, 110°C *in vacuo*) by ion-exchange chromatography using post-column derivatization with ninhydrin.

Uronic acid content was determined by the method of Blumenkrantz & Asboe-Hansen (1973), using *o*-phenylphenol (Sigma) as reagent and galacturonic acid as the standard.

Protein content was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as the standard.

Reduction of uronic acids

Uronic acids were reduced with $NaBH_4$ after activation of carboxyl groups with a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma), according to the method of Taylor *et al.* (1976), scaled to a 1 mg sample. The reduced polysaccharide, recovered by lyophilization instead of precipitation, was hydrolyzed, and the products were converted to alditol acetates for analysis as described above.

Gel filtration chromatography

A column (1·5 cm × 45 cm) of Fractogel TSK HW-65F (Merck) was equilibrated in 0·05 M $(NH_4)_2CO_3$, pH 7·8. Samples (100 mg) of DEAE-cellulose fractions were applied, and 1·4-ml fractions were collected. Aliquots of fractions were assayed for total carbohydrate, uronic acid, protein, neutral sugar, and IOP-lowering activity.

Chromatography on Fractogel TSK HW-65F was performed with three other solvent systems: 0·05 M sodium phosphate–0·2 M LiBr, pH 6·5; 4 M guanidine hydrochloride; and 0·5 M NaCl containing 0·005 M EDTA.

Affinity chromatography

A column containing Concanavalin A–agarose (Sigma) was equilibrated in solvent containing 1 mM $MnCl_2$, 1 mM $MgCl_2$, and 0·1 M NaCl. The pH

was adjusted to 5·3 with acetic acid. A sample of DEAE-cellulose fraction was applied in the same solvent, and the column was eluted with one column volume of equilibrating solvent. The column was then eluted successively with one column volume each of solvents containing 20 mM α-methyl-D-mannoside and 200 mM α-methyl-D-mannoside. The three fractions were dialyzed against distilled water, lyophilized, and analyzed for carbohydrate and IOP-lowering activity.

A column of *Ricinus communis* agarose (RCA-I, E-Y Laboratories) was equilibrated in 0·01 M Tris-HCl, 0·1 M NaCl, pH 7·65. A sample of active fraction 2 was applied in this buffer. The column was eluted with successive column volumes of starting buffer and buffers containing 20 mM and 200 mM β-methyl-D-galactoside. Each of the three fractions was dialyzed against distilled water, lyophilized, and analyzed for IOP-lowering activity.

Chromatofocusing

A column (1·0 cm × 30 cm) was packed with PBE 94 (Pharmacia) and equilibrated in 0·025 M imidazole-HCl buffer, pH 7·35. Polybuffer (Pharmacia) (5 ml), pH 4·0, was passed through the column, and then a sample of a DEAE-cellulose fraction in imidazole buffer was applied. The column was eluted with Polybuffer, pH 4·0, and the effluent was monitored at 280 nm. Fractions were collected, and aliquots were assayed for neutral sugar. The pH of the fractions was also monitored. Washing the column with 1 M NaCl removed material bound at pH 4·0.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to Gabriel (1971).

Pronase digestion

A 6·4 mg sample of a DEAE-cellulose fraction was dissolved in 1 ml of 0·15 M Tris acetate–0·0015 M calcium acetate, pH 8·0. Digestion was carried out at 37°C in the presence of toluene as an antibacterial agent for 72 h with five additions of pronase (Sigma Protease XIV). The total sample volume was then applied to the gel filtration column, and carbohydrate was monitored by the phenol–sulphuric acid assay.

Alkaline borohydride reaction

A 1·4 mg sample of an active fraction obtained from DEAE-cellulose chromatography was dissolved in 1·4 ml of 0·05 M NaOH containing 1 M $NaBH_4$ and heated at 50°C for 15 h. An aliquot was taken for IOP testing, and the remainder was applied to the gel filtration column. Fractions were monitored for carbohydrate by the phenol-sulphuric acid assay.

Intraocular-pressure screening

IOP measurements were made with an Alcon Pneumatonograph on both eyes of an adult rabbit. Baseline values were obtained on readings 30 min prior to and just before the time of intravenous injection of the sample to be tested. Seven hourly measurements were made after delivery of the sample. Measurements taken from four rabbits treated identically at the same time were averaged, and percentage changes in IOP are reported. Additional details of the assay have been described by Deutsch *et al.* (1981) and Green *et al.* (1981).

RESULTS AND DISCUSSION

All water extracts and subsequent fractions obtained from *Cannabis sativa* were tested for IOP-lowering activity by intravenous injection into rabbits (Green *et al.*, 1981; Deutsch *et al.*, 1981). In these assays, activity is expressed as the percentage fall in IOP after administering the sample compared with resting pressure. The maximum percentage fall is about 60%, at which point the IOP is equal to venous pressure.

Water extracts from *Cannabis sativa* were dialyzed and fractionated on DEAE-cellulose. Batchwise elution of DEAE-cellulose using 0·05 M Tris-HCl, pH 6·7, containing 0·25 M NaCl yielded an active material used for subsequent studies to characterize the active component. Attempted elution of extracts at lower ionic strength resulted in material becoming irreversibly bound to the resin.

The active fraction from DEAE-cellulose chromatography was analyzed for carbohydrate and protein content. Carbohydrate analysis of the active DEAE-cellulose fraction showed the presence of about 36% neutral sugar by weight (Table 1), and the relative proportions of

TABLE 1
Composition of Fractions (Weight Per Cent) Obtained from Water Extract of *Cannabis sativa*

Fraction	*Neutral sugar*[a]	*Uronic acid*[b]	*Protein*[c]
DEAE-cellulose	36	12	26
Fractogel			
1	19	3	38[d]
2	22	12	16[d]
3	22	10	23[d]

[a] Determined by phenol-sulphuric acid assay.
[b] Determined by *o*-phenylphenol assay.
[c] Determined by Lowry method.
[d] Determined by amino acid analyses.

TABLE 2
Neutral Sugar Composition[a] (Mol Per Cent of Total Neutral Sugar) of DEAE-Cellulose Fraction Before and After Carbodiimide-Activated Reduction of Uronic Acids

Sugar	*Without reduction of uronic acid*	*With reduction of uronic acid*
Rhamnose	14·2	13·1
Arabinose	31·1	22·5
Xylose	6·6	7·6
Mannose	3·8	5·1
Galactose	35·4	41·8
Glucose	11·0	9·9
Glucosamine	trace	trace

[a] Determined by g.c. analysis of alditol acetates.

neutral sugars were determined by gas chromatography of the alditol acetates (Table 2). The protein content of each fraction, as determined by the Lowry colorimetric test, and the uronic acid content, as determined by the *o*-phenylphenol colorimetric test, are also listed in Table 1.

The type of uronic acids present in the active material was ascertained by reduction of the acids to the corresponding neutral sugars by the method of Taylor *et al.* (1976). A sample of the DEAE-cellulose fraction was treated with a water-soluble carbodiimide and reduced with $NaBH_4$. Reduction of uronic acids was 98% complete, based on the colorimetric response of a control sample before and after reduction using the *o*-phenylphenol reagent. Recovery of material was ascertained by phenol–sulphuric acid assay before and after reduction; according to this method, virtually 100% of the material was recovered. The reduced sample was then hydrolyzed and subsequently converted to the alditol acetates in the normal fashion. The neutral sugar composition showed significant changes in the amounts of only two of the sugars, arabinose and galactose (Table 2). The arabinose content decreased by almost 9%. In our hands, the method used for the reduction of uronic acids has resulted in loss of arabinose in all samples tested, including standards such as gum arabic. The acidic conditions of the reduction may cause this loss of arabinose. The galactose content showed a 6% increase compared with the non-treated control. These results suggest that the predominant uronic acid present in the active sample is galacturonic acid. The degree of esterification of the uronic acids has not yet been determined.

An attempt was made to purify the DEAE-cellulose fraction further by means of the affinity of its carbohydrate moieties for Concanavalin A, a lectin which shows affinity for α-D-mannose and α-D-glucose residues. When a sample was applied to a column of Concanavalin A–agarose, however, the active component passed through the column unbound.

Chromatofocusing was used in an attempt to separate the components of the DEAE-cellulose fraction by their isoelectric points. The active material remained bound to the column and was only eluted with 1 M NaCl, suggesting that the material has an isoelectric point less than 4·0.

Separation of the DEAE-cellulose fraction on a column of Sepharose 6B showed the presence of very high molecular weight components which eluted at or near the void volume. Excluded material on this column would be expected to have a molecular weight near 1×10^6. A column of Fractogel TSL HW-65F, which fractionates polysaccharides in a molecular weight range of 1×10^4 to 1×10^6, was prepared in 0·05 M $(NH_4)_2CO_3$. A sample of the DEAE-cellulose fraction was

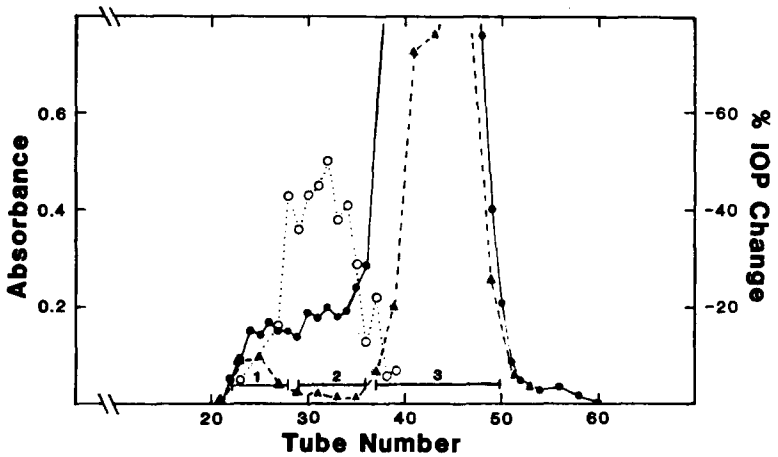

Fig. 1. Gel filtration of DEAE-cellulose fraction on Fractogel TSK HW-65F. A 1·5 cm x 45 cm column was equilibrated in 0·05 M $(NH_4)_2CO_3$, pH 7·7. Neutral sugar was monitored by phenol-sulphuric acid assay and absorbance at 490 nm was measured (●). Protein was assayed by the Lowry method, and absorbance at 660 nm was measured (▲). IOP was monitored as explained in the Experimental section (○).

separated on this column as seen in Fig. 1. The column effluent was monitored by the phenol-sulphuric acid colorimetric test for carbohydrates, and individual tubes were analyzed for neutral sugar and IOP-lowering activity.

Neutral sugar composition of the individual fractions of the Fractogel TSK HW-65F chromatography was determined by g.c. analysis of the alditol acetates. The results are plotted in Fig. 2. The most active fraction correlates with the neutral sugar composition which is highest in rhamnose and galactose as shown in Table 3 and indicated on Fig. 2.

The IOP-lowering activity for the individual fractions obtained after gel filtration chromatography as shown in Fig. 1 indicates that only those fractions in the intermediate area of the fractionation range exhibit activity. These tubes were pooled and designated fraction 2. Fractions 1 and 3 were also pooled as indicated in Fig. 1. The molecular weight range of the active fraction was estimated between 5×10^5 and 1×10^6 by calibrating the Fractogel column with a dextran sample of 5×10^5 average molecular weight. Attempts to assess the homogeneity and molecular weight of each fraction by conventional polyacrylamide

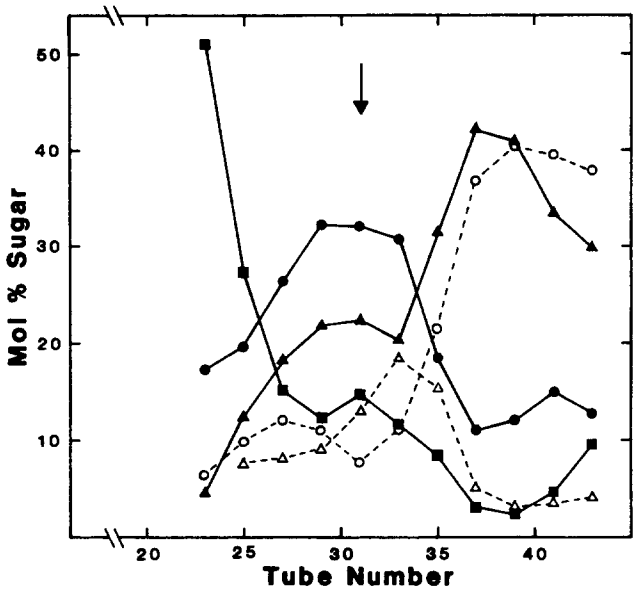

Fig. 2. Neutral sugar composition (mol% of total sugar) of individual fractions obtained from Fractogel TSK HW-65F chromatography of the DEAE-cellulose fraction. The sugars indicated are as follows: rhamnose (●); galactose (▲); glucose (■); arabinose (○); xylose (△).

TABLE 3
Neutral Sugar Composition[a] of Fractogel TSK HW-65F Most Active Fraction

Sugar	*Content (mol %)*
Rhamnose	32·2
Arabinose	7·6
Xylose	11·4
Mannose	5·0
Galactose	23·7
Glucose	15·6
Glucosamine	4·5

[a] Determined by g.c. analysis of alditol acetates.

TABLE 4
Neutral Sugar Composition[a] (Mol Per Cent of Total Neutral Sugar) of Fractogel TSK HW-65F Pooled Fractions

Sugar	*Fraction 1*	*Fraction 2*	*Fraction 3*
Rhamnose	19·8	27·9	12·7
Arabinose	9·8	9·9	38·1
Xylose	7·6	11·5	4·0
Mannose	10·6	6·1	4·5
Galactose	12·2	25·0	29·8
Glucose	27·2	14·1	9·5
Glucosamine	12·8	5·6	1·4

[a] Determined by g.c. analysis of alditol acetates.

TABLE 5
Partial Amino Acid Composition (Mol Per Cent) of Fractogel TSK HW-65F Pooled Fractions[a]

Amino acid	*Fraction 1*	*Fraction 2*	*Fraction 3*
Aspartic acid	12·0	11·6	13·7
Threonine	7·3	7·2	7·0
Serine	6·6	6·6	7·0
Glutamic acid	11·6	10·5	12·3
Glycine	10·6	9·4	9·9
Alanine	10·6	10·1	10·7
Valine	6·6	4·7	5·9
Methionine	1·2	1·1	1·2
Isoleucine	5·3	4·7	4·7
Leucine	12·7	17·9	8·4
Tyrosine	3·7	7·6	5·0
Phenylalanine	4·3	3·9	3·1
Histidine	1·7	0·3	7·9
Arginine	5·8	4·5	3·1

[a] Lysine was not resolved from ammonia. The amino acid analyzer was not equipped with a 440 nm channel for detecting proline and hydroxyproline.

gel electrophoresis (using 3·75% gel) were unsuccessful since the high molecular weight material would not enter the gel matrix. Summaries of the carbohydrate and partial amino acid analyses data for these fractions are given in Tables 4 and 5, respectively. A summary of the purification scheme routinely used and typical yields obtained for the isolation of active material is shown in Fig. 3.

The high content of galactose in the active fraction 2 suggested that further purification might be possible using the *Ricinus communis* lectin which specifically binds β-D-galactose. A sample of fraction 2 was applied to a *Ricin*-agarose affinity column which was successively eluted with the starting buffer, buffer containing 10 mM β-methyl-D-galactopyranoside and buffer containing 200 mM β-methyl-D-galactopyranoside. The sugar composition and IOP-lowering activity of the resulting three fractions are shown in Table 6. The bulk of the material and most of the activity eluted with the starting buffer. Again, the sugar composition of this active fraction shows a high percentage of rhamnose and galactose.

Since the high molecular weight of the active fraction poses problems in purification and characterization, several methods were

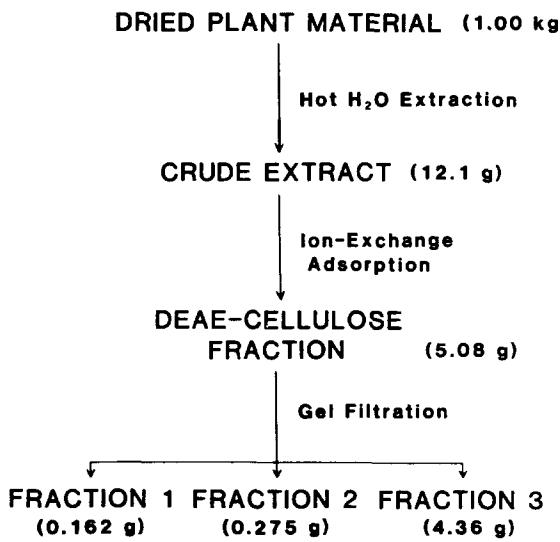

Fig. 3. Purification scheme for isolation of IOP-lowering active material from *Cannabis sativa*.

TABLE 6
Neutral Sugar Composition (Mol Per Cent of Total Neutral Sugar) Obtained from Ricin Affinity Chromatography of Fraction 2

Sugar	*Fraction*			
	Sample applied	*Unbound fraction*	*20 mM Methyl Gal*	*200 mM Methyl Gal*
Rhamnose	27·9	29·5	20·7	10·2
Arabinose	9·9	12·4	21·6	12·6
Xylose	11·5	7·8	3·5	9·4
Mannose	6·1	5·0	1·9	8·7
Galactose	25·0	24·0	43·8	31·5
Glucose	14·1	14·7	6·1	26·0
Glucosamine	5·6	6·7	2·3	1·6
IOP-lowering activity[a]	−47·6	−50·4	−12·8	−9·9

[a] 2 μg dose, 4–7 h average fall.

attempted to reduce the size of the material and yet retain activity. Two methods were tried, one which specifically cleaves amino acid linkages and one which cleaves *O*-glycosidic bonds.

A sample of the DEAE-cellulose fraction was digested extensively with pronase, a mixture of nonspecific proteases which digests peptides to free amino acids. The digested sample was applied to a gel filtration column. Compared with the original elution profile of the DEAE-cellulose fraction, the high molecular weight peaks shifted slightly to lower molecular weight. Also, the low molecular weight peak broadened. The activity profile, however, remained the same.

An active fraction obtained from gel filtration was treated with alkaline borohydride which hydrolyzes *O*-glycosidic bonds. This sample was polydisperse in molecular weight when reapplied to the Fractogel column and was not active.

Our work indicates that the water-soluble component from *Cannabis sativa* that is active in lowering IOP is of high molecular weight (between 5×10^5 and 1×10^6 daltons) and primarily a carbohydrate-containing material. From chromatofocusing studies, the isoelectric point of the active material appears to be less than 4·0. Rhamnose, galactose and

uronic acids are the major sugar constituents. The active material does not bind on columns of Concanavalin A or *Ricinus communis* lectin suggesting that α-D-glucose, α-D-mannose, or β-D-galactose are not present in bindable locations on the material. The composition of the components and its extraction from plant tissue by hot water suggest that the active (IOP-lowering) material is a pectic polysaccharide.

The high molecular weight of this material has posed problems both in separation procedures and in formulating a mode of action for the component. Chaotropic (0·2 M LiBr) and denaturing solvents (4 M guanidine-HCl; 0·5 M NaCl–0·005 M EDTA) have not affected the apparent molecular weight of the material as determined by gel filtration; the amino acid composition also does not show the presence of a large number of nonpolar residues that might enhance aggregation of several discrete components. Digestion with a nonspecific protease does not affect IOP-lowering activity, whereas extensive degradation of carbohydrate with alkaline borohydride destroys activity. These results in conjunction with the sugar composition data suggest that we might be dealing with a cell wall component (Aspinall, 1980; McNeil *et al.*, 1984).

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