Phytochemistry, Vol. 24, No. 11, pp. 2619–2622, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00
Pergamon Press Ltd.

IMMUNOLOGICALLY ACTIVE POLYSACCHARIDES OF *ACANTHOPANAX SENTICOSUS*

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(*Received* 30 *October* 1984)

Key Word Index—*Acanthopanax senticosus*, *Eleutherococcus senticosus*; Araliaceae; glucan; heteroxylan; plant polysaccharides; structure analysis.

Abstract—From the alkaline aqueous extract of *Acanthopanax* (*Eleutherococcus*) *senticosus* two homogeneous polysaccharides, a glucan with a mean M_r of 150 000 and a heteroxylan with a mean M_r of 30 000, have been isolated by DEAE–Sepharose CL-6B and Sephacryl S-400 column chromatography. Their structures were elucidated mainly by permethylation, periodate oxidation, Smith degradation, reduction experiments, partial hydrolysis and ^{13}C NMR. The isolated crude polysaccharide mixture and the heteroxylan enhance phagocytosis in *in vitro* and *in vivo* immunological tests.

INTRODUCTION

Acanthopanax senticosus L. is a small tree, which is widely distributed in the northeastern area of China. The roots are used as a tonic in Chinese traditional medicine [1] and are said to have adaptogenic activity [2]. In order to verify and confirm the medicinal use, a variety of chemical investigations have been undertaken, which led to the isolation of triterpenes, lignans, phenylpropane derivatives and coumarins [3]. While Brekhman [2] claimed that the adaptogenic activity of the drug can be assigned to the lignan fraction, preliminary studies on the polysaccharides of the drug, performed by a Chinese group [4], localized the immunological activity in a polysaccharide fraction of the drug. We have investigated the polysaccharides from *A. senticosus* and have isolated two homogeneous polysaccharides [5].

RESULTS

The dried powder of the roots was extracted with methanol followed by 0.5 M aqueous sodium hydroxide at 4°. A crude polysaccharide mixture was obtained as a brownish powder by precipitation of the aqueous phase with acetone, followed by treatment of the dissolved precipitate with trichloroacetic acid to remove protein. The crude polysaccharides were fractionated by DEAE–Sepharose CL-6B column chromatography with NaCl-gradient elution. The column fractions were monitored polarimetrically as well as by reaction with phenol sulphuric acid [6]. Each fraction was further purified on Sephacryl S-400, using 0.1 M NaCl as the eluent. Two polysaccharides (As II and III) were obtained as white powders. These polysaccharides were homogeneous as judged by HPLC [7] and gel chromatography. They were readily soluble in water and the M_rs were estimated to be in the range of 150 000 and 30 000 for AS II and III, respectively. The specific rotation $[\alpha]_D^{20}$ (*c* 0.1; H_2O) was +167° and −48° for AS II and III, respectively, showing that AS II contained mainly α-linked sugars, whereas AS III consisted of mainly β-sugar linkages.

The polysaccharides were free of nitrogen, based on elementary analysis. According to GC of the corresponding alditol acetates obtained from the sugars by $NaBH_4$ reduction, AS II contained only glucose and should be a glucan, whereas the underivatized hydrolysate of AS III yielded arabinose, xylose and 4-*O*-methyl-glucuronic acid in a molar ratio of 1:11:1, indicating a heteroxylan. AS III was the major component.

AS II was subjected to methylation by the method of Hakomori [8]. The fully *O*-methylated polysaccharide was hydrolysed, and the resulting partially *O*-methylated monosaccharides analysed by GC (OV-225 at 170°) after conversion to the corresponding *O*-methyl-alditol acetates. The *O*-methylated sugars were identified by comparing the RR_ts (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol 1) with those of authentic samples and the values reported in literature [9]. 2,3,4,6-Tetra-*O*-methyl-glucose (RR_t 1), 2,3,4-tri-*O*-methyl-glucose (RR_t 2.24) and 3,4-di-*O*-methyl-glucose (RR_t 4.22) were found to be present in a molar ratio of 1:15:1 for AS II. A sample of AS II was then partially hydrolysed with 0.5 M trifluoroacetic acid at 100° for 1.5 hours, followed by dialysis. The IR spectrum of the degraded AS II showed absorption at 850 cm^{-1} due to the presence of α-glycosidic linkages [10]. After conversion of the degraded polysaccharide into the corresponding methylated alditol acetates, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol was the only component detectable by GC.

According to these results, AS I should be a (1 → 6)-α-glucan with single, terminal α-linked glucose residues in position 2 of about every 15th glucose unit of the backbone.

The main component of the isolated polysaccharide fraction was AS III. Since this component was only slightly soluble in dimethylsulphoxide, the fully *O*-acetylated AS III was used as a starting material for permethylation. Since AS III contained glucuronic acid,

the carboxyl-reduced derivative was prepared by reduction of the fully methylated AS III with lithium aluminium hydride. Methylation and conversion of the partially methylated sugars into their corresponding alditol acetates as well as the GC and GC–MS analysis were performed as described above. 2,3-Di-*O*-methyl-xylose, 3-*O*-methyl-xylose, 2,3,5-tri-*O*-methyl-arabinose and 2,3,4-tri-*O*-methyl-glucose were identified and determined in a molar ratio of 15:6:2:2. A trace of 2,4-di-*O*-methyl-galactose was also detected. 2,3,4-Tri-*O*-methyl-glucose was only observed after carboxyl reduction and was obviously derived from the terminal 4-*O*-methyl-glucuronic acid residues. As 3-*O*-methyl-xylitol and 2-*O*-methyl-xylitol do not separate by GC on the phase used, the ion-chromatogram of the whole area of the peak was examined, but no evidence was found of other xylitol derivatives which would indicate the presence of a second branching point in position 3 of the xylose backbone (Table 1).

Periodate oxidation of AS III resulted in a maximum value of 0.40 mol periodate consumed per anhydrohexose unit. After further Smith degradation [11] of the periodate-oxidized AS III and analysis of the polyalcohols, xylose, galactose, ethyleneglycol and glycerol were detected by GC after conversion to the corresponding alditol acetates. The large amount of ethyleneglycol and glycerol detected demonstrated the presence of a high percentage of 1,4-linked xylosyl residues in the molecule. The presence of xylose might be due to oxidation resistant xylose residues such as 1,2,4-linked xylopyranose.

In another experiment, AS III was subjected to partial acid hydrolysis with 0.05 M trifluoroacetic acid at 100° for 1.5 hr. The hydrolysate dialysed against distilled water and the dialysable fraction analysed by TLC. Arabinose, xylose and two other unknown components were detected. The dialysable fraction was then subjected to column chromatography on Sephadex G-25, and the substances obtained from the first and second peak methylated. GC analysis of the first peak revealed the presence of 2,3,4-tri-*O*-methyl-xylose and 2,3-di-*O*-methyl-xylose in a molar ratio of 1:2, suggesting the presence of a 1,4-linked xylotriose. The second peak contained 2,3,4-tri-*O*-methyl-xylose and 2,3-di-*O*-methyl-xylose in a molar ratio of 1:1, confirming the presence of 1,4-linked xylobiose. The non-dialysable fraction, AS III-1B, was methylated, hydrolysed and the resulting hydrolysate analysed by GC as alditol acetates. The carboxyl-reduced AS III-1B hydrolysate gave 2,3-di-*O*-methyl-xylose, 3-*O*-methyl-xylose, 2,3,4-tri-*O*-methyl-glucose and 2,3,4-tri-*O*-methyl-xylose in a molar ratio of 5:2:1:1.

AS III-1B was further hydrolysed with 0.5 M trifluoroacetic acid at 100° for 2 hr and then dialysed. TLC analysis of the dialysable fraction revealed the presence of xylose, a 1,4-linked xylobiose, 1,4-linked xylotriose and three additional unknown components. Chromatography of the non-dialysable fraction on a Sephadex G-25 column resulted in the separation of one non-overlapping peak. Methylation of this substance followed by carboxyl reduction revealed the presence of 2,3,4-tri-*O*-methyl-glucose and 2,3-di-*O*-methyl-xylose in a molar ratio of 1:3. No 3-*O*-methyl-xylitol could be detected as in the methylation analysis of the original polysaccharide AS III. However, since 2,3,4-tri-*O*-methyl-glucitol was obtained as the reduction product from glucuronic acid, it is likely that 3,4-di-*O*-methyl-xylitol was present. According to the literature this methylation product overlaps with 2,3-di-*O*-methyl-xylitol in GC. The other peaks were not analysed due to peak overlapping.

In the ^{13}CNMR spectrum of AS III (Table 2), the chemical shifts of δ104.58 and 104.21 for the C-1, 64.91 and 64.24 for the C-5 signals of the xylopyranosyl residues suggested that the D-xylose residues were β-linked. The same β-linkage can be deduced for 4-*O*-methyl-glucuronic acid from the signal at δ100.52 [12, 13]. Chemical shifts of δ179.1 and 62.68 for the carbonyl and methyl groups, respectively, also indicated the presence of 4-*O*-methyl-glucuronic acid [14]. This was confirmed by the presence of a marked signal of a methyl group at δ3.4 in ^{1}H NMR spectrum.

Based on these results, it can be concluded that the backbone chain of AS III is predominantly composed of β-(1 → 4) linked xylopyranosyl residues, possessing branching points at position 2 and arabinofuranose and 4-*O*-methyl-glucuronic acid as non-reducing terminal residues.

DISCUSSION

Although previous investigations of *Acanthopanax* polysaccharides have not reported exact chemical structures for any of the components isolated [4], it is obvious that they are not identical to those of our investigations, since the qualitative and quantitative sugar compositions are quite different. This might be due to the use of drugs from different sources or to the differences in the extraction and fractionation procedure used by the Chinese group and ourselves.

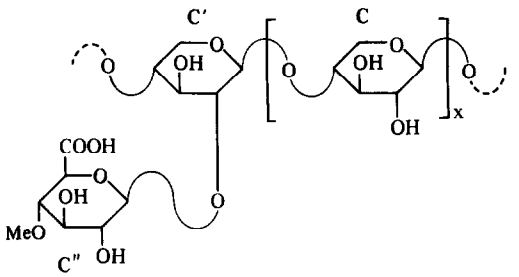
The isolated glucan and heteroxylan are the second chemically investigated polysaccharides of the Araliaceae family. The first polysaccharides studied were those of

Table 1. Alditol acetates from the methylated polysaccharide AS III

Partially methylated sugar	RR_t*	Mol ratios AS III	AS III (carboxyl reduced)	AS III-1B	MS (*m/z*)
2,3,5-Tri-*O*-methyl-arabinitol	0.46	2	2	—	45, 101, 117, 129, 161
2,3-Di-*O*-methyl-xylitol	1.18	15	15	5	43, 101, 117, 129, 161, 189
3-Mono-*O*-methyl-xylitol	2.07	6	6	2	43, 129, 189
2,3,4-Tri-*O*-methyl-xylitol	0.54	—	—	1	43, 101, 117, 161
2,3,4-Tri-*O*-methyl-glucitol	2.24	—	2	1	43, 101, 117, 129, 161, 173, 189, 233

*Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol on OV-225 at 170°.

Table 2. ^{13}C NMR data for AS III (D_2O)

Compound	Ring	C-1	C-2	C-3	C-4	C-5	C-6	OMe
	C	104.58	75.61	76.61	79.31	64.91		
	C′	104.21	79.02	75.14	79.72	64.24		
	C″	100.52	74.18	74.83	85.17	72.12	179.10	62.68

Panax ginseng which were separated into a starch-type of glucan, an arabino-3,6-galactan (pectin) and an acidic heteropolysaccharide, containing galactose, galacturonic acid, xylose and rhamnose [15].

The polysaccharides of *A. senticosus* described are related to similar structural types found in lower and higher plants. (1 → 6)-α-D-Glucans such as AS II have been detected in lichens, mosses and fungi, whereas 1,4-α-D-glucans are to be found predominantly in higher plants [16, 17]. AS III belongs structurally to the hemicellulose class and shows great similarity with the heteroxylans of the Gramineae family and hard wood [18].

As far as the immunostimulating activity of our polysaccharides is concerned, we found a strong phagocytosis enhancing effect (*ca* 30%) in two immunological test systems, mainly for the AS III polysaccharide. This activity is of the same order as that described for other acidic heteroglycans, which were isolated by us from some higher plants [19]. The details of the immunological results will be published elsewhere.

EXPERIMENTAL

Analytical methods. Evaporations were carried out under red. pres. below 40°. TLC: silica gel 60 F_{254}, $CHCl_3$–MeOH–H_2O, 8:5:1. Sugars were detected by spraying with diphenylamino-phosphoric acid reagent [20]. GC: glass column 0.3 × 200 cm, packed with 3% OV-225 on Gas Chrom Q, 80–100 mesh, oven temp. 215° for sugar analysis, 170° for methylation analysis, 150° for ethyleneglycol and glycerol analysis. ^{13}C NMR (Bruker WP 200 S4): chemical shifts are given in δ values; MS: Varian CH 7A coupled with a Varian 188-MS data bank; GC–MS with Carlo Erba (Milano)-Fractovap 2110 on a quarzcapillary 30 m × 0.25 mm with OV 1701.

Plant material. The roots of *A. senticosus* were collected in the Chinese Province of Shansi in 1982. A voucher specimen is available at the Herbarium of the Academica Sinica Materia medica, Shanghai, People's Republic of China.

Isolation and purification. The dried root powder (200 g) was first refluxed with MeOH for 6 hr. The residue (*ca* 190 g) was then extracted with 0.5 M NaOH (1000 ml) at 4° overnight and filtered. This process was repeated twice. The combined filtrates were precipitated with 2 vols of Me_2CO and then centrifuged. The precipitate was dissolved in H_2O (900 ml) and cold 15% TCA (900 ml) added with stirring. The soln was allowed to stand for 1 hr, after which the precipitate was collected by centrifugation. The supernatant was reprecipitated with Me_2CO (3600 ml), kept overnight and then centrifuged. The precipitate was dissolved in H_2O (380 ml), dialysed against running tap water for 2 days, followed by dialysis against deionized water for 1 day. The soln was then lyophilized to yield a crude polysaccharide fraction (3.5 g) as a brownish powder. The crude polysaccharide (250 mg) was dissolved in H_2O (8.5 ml) and applied to a column (2.6 × 48 cm) of DEAE–Sepharose CL-6B. The column was eluted first with H_2O (200 ml), followed by gradient elution with NaCl soln (0.0–0.7 M) (500 ml). Each tube contained 4 ml. Fractions II (Nos 65–70) and III (Nos 80–83) were collected and analysed polarimetrically and by the phenol–sulphuric acid method. Both fractions were dialysed against deionized water and then lyophilized. Amorphous powders were obtained (fract. II 11 mg, fract. III 81 mg). For further purification each fraction was dissolved in 0.1 M NaCl, applied to a column (2.6 × 48 cm) of Sephacryl S-400 and eluted with 0.1 M NaCl. The main peaks determined by refractive index, optical rotation and the phenol–sulphuric acid method were collected, dialysed and lyophilized to yield AS II and AS III as white powders (yield: AS II 5%, AS III 32%).

Homogeneity and M_r. These were determined by HPLC on μ Bondagel 125 E, 500 E and 1000 E columns (Waters Assoc., Milford, MA, U.S.A.) [7, 21]. The calibration curves were made with dextrans T 2000, T 110, T 70 and T 40. NaPi (0.2 M) buffer, pH 7.0, was used as a mobile phase, and the flow rate was maintained at 0.5 ml/min. All samples were prepared as 0.1% (w/v) solns. 15 μl of soln were injected in each run. Peaks were detected with a UV and an RI detector.

Sugar analysis. Polysaccharides (10 mg) were hydrolysed with 2 M TFA (3 ml) at 120° for 1 hr in a sealed tube. After the TFA was removed, the hydrolysate was analysed by TLC. One part of the hydrolysate was dissolved in H_2O (2 ml) and reduced with $NaBH_4$ (*ca* 6 mg) to the corresponding alditols. The reaction product was subdivided in two portions. One portion was acetylated whilst the other was reduced according to ref. [22], acetylated and analysed by GC.

Methylation analysis. Since AS III (8 mg) was only slightly soluble in DMSO, it was acetylated with a mixture of C_5H_5N (0.2 ml) and Ac_2O (0.15 ml) in formamide (0.4 ml) [23] and then methylated. Methylation was performed according to the method of Hakomori [8], followed by GC and GC–MS analysis of 4 mg of the partially methylated alditol acetate derivatives [9].

The remainder (5 mg) of the methylated AS III was dissolved in tetrahydrofuran (THF), and the soln added to a 1.25% suspension of $LiAlH_4$ in THF [24]. The mixture was stirred for 24 hr, the excess hydride destroyed by dropwise addition of H_2O, the soln filtered, and the residue extracted three times with $CHCl_3$

(3 ml). The combined extracts were evaporated to dryness. The methylated carboxyl reduced AS III was hydrolysed, acetylated and analysed by GC as described above [9].

Periodate oxidation of AS III. AS III (10 mg) was oxidized with 0.01 M $NaIO_4$ (20 ml) at 4° in the dark for 6 days. The periodate consumption was measured by a spectrophotometric method [25].

Smith degradation of AS III. Ethyleneglycol (0.2 ml) was added to the soln of the oxidized AS III, which was then dialysed against deionized H_2O for 24 hr. The soln was then reduced with $NaBH_4$ (8 mg) for 12 hr at room temp, after which it was acidified with 0.1 M HAc to pH 5.0 and dialysed for 24 hr. The non-dialysable fraction was lyophilized, then hydrolysed with 1 M TFA at 100° for 12 hr. Alditol acetate derivatives of the hydrolysate were analysed by GC.

Partial acid hydrolysis of AS III. Partial acid hydrolysis of AS III (50 mg) was carried out with 0.05 M TFA (14 ml) at 100° for 1.5 hr. TFA was removed from the hydrolysate and the soln dialysed against distilled H_2O. The dialysable fraction (AS III-1A) was analysed by TLC. AS III-1A was separated by gel filtration on a column (1.5 × 50 cm) of Sephadex G-25. The column was eluted with H_2O and each peak collected according to refractive index. The first and second peaks were methylated and analysed by GC. The homogeneity of the non-dialysable fraction (AS III-1B) was determined by HPLC. AS III-1B was methylated and analysed by GC.

AS III-1B was further hydrolysed with 0.5 M TFA at 100° for 2 hr. After removal of TFA the soln was dialysed against distilled H_2O. Only the dialysable fraction was analysed by TLC. The fraction was separated by gel filtration on a Sephadex G-25 column (1.5 × 50 cm) and eluted with H_2O. One non-overlapping peak was methylated, carboxyl reduced and analysed by GC.

Acknowledgements—We wish to thank Prof. Schäfer (Max Planck Institute of Biochemistry, Munich) for GC–MS analysis and Dr. J. Harangi (Debrecen) for measurement of ^{13}C NMR and ^{1}H NMR spectra.

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