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Structural features and biological activity of an acidic polysaccharide complex from *Mahonia aquifolium* (Pursh) Nutt

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Abstract

The crude hemicelluloses, isolated from stems of *Mahonia aquifolium*, on ion-exchange and gel chromatography gave an acidic polysaccharide (AP) which eluted as a broad band in high-pressure gel-permeation chromatography. The average apparent molecular mass (M_{app}) was determined to be 5.8 kDa. Compositional and linkage analyses, supported by 1D and 2D NMR spectroscopy measurements of the polysaccharide before and after modification using partial acid hydrolysis and digestion with pectinase led to the conclusion that AP was a complex of two closely associated polysaccharides. The first was a (4-*O*-methylglucurono)xylan with the molar ratio Xyl/MeGlcA = 6.7 and the second one a pectin with a very low proportion of neutral side chains. The pectin component contained homogalacturonan and rhamnogalacturonan RG-I regions in the ratio of about 2:1. Though the HMBC spectrum of AP indicated that some of the xylan chains might be linked to GalA of PGA, unequivocal proof for the existence of the linkage was not found. When tested for antitussive activity on a mechanically induced cough in cats, AP exhibited a pronounced cough-suppressive effect, much higher than that of some drugs used in clinical practice. The polysaccharide also showed mitogenic and comitogenic activities comparable to those of the commercial immunomodulator Zymosan—a beaker's yeast β-glucan.

Keywords: *Mahonia aquifolium*; Glucuronoxylan; Pectin; Structural features; Biological activity

1. Introduction

Mahonia aquifolium (Pursh) Nutt. (*Berberidaceae*) is a shrub widespread in the forests of the North American Pacific coast and is known also as an ornamental plant in gardens and parks called mahonia. It contains many pharmacologically important alkaloids which justify its use in traditional medicine for treatment of skin disorders, such as psoriasis, dermatitis and eczema (Gieler, Von der Weth, & Heger, 1995; Müller, Ziereis, & Gawlik, 1995). At present, a mahonia ointment is available as a topical antipsoriatic drug. In a previous paper (Košťálová, Kardošová, & Hajnická, 2001) the aqueous-ethanolic extract from mahonia and one of its polysaccharide component were shown to be active in tests for production of interleukin-8 (IL-8) by human acute monocytic leukemia cell line THP-1. Recently, we isolated and structurally characterized the most abundant component of the crude hemicellulose obtained from mahonia, a glucuronoxylan, which showed high cough-suppressing activity (Kardošová, Malovíková, Pätoprstý, Nosál'ová, & Matáková, 2002) and immunomodulatory properties (Ebringerová, Kardošová, Hromádková, Malovíková, & Hřibalová, 2002).

The aim of the present paper was to investigate the structure of the other, rather abundant polysaccharide component of the hemicellulosic material, particularly in relation to the positive responses in the above mentioned biological activity tests.

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2. Experimental

2.1. Materials

The plant mahonia was collected in the Arboretum Tesárske Mlyňany (Slovakia) and identified at the Department of Pharmacognosy and Botany, Faculty of Pharmacy, Comenius University, Bratislava where the voucher specimen is deposited. DEAE-Sephadex A-50 was obtained from Pharmacia (Sweden), Bio-Gel P-2 and P-4 from Bio-Rad (USA), and Sep-pak C_{18} cartridges from Waters Associates (USA). The α-1,4-D-polygalacturonase (pectinase, Gammapect LC) was a product of Gamma Chemie GmbH, Munich, Germany). ^{3}H-Thymidine ([6-^{3}H]-TDR, specific activity 960 GBq mmol^{-1}) was obtained from the Institute for Research, Production and Application of Radioisotopes (Prague, Czech Republic), Zymosan from Likospol Ltd (Bratislava, Slovak Republic), Phytohaemagglutinin (PHA) from Murex Biotech Ltd (England), and Polymyxin B from Wellcome (England). All chemicals used were of analytical grade.

2.2. General methods

Concentrations were performed under diminished pressure at a bath temperature not exceeding 45 °C. Free-boundary electrophoresis of polysaccharide solution (10 mg ml^{-1}) was performed in 0.05 M sodium tetraborate with a Zeiss 35 apparatus at 150 V and 8 mA for 30 min. Optical rotation of the polysaccharide (10 mg ml^{-1}, H_2O) was measured at 20 ± 1 °C with a Perkin–Elmer Model 141 polarimeter.

High-pressure gel-permeation chromatography (HPGPC) was performed using a commercial instrument (Laboratorní přístroje, Prague, Czech Republic) equipped with two Labio Prague Biospher GM 300 and 1000 exclusion columns (8 × 250 mm) and using aqueous 0.1 M $NaNO_3$ as solvent (0.4 ml min^{-1}). The eluate was monitored by RI detector. The columns were calibrated with pullulan standards P5–P50 (Shodex Standard P-82, Macherey-Nagel GmbH, Germany). IR spectra were measured using a Nicolet-Magna 750 spectrophotometer.

Descending paper chromatography (PC) was performed on Whatman No. 1 paper in the following solvent systems: S1, 8:2:1 EtOAc–pyridine–water; S2, 18:3:1:4 EtOAc–AcOH–formic acid–water, the sugars being detected with anilinium hydrogen phthalate. Carbohydrates were determined by the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Total hydrolysis of polysaccharides was effected with 2 M CF_3CO_2H (TFA) at 120 °C for 2 h. The sugars in the hydrolyzate were converted to alditol trifluoroacetates and analyzed by gas chromatography on a Hewlett-Packard 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m) at a temperature program of 110–125 °C (2 °C min^{-1}) to 165 °C (20 °C min^{-1}) and a flow rate of hydrogen 20 ml min^{-1}. GC–MS analysis of partially methylated alditol acetates of saccharides was effected on a Finnigan Mat SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm × 30 m) at 80–240 °C (6 °C min^{-1}), 70 eV, 200 μA, and ion-source temperature 150 °C. Spectrophotometric measurements were carried out on a Specol 11 (Zeiss, Jena, Germany) spectrometer.

NMR spectra were recorded in deuterated water at 25 or 60 °C on an FT NMR Bruker AVANCE DPX 300 spectrometer (^{1}H at 300.13 MHz and ^{13}C at 75.46 MHz) equipped with a selective unit and gradient enhanced spectroscopy kit (GRASP) for generation of *z*-gradients up to 50 G cm^{-1} in a 5 mm inverse probe kit. Chemical shifts of signals were referenced to internal acetone (2.225 and 31.07 ppm for ^{1}H and ^{13}C, respectively). The data matrix for HSQC experiment was processed according to Schleucher et al. (1993) with squared sine function, using Bruker software XWIN-NMR version 1.3.

2.3. Isolation of the acidic heteropolysaccharide (AP)

The methanol-pretreated, cold- and hot-water extracted, air-dried mahonia stems (400 g) were extracted with 0.25 M NaOH (81) for 24 h at room temperature. The plant residue was separated from the supernatant by filtration. The supernatant after centrifugation (5000*g*) was precipitated with four volumes of ethanol and the precipitate, collected by centrifugation, was suspended in water, exhaustively dialyzed, and freeze-dried. The brownish product was further purified by washing with 80% aqueous ethanol acidified with HCl (1 vol%) to give 11.2 g of crude hemicellulose (CHC). CHC (200 mg) was loaded onto a column (5 × 100 cm) of DEAE-Sephadex A-50 (carbonate form) and irrigated successively with water (W) and 0.25 M (C1) and 0.5 M (C2) carbonate solutions. The largest, water-eluted fraction W gave on gel filtration on a column (2.5 × 130 cm) of Bio-Gel P-4 the glucuronoxylan MahX already described (Kardošová et al., 2002). The fraction C1, representing 36% of the crude hemicellulose, was further purified on Biogel P-2 to give a polysaccharide (AP) homogeneous by free-boundary electrophoresis.

2.4. Methylation analysis

The polysaccharide AP (50 mg), pretreated with $NaBH_4$, was methylated according to the method of Ciucanu and Kerek (1984) to give a fully methylated product, as evidenced by absence of IR absorption for hydroxyl. The methylated sample was recovered using a Sep-pak C_{18} cartridge by the procedure of Waeghe, Darvill, McNeil, and Albersheim (1983). The product was then converted into partially methylated alditol acetates by hydrolysis, reduction with $NaBD_4$, and acetylation, and was subjected to linkage analysis by GC–MS (Jannson, Kenne, Liedgren, Lindberg, & Lönngren, 1976).

2.5. Partial acid hydrolysis of AP

The polysaccharide (150 mg) was hydrolyzed with 1 M TFA (100 ml) for 1 h at 100 °C. After cooling, the solution was made neutral, concentrated to a smaller volume, and added slowly into four volume of ethanol to precipitate the high-molecular-weight portion. After washing with EtOH on a sintered glass filter, the precipitate was dissolved in distilled water, exhaustively dialyzed, and lyophilized. This product was subsequently purified on a column (2.5 × 150 cm) of Biogel P-2 by water elution, collecting 5 ml fractions. The polysaccharide (P3), separated from the pooled fractions 17–23, represented 7% of AP. The mixture of low molecular hydrolytic products was percolated through Dowex 1 × 2 (CH_3COO^- form) and subsequently eluted with water and 6 M acetic acid (Ebringerová & Kramár, 1968) yielding the neutral and acidic carbohydrates, respectively, which were further analyzed by PC using solvent systems S1 and S2.

2.6. Enzymic hydrolysis of AP

The polysaccharide (190 mg), dissolved in acetate buffer of pH 4.6 (40 ml), was treated with pectinase (1 ml) for 4 h at room temperature. After inactivation of the enzyme by 10 min boiling, the solution was diluted and dialyzed to constant conductivity of the dialyzate. The retentate was freeze-dried to give the polymeric product EHAP in 27.6% yield (related to AP) and the dialyzate containing mono- and oligosaccharides was concentrated and analyzed as described in Section 2.5.

2.7. Antitussive activity tests

The experiments, approved by the Ethics Committee of Jessenius School of Medicine, Comenius University and Faculty Hospital in Martin (Slovakia), were performed on adult non-anaesthetized cats of both sexes weighing 1500–2500 g (10 in each set). After several days' quarantine, the animals were surgically implanted with a tracheal cannula. This enabled mechanical stimulation of airways and the recording of the side tracheal pressure. The mucous membranes of the laryngopharyngeal (LP) and tracheobronchial (TB) areas were stimulated consecutively five times with 0.35 mm diameter nylon fibre. The cough parameters, i.e. the number of efforts (NE), intensity of cough attacks in expirium (IA^+) and inspirium (IA^-), cough frequency (NE min^{-1}), and intensity of maximal cough efforts in expirium (IME^+) and inspirium (IME^-), were evaluated on the basis of the pressure values recorded on a Biograph 12-03 electromanometer. The water solution of the tested compound was administered perorally in a dose of 50 mg kg^{-1} b.w. The values of cough parameters obtained prior to administration of the tested compound represented the normal value (*N*). For comparative purposes commercial products generally used in clinical practice to treat coughing, i.e. prenoxdiazine (P), dropropizine (D), and codeine (C), were tested along with the polysaccharide. The effect of drugs was monitored in time intervals 0.5, 1, 2, and 5 h. Statistical evaluation of the results was carried out by the method of Wilcoxon and Wilcox (1964). The doses of the individual comparative drugs used herein, i.e. P = 30 mg kg^{-1} b.w., D = 100 mg kg^{-1} b.w., C = 10 mg kg^{-1} b.w., represented the amounts which, in earlier experiments, exhibited the highest antitussive effect.

2.8. Mitogenic and comitogenic activity assay

The mitogenic and comitogenic activities were tested according to a slightly modified method described previously by Iribe and Koba (1984). Briefly, the rat (strain Wistar, males weighing 200 g) thymocytes in medium RPMI-1640 supplemented with 10% fetal calf serum, used at concentration of 1.5×10^6 cells per 0.2 ml per well, were stimulated by the test compound at concentrations 1, 10, 100, or 1000 μg ml^{-1} either in absence (mitogenic activity) or in presence (comitogenic activity) of PHA added to a final concentration 25 μg ml^{-1}. Possible contamination of the tested polysaccharide by endotoxin was checked by cultivation in the presence of polymyxin B (20 μg ml^{-1}) which inhibits, dose dependently, the biological effects of endotoxin, including its mitogenic activity (Jacobs & Morrison, 1977). After 72 h cultivation, DNA synthesis, as measured by the method of [^{3}H]-Td incorporation, was determined. The activity of polysaccharides was compared using the arithmetic means of the stimulation indices (SI) from four experiments. In each experiment, the geometric means of counts per min (cpm) for each set of four replicas were used for calculation of the SI. The direct mitogenic effect of the compound tested was expressed as: SI_{mit} = mean cpm for test compound/mean cpm without stimulant. The comitogenic effect was expressed as SI_{comit} = mean cpm (PHA + test compound)/mean cpm for PHA. In the repeated experiments, the geometric means of cpm for control cultures without any additive ranged from 258 to 502, and for cultures stimulated with PHA from 456 to 714. The means of SI in repeated testing were compared by the analysis of variance (ANOVA).

3. Results and discussion

From the methanol-insoluble, cold- and hot-water extracted mahonia stems, a crude hemicellulosic material (CHC) was isolated by alkaline extraction in 2.8% yield and further fractionated by anion-exchange chromatography. The main acidic polysaccharide component, obtained in 60% yield, was previously reported (Kardošová et al., 2002) to be a 4-*O*-methyl-D-glucurono-D-xylan (MahX). Another fraction obtained in the 0.25 M ammonium carbonate eluate, represented 36% of the loaded material. Its purification by gel filtration yielded the acidic polysaccharide AP

Table 1
Yields and characteristics of the acidic polysaccharide complex (AP), the polymeric residue from partial acid hydrolysis (P3) and that from the pectinase treatment (EHAP)

	AP	P3	EHAP
Yield (%)	36[a]	7[b] (2.5)[a]	27.6[b] (9.9)[a]
Neutral sugar composition (mol%)			
Rhamnose	14.5	63.7	35.8
Fucose	3.0	3.0	3.0
Arabinose	9.5	3.3	12.1
Xylose	59.1	9.6	19.3
Mannose	2.5	7.9	6.7
Glucose	4.1	7.8	7.7
Galactose	7.3	4.7	15.4
Uronic acid[c]	+	++	+
Xyl:MeGU (mole ratio)[d]	6.7:1		
M_{app} (kDa)[e]	5.8	2.1	3.2
$[\alpha]_D^{22}$ (Degrees)	+29.35		

[a] Related to the crude hemicellulose fraction (CHC).
[b] Related to polysaccharide AP.
[c] Detected by PC.
[d] Calculated from the integrated peak areas corresponding to the anomeric carbons of all Xyl*p* residues and MeGlcA and of C-5 carbons of all Xyl*p* residues and C-4 of MeGlcA.
[e] Mean molecular mass estimated by HPGPC on pullulan-calibrated Biospher columns.

with analytical characteristics given in Table 1. As seen, the dominating neutral sugar component of AP was xylose (59.1 mol%), followed by rhamnose, while arabinose, galactose, glucose, mannose, and fucose were minor constituents. As determined by PC, the hydrolyzate of AP contained a mixture of 4-*O*-methylglucuronic acid and galacturonic acid. The low positive value of optical rotation (+29.35°) indicated the presence of both α- and β-glycosidic linkages.

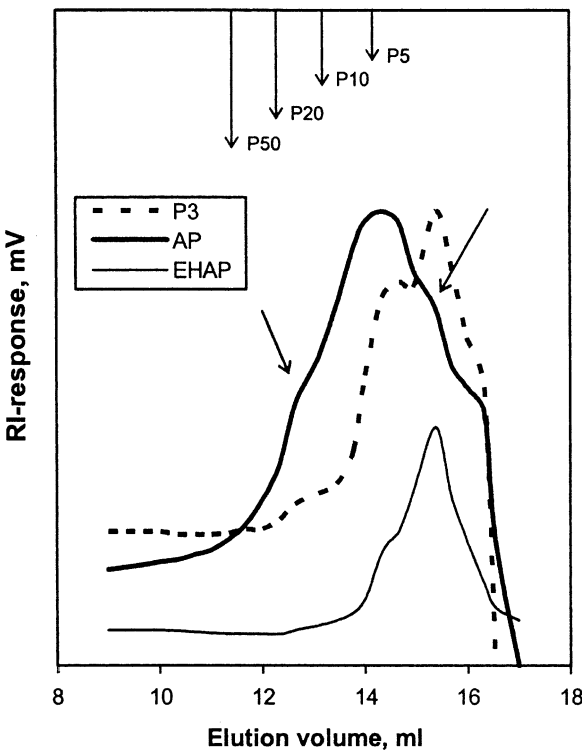


Fig. 1. HPGPC records of AP, P3 and EHAP obtained from Biospher columns calibrated with pullulan standards P5–P50. The arrows indicate the shoulders in the regions of M_{app} 25.5 and 1.8 kDa.

On HPGPC (Fig. 1), AP gave a broad elution curve. Based on pullulan calibration, the average molecular mass (M_{app}) was determined to be 5.8 kDa and the polydispersity $M_w/M_n = 3.2$. Except for a major molecular peak at 3.9 kDa, shoulders are seen at the lower and upper ends of the elution curve at 25.5 and 1.8 kDa, respectively, indicating the presence of two minor molecular populations. On the other hand, on free-boundary electrophoresis, AP appeared as a homogeneous substance. It seems that there are no major differences in the charge of the various molecular populations of AP. The sugar composition and the broad elution curve on HPGPC suggested the presence of a mutually associated xylan and pectin components all the more that also other separation methods (gel filtration on Sephadex G-50 and ion-exchange chromatography on DEAHP-Ostsorb, not described) resulted in coelution of both components.

Methylation analysis of AP (Table 2) gave information about the linkage composition. The main product

Table 2
Methylation analysis data of the acidic polysaccharide complex AP

Derivative[a]	Mole%	Linkage indicated
2,3,5-Me_3-Ara	0.5	Ara*f*-(1 →
2,3-Me_2-Ara	0.3	→ 5)-Ara*f*-(1 →
5-Me-Ara	0.5	→ 2,3)-Ara*f*-(1 →
Total	*1.3* (*9.5*)	
2,3,4-Me_3-Xyl	2.5	Xyl*p*-(1 →
2,3-Me_2-Xyl	82.7	→ 4)-Xyl*p*-(1 →
3-Me-Xyl	9.6	→ 2,4)-Xyl*p*-(1 →
Total	*94.8* (*59.1*)	
2,3,4-Me_3-Rha	0.1	Rha*p*-(1 →
2,3-Me_2-Rha	0.4	→ 4)-Rha*p*-(1 →
3,4-Me_2-Rha	0.2	→ 2)-Rha*p*-(1 →
3-Me-Rha	0.4	→ 2,4)-Rha*p*-(1 →
Total	*1.1* (*14.5*)	
2,3,4,6-Me_4-Gal	0.8	Gal*p*-(1 →
2,3,6-Me_3-Gal	0.3	→ 4)-Gal*p*-(1 →
2,3,4-Me_3-Gal	1.0	→ 6)-Gal*p*-(1 →
2,6-Me_2-Gal	0.2	→ 3,4)-Gal*p*-(1 →
Total	*2.3* (*7.3*)	
2,4,6-Me_3-Glc	0.1	→ 3)-Glc*p*-(1 →
2,3,6-Me_3-Glc	0.2	→ 4)-Glc*p*-(1 →
2,3,4-Me_3-Glc	0.1	→ 6)-Glc*p*-(1 →
2,6-Me_2-Glc	0.1	→ 3,4)-Glc*p*-(1 →
Total	*0.5* (*4.1*)	

[a] 2,3,5-Me_3-Ara = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylarabinose, etc. The values in brackets are from Table 1.

(~83 mol%) was 2,3-di-*O*-methyl-D-xylose, arising from the (1 → 4)-linked xylose chain units, while the 3-*O*-methyl-D-xylose derivative indicated *O*-2 substitution originating from the branching by 4-*O*-methyl-D-glucuronic acid residues. The proportion of terminal xylose units was very low indicating a DP about 40, in accord with the mean molecular mass (M_{app} = 5.8 kDa) of the main molecular population estimated by HPGPC (Table 1). The presence of pectic-type polysaccharide was inferred from the occurrence of variously linked rhamnose residues, i.e. terminal, 1,4-, 1,2-, and 1,2,4-linked. Rhamnose is involved probably in the backbone and may constitute the point of attachment of the two neutral sugar residues, i.e. galactose (terminal, 1,6-, 1,4-, and 1,3,4-linked) and arabinose (terminal, 1,5-, and 1,2,3-linked) as side chains in the so-called hairy region of the rhamnogalacturonan I (RG-I) backbone (De Vries, den Ujil, Voragen, Rombouts, & Pilnik, 1983). Most of these units were easily accessible to acid hydrolysis in contrast to xylose and rhamnose linked to uronic acids, which resist in form of aldobiouronic acids. This might cause underestimation of the mentioned monosaccharides and explain the differences in neutral sugar proportions determined before and after methylation analysis.

The ^{13}C NMR spectrum of AP (Fig. 2A) showed dominating signals C-1–C-5 of 4-linked β-Xyl*p* residues at δ 102.54, 73.59, 74.54, 77.23, and 63.84 and the C-1–C-6 signals of MeGlcA residues of the 4-*O*-methylglucuronoxylan component (GX) at δ 98.47, 72.07, 73.14, 83.14, 72.60, and 177.5. The ^{1}H/^{13}C chemical shifts of the Xyl and MeGlcA residues were assigned according to previously reported spectral data (Cavagna, Deger, & Puls, 1984; Kardošová, Matulová, & Malovíková, 1998), supported by the 2D-COSY (not shown) and 2D-HSQC NMR spectra (Fig. 2B), and summarized in Table 3. The marked ^{1}H/^{13}C cross-peaks in the HMBC spectrum (Fig. 2C) at 4.54/77.23

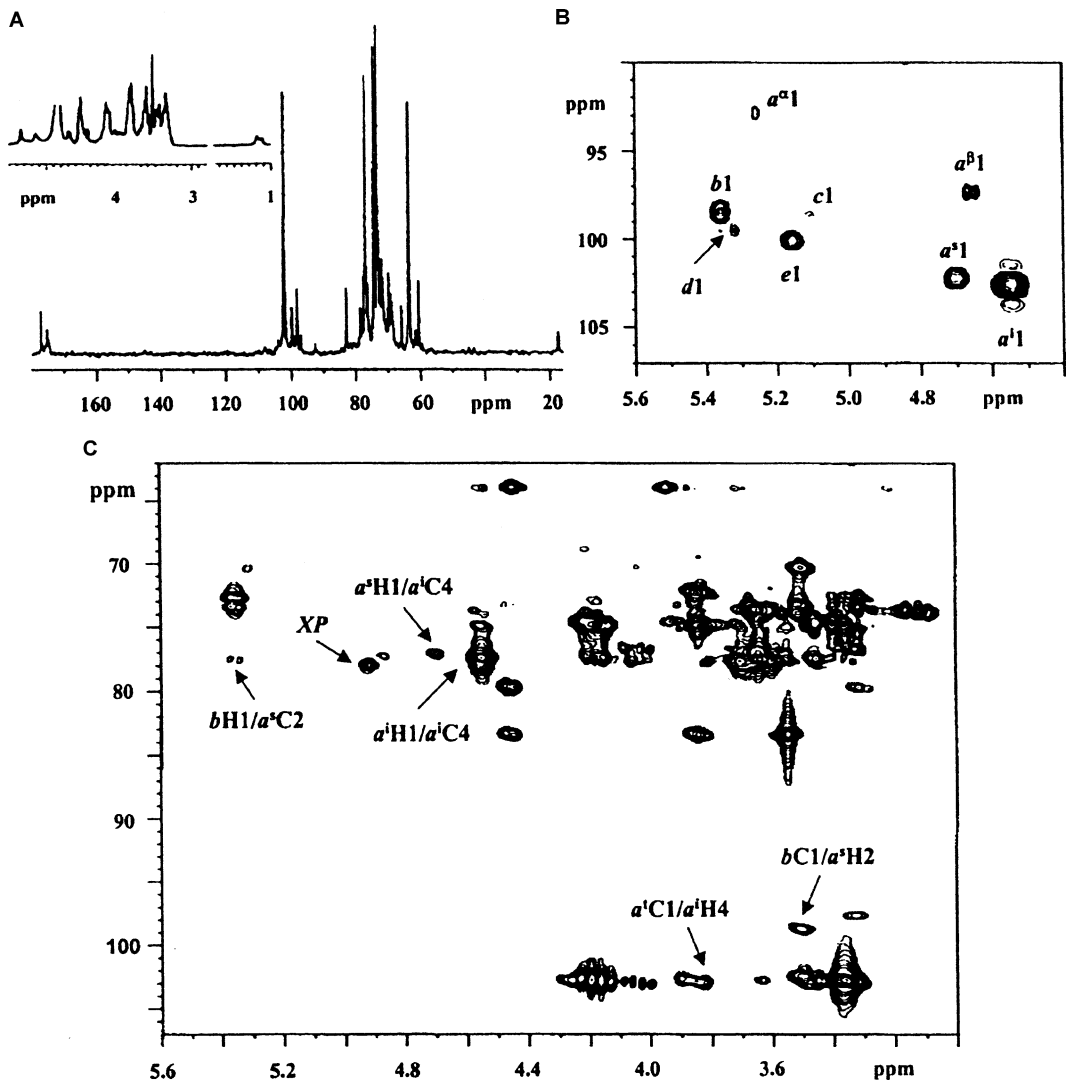


Fig. 2. NMR spectroscopy analysis of AP: (A) ^{13}C NMR spectrum with inserted partial ^{1}H NMR spectrum, (B) ^{1}H/^{13}C HSQC spectrum with marked main anomeric atom cross-peaks **a**–**d** of the glucuronoxylan and pectin components, and (C) HMBC spectrum with marked inter-residual connectivities. For labels see Schemes 1 and 2.

Table 3
$^{13}C/^{1}H$ chemical shifts (in D_2O) of the 4-*O*-methylglucuronoxylan and pectin components of AP

Sugar unit	Chemical shift, δ (ppm) C,H					
	1	2	3	4	5	6
4-O-Methylglucuronoxylan						
a^{i}	102.54, 4.54	73.59, 3.35	74.54, 3.62	77.23, 3.84	63.84, 4.19[e], 3.47[a]	
a^{s}	102.25, 4.69	77.65, 3.49	72.14, 3.68	76.88, 3.52	63.92, 4.16[e], 3.44[a]	
a^{t}	102.69, 4.53	73.20, 3.30	76.49, 3.53	70.08, 3.74	66.10, 4.04[e], 3.40[a]	
a$^{\alpha}$	93.00, 5.26	72.60, 3.60			59.90, 3.83[e], 3.75[a]	
a$^{\beta}$	97.39, 4.63	73.59, 3.31			63.84, 4.14[e], 3.43[a]	
b	98.47, 5.34	72.07, 3.65	73.14, 3.73	83.14[*], 3.32	72.60, 4.45	177.5
Pectin						
c	98.60, 5.10		69.12, 4.00	78.84, 4.50	71.89, 4.87	175.4
d	99.61, 5.31	77.31, 4.27	72.10, 3.83	73.40, 3.37	69.00, 3.73	17.46 (d), 1.32/1.37
e	100.14, 5.14	69.01, 3.85	69.50, 4.09	78.89, 4.51	72.07, 4.92	174.8

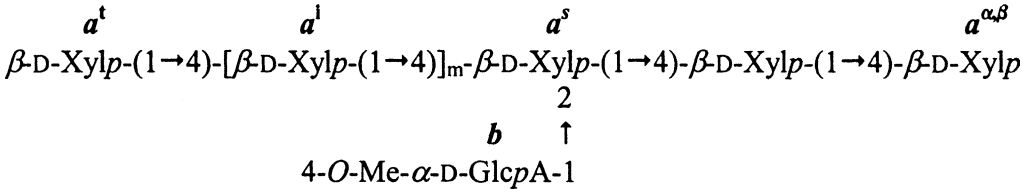
e, equatorial; a, axial, (d) doublet. The letter **a** and **b** refer to corresponding sugar residues in the structure of 4-*O*-methylglucuronoxylan (Scheme 1). Letters **c** and **d** belong to those of RG-I and letter **e** to PGA sequences of pectin (Scheme 2).
[*] $^{13}C/^{1}H$ chemical shifts of OCH_3 at C-4: 60.78/3.54.

and 5.36/77.65 confirmed the 1,4-β-glycosidic linkages of the xylan backbone and that of the α-MeGlcA residues at position 2, respectively (Scheme 1). The presence of reducing Xyl residues indicated the admixture of xylooligosaccharide chains which contribute to the minor low-molecular mass population in AP (Fig. 1).

The pectin component of AP (Scheme 2) is manifested by the C-1 chemical shifts of 4-linked α-GalA at δ 100.14 (**e**) and 98.60 (**c**) and 2-linked α-Rha residues at δ 99.61 (**d**) (Fig. 2B). Based on the intensities of the corresponding peak areas in the ^{13}C NMR spectrum (Fig. 2A), the estimated GalA to Rha ratio of ~3:1 indicated the presence of poly-(1,4)-α-D-galacturonan (PGA) as well as rhamnogalacturonan (RG-I) sequences in the pectin component. The multiple signals at δ ~174.85 and the broad $^{1}H/^{13}C$ cross-peak of the anomeric atom at δ 5.16/100.14 indicate the variously located GalpA residues (**e**) in PGA molecules. Rhamnose showed two C-6 signals at δ ~17.46 and splitting of the H-6 resonance at δ 1.32 and 1.37, reflecting different environments as well. The $^{1}H/^{13}C$ cross-peaks of GalA residues of PGA and that of the Rha residue of RG-I were assigned (Table 3) in accordance with recent literature data for models comprising 4-linked α-GalpA units of PGA (Ló, Hahn, & van Halbeek, 1994; Mort, Qiu, & Maness, 1993; Tjan, Voragen, & Pilnik, 1974) and the [-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-] sequence of the RG-I regions (An, O'Neill, Albersheim, & Darvill, 1994; Capek, Matulová, & Kardošová, 1997; Colquhoun, de Ruiter, Schols, & Voragen, 1990; Zhan, Janssen, & Mort, 1998). The cross-peaks at δ 4.50/78.4 and 4.51/78.9 were assigned to C-4 atoms of the GalA residues **c** and **e** of the PGA and RG-I polymers, respectively (Capek et al., 1997; Colquhoun et al., 1990), thus confirming the methylation analysis data. Probably due to the low abundance of these components, the corresponding inter-glycosidic cross-peaks were not seen in the HMBC spectrum of AP (Fig. 2C).

The area integration of the anomeric signals of Xyl (all residues **a**), MeGlcA (**b**), GalA (**e**), and Rha (**d**) residues in the ^{13}C NMR spectrum of AP (Fig. 2A) gave the mole ratios Xyl to MeGlcA = 6.7:1 for GX and GalA to Rha = 3:1 for the pectin component which represents about 10% of AP. This is in accord with the methylation analysis data (Table 2). Though the integrals of signals are not strictly proportional to the relative proportions of the respective carbons due to their different T_1's and NOEs, the values obtained can be considered as semiquantitative estimates (Shashkov & Chizhov, 1976).

The above mentioned results confirmed the preponderance of GX in AP and suggested that AP is a closely associated complex of both GX and P components. The earlier reports on the occurrence of xylose-rich polymers associated with pectic substances by covalent linkages were based on methylation analyses data only. Also short xylooligosaccharide chains attached to galacturonan chains



Scheme 1.

c ***d*** ***e***

→4)-[α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→4)]$_m$-[α-D-Gal*p*A-(1→4)-α-D-Gal*p*A-(1→]$_n$-

Scheme 2.

have been suggested from the methylation analysis data of the xylose-rich pectin (Goff, Renard, Bonnin, & Thibault, 2001; Oechslin, Lutz, & Amadò, 2003). Recently, 2D-NMR spectroscopy analyses confirmed the presence of such linkages in the subunits isolated from apple pectin (Schols, Bakx, Schipper, & Voragen, 1995) and watermelon (Mort, Qiu, Nimtz, Stark, & Bell-Eunice, 2002). This prompted us to look for possible xylan–pectin linkages in AP. According to the above mentioned reports, xylogalacturonan contains terminal Xyl*p* residues attached to the α-1,4-galacturonan backbone at position 3.

The HMBC spectrum of AP (Fig. 2C) showed the $^{1}H/^{13}C$ cross-peak (XP) at δ 4.92/77.8. The ^{13}C chemical shift resembles the resonances of C-2 of 2-linked or C-4 of 4-linked Xyl*p* residues (Scheme 1). These residues are very frequent in AP and gave broad cross-peaks. As the ^{1}H chemical shift had no anomeric carbon counterpart, it could be assigned to the H-5 resonance of GalA residues **c** and **e** of the pectin component (Scheme 2). This implies that some 4-linked GalA residues could be 2- and/or 4-linked to the xylan backbone. However, a corresponding inter-glycosidic cross-peak substantiating a possible linkage between both xylan and pectin components of AP was not observed.

The reported C-3/H-3 resonances of the GalA residues substituted with single Xyl*p* residues were found at δ 78.2/3.85 (Mort et al., 2002; Schols et al., 1995). Though all NMR spectra of AP revealed the presence of a small amount of non-reducing terminal Xyl residues in accord with the methylation analysis data (Table 2), none of the techniques we used gave evidence of the substitution of GalA at position 3.

The pectin network has been considered to be independent of the cellulose/hemicellulose network. However, pectins are frequently isolated together with hemicelluloses and their separation is usually difficult. It might be explained by strong physical interactions and/or covalent bonding. It is to be noticed that the aldobiouronic acid 4-*O*-(α-D-Gal*p*A)-D-Xyl*p* was several times isolated from partial hydrolyzates of woody plants (Ebringerová and Kramár, 1968; Shimizu et al., 1998) and hemicelluloses (Andersson & Samuelson, 1983; Johansson & Samuelson, 1977) where it was suggested to be located at the reducing end of 4-*O*-methylglucuronoxylan chains. Nevertheless, there is still the possibility that such structural unit might also represent a connection between the pectin and xylan components of the cell wall network.

In order to get further information on the existence of chemical links between the xylan and pectin components, AP was subjected to two hydrolytic treatments, i.e. partial acid hydrolysis cleaving preferentially the xylan backbone and the neutral side chains of the hairy region of pectin, and hydrolysis with pectinase attacking preferentially the homogalacturonan chains. The effect of both treatments on the molecular mass distribution of AP is shown in Fig. 1. Interestingly, the trimodal nature of the elution curve persisted in both resistant polymeric fractions resulting from the acid (P3) and enzymic (EHAP) hydrolytic treatments in 7 and 26% yields, respectively. However, the proportions of the three molecular populations changed.

As seen in Table 1, the neutral sugar composition of the polymeric residues showed major changes. The most susceptible to acid hydrolysis were the glycosidic bonds of the xylan backbone as well as of the neutral side chains of the pectin component built up by galactose and arabinose, resulting in lowered proportions of these sugars and a substantial increase of rhamnose in P3. In EHAP, the proportions of mainly rhamnose and galactose increased due to splitting of the PGA backbone. However, a part of xylose was also lost, indicating the presence of xylan-degrading enzymes in the commercial pectinase.

The NMR spectroscopy analyses of P3 are shown in Fig. 3A and B, and the derived $^{1}H/^{13}C$ chemical shifts of GalA and Rha residues are summarized in Table 4. No signals and cross-peaks corresponding to xylose residues were observed in the spectra, indicating the destruction of the xylan backbone to oligomers removed by dialysis from P3. However, also the PGA and RG-I regions of the pectin component suffered from the acid hydrolysis, PGA in particular. This was confirmed by the high proportions of reducing 2-Rha*p* and 4-Gal*p*A units. The HMBC spectrum (Fig. 3C) afforded only the inter-glycosidic $^{13}C/^{1}H$ cross-peaks *c*C-1/*d* H-2 and *d*C-1/*c* H-4 at δ 98.44/4.19 and 99.36/4.50, respectively, corresponding to the [-α-Gal*p*A-(1 → 2)-α-Rha*p*-(1 → 4)-] sequence of the RG-I regions. In spite of the much higher proportion of PGA in P3, when compared to that in AP, the inter-glycosidic cross-peak of the backbone was not observed either.

The HSQC spectrum of EHAP (shown for the anomeric region in Fig. 4B) indicated a considerable degradation of the GX component, documented by the variety of $^{13}C/^{1}H$ cross-peaks originating from reducing β-Xyl*p* residues at δ 96.8–97.6/4.47–4.58. The derived $^{13}C/^{1}H$ chemical shifts of EHAP are summarized in Table 5. As seen in the HMBC spectrum (Fig. 4C), the resistant GX chains gave inter-glycosidic connectivities of its basic constituents ($\mathbf{a^i}$) at δ 4.46/77.27 and (**b**) at δ 98.20/3.39. The preponderant RG-I moiety of the pectin component is indicated by the interglycosidic cross-peaks at δ 4.43/99.41 and 5.23/78.05. The HSQC spectrum of EHAP revealed the chemical shifts of the 4-deoxy-hexenouronic acid (Table 5) resulting from the β-eliminative cleavage of the PGA chains by pectinase. As shown in Fig. 4C, residue ($\mathbf{e^b}$) gave long-range

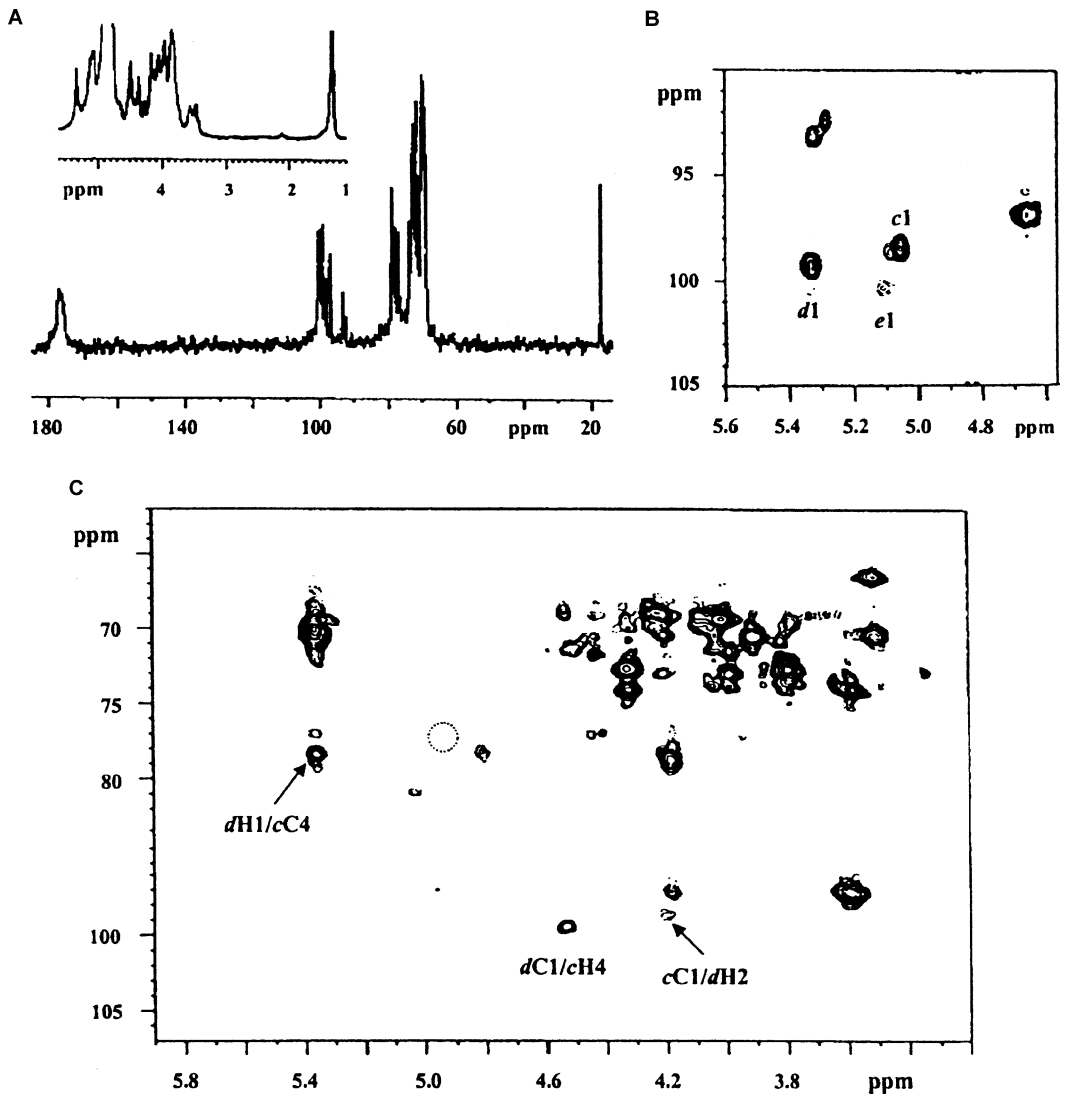


Fig. 3. NMR spectroscopy analysis of P3: (A) ^{13}C NMR spectrum with inserted partial ^{1}H NMR spectrum, (B) $^{1}H/^{13}C$ HSQC spectrum with marked main anomeric atom cross-peaks, and (C) HMBC spectrum with marked inter-residual connectivities. For label data see Scheme 2. The dotted circle indicates the region of cross-peak XP.

Table 4
$^{13}C/^{1}H$ chemical shifts (in D_2O) of the acid-resistant fraction P3

Sugar residue	Chemical shift, δ (ppm) C/H					
	1	2	3	4	5	6
Rhamnogalacturonan						
c	98.45, 5.07	68.90, 3.97	71.10, 4.18	78.80, 4.47	72.31, 4.78	176.8 (m)
d	99.36, 5.33	76.96, 4.13	70.40, 3.98	72.80, 3.48	69.41, 3.78	17.47, 1.32
$\mathbf{d}^{\alpha}$	92.50, 5.28	77.60, 4.02			69.58, 3.87	18.10, 1.26
Polygalacturonan						
$\mathbf{e}^{i}$	100.31, 5.11 (m)	69.41, 3.87	69.90, 4.06	78.80, 4.49	72.31, 4.82	175.9 (m)
$\mathbf{e}^{t}$	100.40, 5.07			71.7, 4.40		
$\mathbf{c},\mathbf{e}^{\alpha}$	93.23, 5.31			77.8, 4.41		
$\mathbf{e},\mathbf{c}^{\beta}$	96.94, 4.66	72.58, 3.60	73.16, 3.83	73.78, 3.76	71.21, 4.28	

(m), Multiplet. For annotation letters **c**–**e**, see Scheme 2.

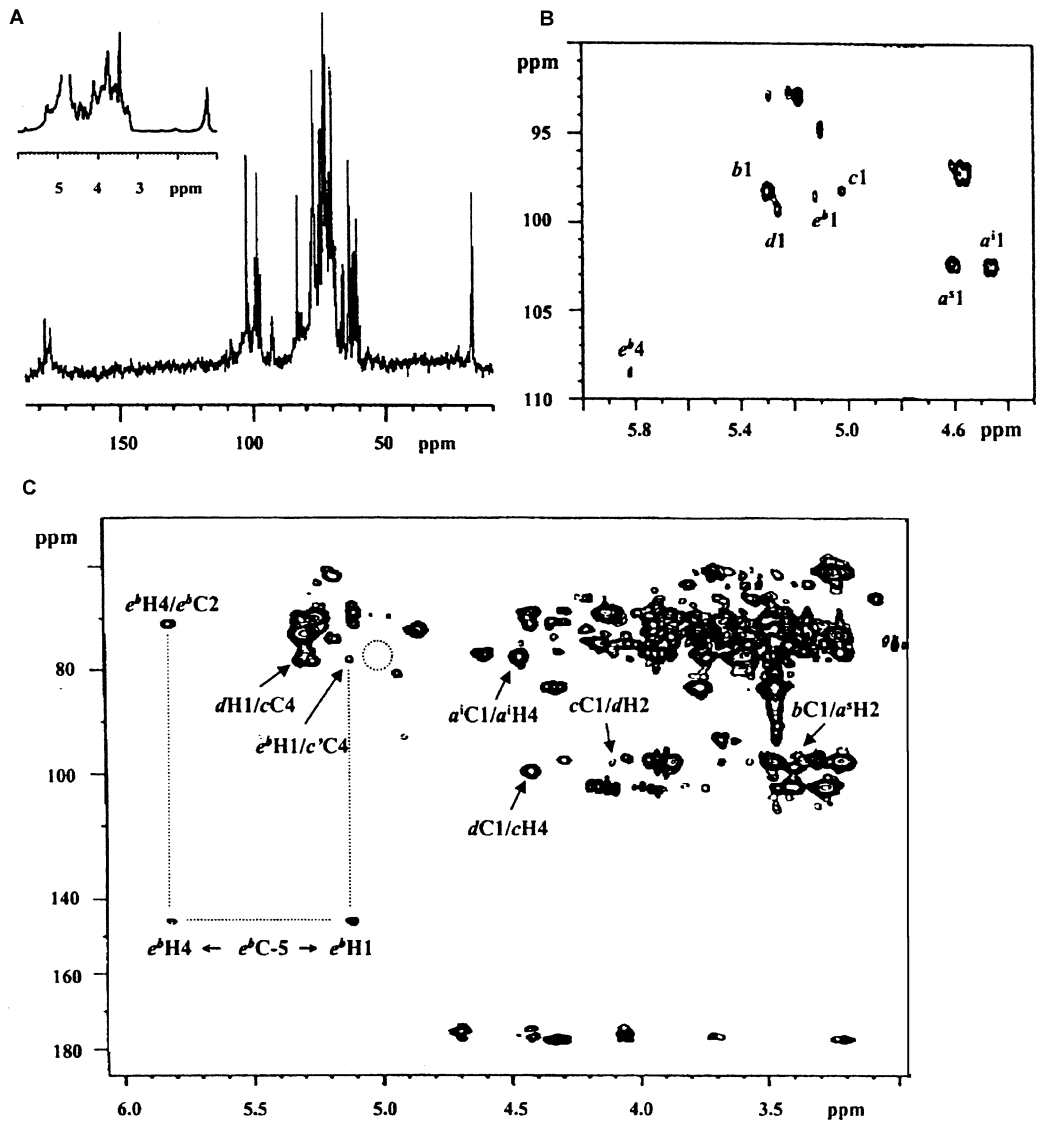


Fig. 4. NMR spectroscopy analysis of EAHP: (A) ^{13}C NMR spectrum with inserted partial ^{1}H NMR spectrum, (B) $^{1}H/^{13}C$ HSQC spectrum with marked main anomeric atom cross-peaks, and (C) HMBC spectrum with marked inter- and intra-residual connectivities. For labels see Schemes 1 and 2 and Table 5. The dotted circle indicates the region of cross-peak XP.

Table 5
$^{13}C/^{1}H$ chemical shifts (in D_2O) of the pectinase-resistant fraction EHAP

Sugar residue	Chemical shift δ (ppm) C/H					
	1	2	3	4	5	6
a^{i}	102.55, 4.46	73.57, 3.26	74.60, 3.57	77.27, 3.78	63.77, 4.14, 3.45	
a^{s}	102.16, 4.58	77.49, 3.39	72.41, 3.74	76.53, 3.46	63.49, 4.06, 3.36	
b	98.2, 5.27	72.15, 3.57	73.61, 3.67	83.32*, 3.26	73.0, 4.32	177.59
c	98.40, 5.01	68.79, 3.82	71.10, 4.11	78.05, 4.43	72.0, 4.70	176.9
c$^{/}$	98.41, 5.05		71.20, 4.20	77.9, 4.32		
d	99.41, 5.23	76.35, 4.07	70.1, 3.90	72.90, 3.39	69.55, 3.92	17.51, 1.23
d$^{\alpha}$	92.9, 5.26	77.6, 3.97				
d^{t}	101.70, 5.21	4.07				
e^{b}	98.6, 5.12	71.05, 3.81	67.01, 4.34	108.25, 5.79	145.61	

e^{b}, $^{13}C/^{1}H$ chemical shifts of the C4=C5 of unsaturated GalA residue. For annotation letters see Schemes 1 and 2; **c$^{/}$** corresponds to GalA having **e^{b}** attached at position 4.

* $^{13}C/^{1}H$ chemical shifts of OCH_3 at C-4: 60.80/3.48 ppm.

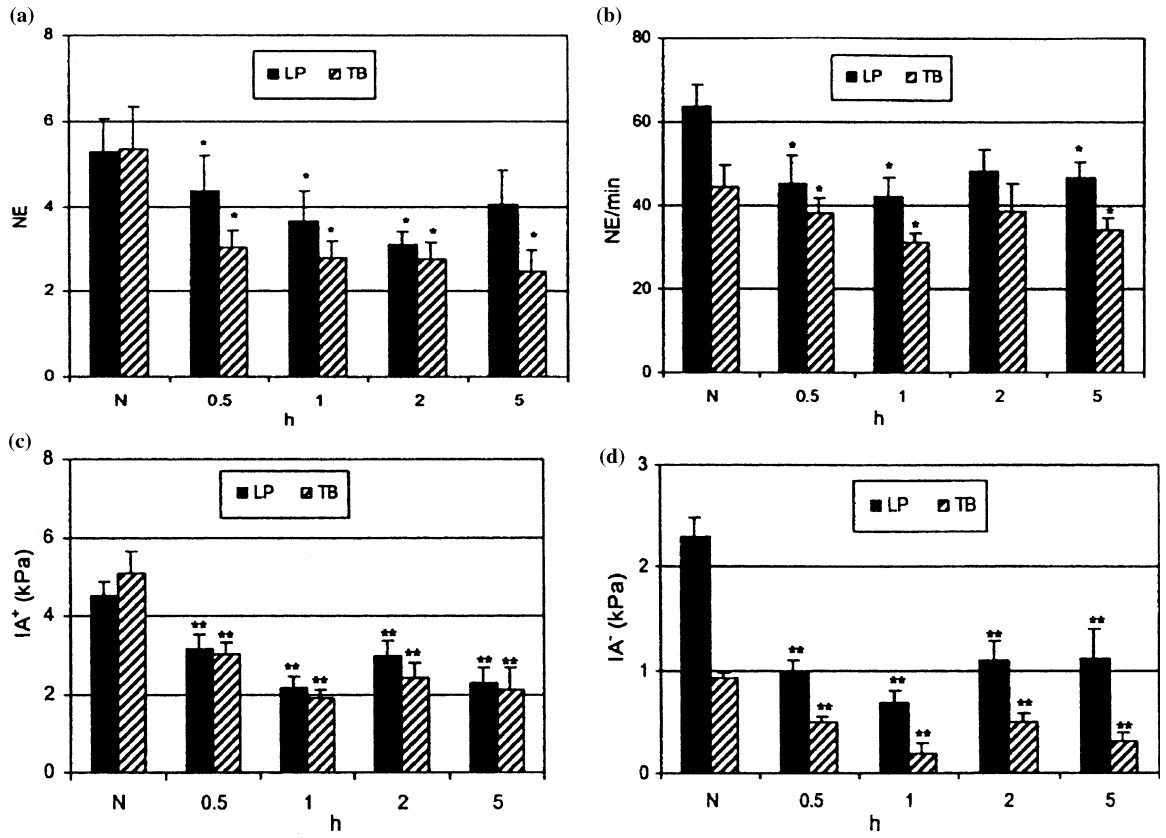


Fig. 5. Antitussive effects of AP. Effect (a) on the number of cough efforts (NE), (b) cough frequency (NE min^{-1}), and intensity of cough attacks, (c) in expirium (IA^+) and (d) inspirium (IA^-). LP, Laryngopharyngeal area; TB, tracheobronchial area; N, normal value (control). The columns represent the mean, values of cough parameters, the range denotes standard error of means, * 5% significance, ** 1% significance.

intra-residual connectivities of C-5 (δ 146.5) with H-1 at δ 5.12 and H-4 at δ 5.79, the last proton also with C-2 (δ 71.0), and the inter-glycosidic cross-peak δ 5.12/77.9 indicating the linkage of $\mathbf{e^b}$ to C-4 of an intact GalA residue ($\mathbf{c'}$).

The cross-peak XP, considered to reflect potential xylan–pectin linkage sites in AP, could not be observed in either of the HMBC spectra of P3 and EHAP. The respective linkages (if existing) were either cleaved during the hydrolytic treatments or retained in some of the resulting oligosaccharides. Unfortunately, we failed to detect the aldobiouronic acid consisting of GalA and Xyl in both acid and enzymic hydrolyzates probably due to its minute amount, when considering the relatively high molecular mass of AP. As the RG-I regions were essentially preserved, the results indirectly implied that xylan was not linked to the RG-I region of the pectin component of AP. This is in accord with the hitherto published data on xylan–pectin linkages.

4. Antitussive activity of the polysaccharide complex AP

The results of tests showed that peroral administration of AP brought about a statistically significant decrease in the number of cough efforts (NE) (Fig. 5a) and intensity of cough attacks in expirium and inspirium (IA^+ and IA^-; Fig. 5c and d) from both stimulated regions (LP and TB) of the airways. The significant inhibition of cough attacks contributes to positive assessment of the antitussive effect of the tested compound because increase of intrathoracic pressure may have a negative impact on the patient.

The frequency of coughing (Fig. 5b) was also reduced significantly from both LP and TB regions. It is noticeable that the number of efforts and frequency of coughing decreased already 30 min after administration of the compound and lasted throughout the whole experiment. It means that the tested compound showed prompt onset of the effect, important for clinical practice.

The intensity of maximum cough efforts (not shown) from the TB region was not affected significantly. This fact indicates that expectoration was not suppressed and consequently, the mucus does not cumulate in the airways. Comparison of cough parameters from TB and LP regions revealed different abilities to influence the mechanism regulating the quality and quantity of coughing. The tests confirmed our earlier finding (Korpáš & Nosál'ová, 1991) that compounds with dominant peripheral mechanisms reduce the frequency of coughing but have much less influence on its amplitude. The frequency of coughing depends probably on the condition of the cough receptors, while the amplitude is determined by the condition of the cough centre.

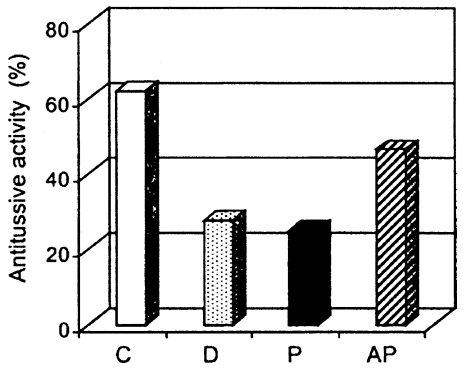


Fig. 6. Antitussive activity (%) of AP compared to that of codeine (C), dopropizine (D) and prenoxdiazine (P).

Comparative tests (Fig. 6) performed at the same conditions with some drugs used in clinical practice revealed that the acidic polysaccharide complex AP was less active (43.5%) than the most frequently used opioid antitussive drug codeine (61.8%), but much more active than the two non-narcotic synthetic drugs dropropizine (28.3%) and prenoxdiazine (24.7%).

It may be concluded that the high antitussive activity of this natural polymer without adverse effects on the tested animals may rank this polymer among prospective antitussive agents.

5. Immunomodulatory activity

The immunomodulatory activity of AP was examined in the in vitro mitogenic and comitogenic thymocyte tests and compared to that of the commercial immunomodulator Zymosan and the previously reported biologically active xylan (MahX) isolated from the same *Mahonia* drug (Kardošová et al., 2002). This test was originally developed for muramyl glycopeptides (Iribe & Koba, 1984), which as such, do not stimulate markedly thymocyte proliferation. On the other hand, some plant polysaccharides are directly mitogenic for rat thymocytes. Thus, it is possible to detect both mitogenic as well as comitogenic activities. The negative polymyxin B test indicated the absence of endotoxin contamination in AP. The results of the mitogenic and comitogenic tests are presented in Fig. 7. As seen, AP showed a dose-dependent stimulatory activity similarly as MahX. The stimulatory effects of both acidic polysaccharides were comparable to those of Zymosan, but achieved at about 10 times higher doses. Zymosan at such doses showed an inhibition effect. The observed differences between the compared polysaccharides were not statistically significant ($p > 0.05$).

In accord with the interpretation of the comitogenic test (Iribe and Koba, 1984; Rovenský, Pekárek, & Mlynárčik, 1990), the SI_{comit}/SI_{mit} ratio could be suggested as an estimate of the adjuvant property of the tested polysaccharides. The ratios of approximately 4 that we have found for

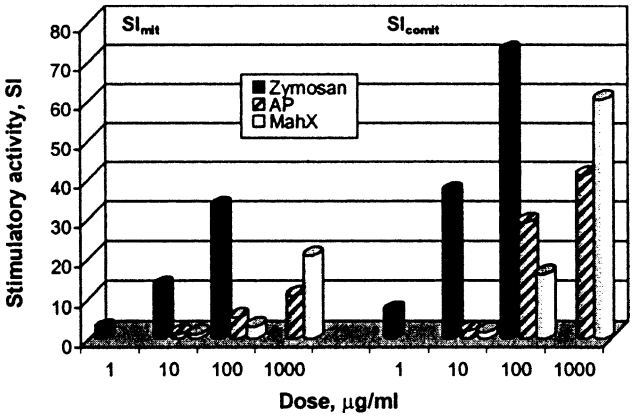


Fig. 7. Immunostimulatory activity in the mitogenic and comitogenic thymocyte tests of AP and the glucuronoxylan MahX isolated from *Mahonia aquifolium* (Kardošová et al., 2002) in comparison to the standard—Zymosan.

both acidic polysaccharides indicates significant adjuvant properties.

The results of both antitussive and mitogenic activity tests point to the fact that polymeric cell wall components contribute essentially to the overall biological activity of the mahonia drug and substantiate its further application in the field of pharmaceutics.

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