Polysaccharides from the Leaves of *Plantago lanceolata* L., var. LIBOR: an α-D-Glucan

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A crude mucilage, composed of L-arabinose (26.0 %), D-galactose (35.8 %), D-glucose (21.9 %), D-mannose (4.6 %), L-rhamnose (4.6 %), and uronic acids (6.9 %), has been isolated from the leaves of *Plantago lanceolata* L., var. LIBOR. From this mucilage a homogeneous, water-soluble α-D-glucan has been separated by successive chromatography on DEAE-Sephadex A-50 and Sephadex G-75. The results of methylation analysis, periodate oxidation, and ^{13}C NMR measurement indicated a linear character of the polysaccharide chain composed of α-D-glucopyranose units, linked by (1→6) glycosidic bonds.

The use of various polysaccharides for pharmaceutical purposes has increased considerably during the last decades. This is not only the case of polysaccharides used as excipients, but also of a series of carbohydrate polymers which have proved to be most useful as direct drugs [1].

Our previous works were concerned with isolation of mucilages from the leaves of marsh mallow (*Althaea officinalis* L., var. *Robusta*) and structure determination of their polysaccharide components [2, 3]. The investigation was motivated by therapeutical effects of the drug in treatments of catarrhs of the respiratory system, gastritis, and inflammations of nasal and oral cavities [4]. *Plantago* leaves were reported to exhibit similar therapeutic effects [4] and have been used also as demulcent, since they contain viscous polysaccharides [5]. While the seeds of different *Plantago* species, a drug used as diuretic and cough medicine, have been intensively analyzed [6—8], little is known about the polysaccharides from the *Plantago* leaves. Lately, *Bräutigam* and *Franz* [9] described the water-soluble polysaccharides of *Plantago lanceolata* leaves as a mixture of an arabinogalactan, a glucomannan, and an acid rhamnogalacturonan.

The present work deals with isolation of the crude mucilage from the leaves of *Plantago lanceolata* L., var. LIBOR, a medicinal plant improved and cultivated in CSFR, and with separation and structure determination of a homogeneous polysaccharide component, a linear α-D-glucan.

EXPERIMENTAL

Material and Methods

The medicinal plant *Plantago lanceolata* L., var. LIBOR was improved in CSFR at the Cultivation Station, Libochovice. The mature leaves were collected in 1990.

Solutions were concentrated under diminished pressure at temperature below 40 °C. Paper chromatography was performed by the descending method on Whatman No. 1 paper in the systems S_1, ethyl acetate—pyridine—water (φ_r = 8 : 2 : 1) and S_2, ethyl acetate—acetic acid—water (φ_r = 18 : 7 : 8). Saccharides were detected with anilinium hydrogen phthalate. Preparative paper chromatography was performed on Whatman 3 MM paper in system S_1. Determination of the protein content was carried out by the method of *Lowry et al.* [10]. The amino acid composition was established with an Automatic Amino Acid Analyzer, type 6020 (Mikrotechna, Prague) after hydrolysis of the polysaccharide with 6 M-HCl for 20 h at 100 °C. The uronic acid content was determined by the carbazole method [11].

The crude mucilage was completely hydrolyzed with 0.5 M-H_2SO_4 for 20 h and the glucan with 2 M trifluoroacetic acid for 6 h at 100 °C. Partial hydrolysis of the glucan (200 mg) was carried out with 0.25 M-H_2SO_4 (90 cm^3) on a steam bath under reflux for 4 h.

The glucan (20 mg) was oxidized with sodium periodate (20 cm^3, c = 15 mmol dm^{-3}) at 4 °C in the dark, the consumption of periodate being spectrophotometrically monitored [12] on aliquots at λ = 223 nm. The reaction was complete after 30 h.

Both the glucan and the disaccharide were methylated by the *Hakomori* method [13]. The efficiency of methylation was checked by the absence of IR absorption for hydroxyl. The fully methylated glucan was hydrolyzed, converted into alditol acetates [14], and analyzed by GLC—MS. The methylated disaccharide (5 mg) was methanolyzed with 4 vol. % methanolic hydrogen chlo-

ride (5 cm^3) in a sealed glass capillary at 105 °C for 7 h, neutralized with silver carbonate, and centrifuged. The supernatant was vacuum-dried and analyzed by GLC.

Free-boundary electrophoresis was effected with a Zeiss 35 (Jena) apparatus, on the polysaccharide (ρ = 10 mg cm^{-3}) in 0.05 M sodium borate buffer of pH 9.2, for 30 min at 10 V cm^{-1} and 6 mA. Optical rotation was measured with a polarimeter 141 (Perkin–Elmer) in an 0.5 mass % aqueous solution at 22 °C. The number average molecular mass ($\bar{M}_N$) was determined osmometrically at 30 °C, using a Knauer Vapour Pressure Osmometer. Infrared spectrum of the methylated products was recorded with a spectrometer 9836 (Perkin–Elmer).

GLC was conducted with a chromatograph 5711 (Hewlett–Packard) and a column *A* (200 cm x 0.3 cm) of 3 % OV-225 on 150—175 μm Chromosorb W (AW-DMSC), programmed to hold a temperature of 120 °C for 4 min, then to increase it to 170 °C at 2 °C min^{-1}; and a column *B* (200 cm x 0.3 cm) of 3 % SP-2340 on 125—150 μm Supelcoport at 110 °C for 2 min, then increasing the temperature to 210 °C at 4 °C min^{-1} Column *A* was used for quantitative determination of sugars in the form of trifluoroacetates [15]. GLC–MS of alditol acetates of methylated saccharides was carried out on the same column as *B*. The inlet helium pressure was 101.3 kPa, temperature programmed from 160 °C to 240 °C min^{-1}. The spectra were measured with a spectrometer JMS-D 100 (Jeol) at the ionization potential of 23 eV.

^{13}C NMR spectrum of the glucan solution (3 mass % in D_2O) was taken with a Bruker AM-300 (75 MHz) spectrometer at 30 °C with suppression of NOE effect. The spectral width was 16.380 Hz; acquisition time 0.5 s; relaxation delay 3 s; data points 16 K; pulse width 19 μs (90°). Chemical shifts were measured relative to internal methanol (δ = 50.16).

Isolation of the Mucilage

The air-dried leaves (60 g) were preextracted with methanol (2 dm^3) and then macerated in cold water (2.5 dm^3) for 24 h at room temperature. The extract was filtered, concentrated to 600 cm^3 and poured into ethanol (3.6 dm^3, 96 vol. %) containing acetic acid (1 %). The precipitate was allowed to settle overnight in the cold. The crude polysaccharides were collected by centrifugation (2000 *g*, 15 min), washed with diluted aqueous ethanol (70 vol. %), suspended in water, and dialyzed against distilled water for 3 d in the cold. The solution in the dialysis bag was concentrated under reduced pressure and freeze-dried to give a brownish product (0.9 g).

Fractionation of the Crude Mucilage

For fractionation of the crude mucilage, batch separation on an ion-exchanger was employed. It was carried out by stirring DEAE-Sephadex A-50 (500 g), previously equilibrated with 0.5 M ammonium carbonate, with aqueous solution of the crude mucilage (5 g, 500 cm^3) for 2 h. The slurry was then filtered and the residue was washed with the buffer solution. This procedure was repeated with a new batch of the ion-exchanger. The water-filtrate was concentrated and freeze-dried to give a yellowish product *P* (1.9 g).

A part of product *P* (200 mg) was dissolved in water and applied to a column (150 cm x 4 cm) of Sephadex G-75. The column was eluted with water, and fractions of 15 cm^3 were collected and analyzed by the phenol–sulfuric acid assay [16]. The elution profile is illustrated in Fig. 1. Peak I, eluted at lower elution volume, was heterogeneous and on complete acid hydrolysis gave rise to a mixture of sugars. Peak II, eluted at higher elution volume, was composed of D-glucose (dominating) and small amounts of D-galactose and L-arabinose. Rechromatography of this fraction at the same conditions provided a white product (65 mg), homogeneous upon electrophoresis and containing D-glucose as the only component sugar.

RESULTS AND DISCUSSION

A crude mucilage was obtained from the leaves of *Plantago lanceolata* L., var. LIBOR by water extraction, followed by ethanol precipitation and dialysis. The brownish product, obtained in 1.5 % per dry mass of the plant material, contained 5.6 % protein. The amino acid composition of the protein moiety is presented in Table 1. The sugar composition of the crude mucilage, presented in Table 2, suggested a mixture of different polysaccharides. The first method used for fractionation was a batch separation of aqueous solution of the mucilage with DEAE-Sephadex A-50 (CO_3^{2-}) to a neutral and an acid portion. In this step, most of the accompanying colour material was removed. The neutral portion consisted of (*x*/%) D-glucose (15.8), D-galactose (41.4), D-mannose (1.8), L-rhamnose (4.2), L-arabinose (29.1), and D-xylose (7.4). Subsequent gel chromatography of this material on Sephadex G-75

Table 1. Amino Acid Composition of the Crude Mucilage from the Leaves of *Plantago lanceolata* L., var. LIBOR

Amino acid	x/%
Lysine	5.51
Histidine	1.90
Arginine	2.61
Aspartic acid	10.05
Threonine	6.62
Serine	7.93
Glutamic acid	10.73
Proline	7.20
Glycine	9.59
Alanine	9.78
Valine	8.75
Methionine	1.50
Isoleucine	3.97
Leucine	8.07
Tyrosine	2.81
Phenylalanine	2.98

resulted in two peaks (Fig. 1), differing in carbohydrate composition. Peak I, eluted at lower elution volume, contained (*x*/%) D-glucose (5.0), D-galactose (42.0), D-mannose (0.9), L-rhamnose (11.4), L-arabinose (38.1), and D-xylose (2.4). In peak II, D-glucose predominated over small amounts of D-galactose and L-arabinose. Rechromatography of this fraction at the same conditions afforded a homogeneous compound containing D-glucose as the only component sugar. The glucan was readily soluble in water, showed a positive optical rotation + 84°, and had a number average molecular mass $\overline{M}_N$ = 19 479 (DP = 120). The iodine colour reaction with this glucan was negative.

Partial hydrolysis of the glucan provided beside the sugar monomer a disaccharide of chromatographic mobility identical with that of the isomaltose standard. The disaccharide was separated by preparative paper chromatography and identified as isomaltose on the basis of its optical rotation + 99° and methanolysis products of the methylated disaccharide, shown by GLC to be methyl 2,3,4,6-tetra-*O*-methyl-D-glucopyranoside and methyl 2,3,4-tri-*O*-methyl-D-glucopyranoside.

The hydrolytic products of the fully methylated polymer, converted to the corresponding alditol acetates, were identified by GLC–MS analysis as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, indicating a linear polymer with (1 → 6) glycosidic bonds. This fact was supported also by the periodate consumption (1.9 mol per glucose unit) within 30 h oxidation of the polysaccharide, which is very close to the theoretical 2 mol for a hexose unit linked by (1 → 6) bonds.

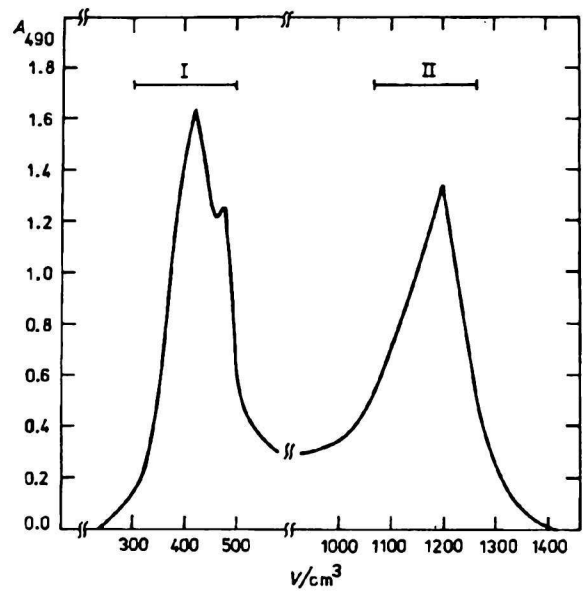

Fig. 1. Gel chromatography elution profile of product *P*.

Table 2. Characterization of the Crude Mucilage Isolated from the Leaves of *Plantago lanceolata* L., var. LIBOR

Yield /%[a]	1.5
w(Protein)/%	5.6
Sugar composition (*x*/%)	
D-Glc	21.9
D-Gal	35.8
D-Man	4.6
L-Rha	4.6
L-Ara	26.0
D-Xyl	Traces
L-Fuc	Traces
Uronic acid	6.9

a) Relative to dry mass of the leaves.

The ^{13}C NMR spectrum of the polysaccharide is a further evidence for structure identification (Fig. 2). Each signal in the spectrum could be assigned unambiguously [17, 2]. The signal belonging to anomeric carbons was observed at δ = 98.9, suggesting the α-anomeric character of the glucosidic bonds. The only other signal shifted downfield relative to its position in glucopyranose was that for C-6 at δ = 66.7, evidencing the involvement of this carbon in a linkage. Deviations due to the ring size or conformational differences of anomeric bonds were not observed in the spectrum. The additional, minor resonances observable in the spectrum are evidently due to the residual, accompanying arabinogalactan.

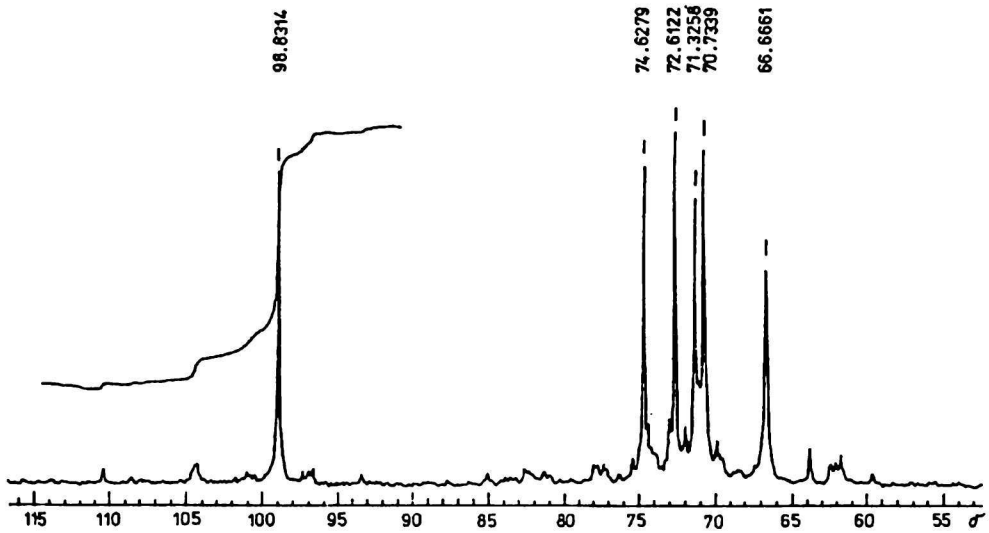

Fig. 2. ^{13}C NMR spectrum of the α-D-glucan.

The water-soluble, linear α-(1 → 6)-D-glucan described herein resembles the glucan isolated from the leaves of the medicinal plant *Althaea officinalis* L., var. *Robusta* [2]. Separation of the other neutral polysaccharide component of the mucilage under study and its structure determination will be the subject of our further work.

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