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Studies on Pectins From the Leaves of *Tussilago farfara* L.

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During previous work we have shown that the main fraction of the water soluble polysaccharides from the leaves of *Tussilago farfara* L. is a pectin, containing galacturonic acid (67 %) and galactose, glucose, arabinose, and rhamnose, together with small amounts of xylose and ribose. The present paper gives evidence of three further components of this pectin: D-glucuronic acid, L-fucose, and 2-*O*-methyl-D-xylose.

Further yield of a pectin of similar composition (galacturonic acid, 66 %) may be obtained by extracting with an ammonium oxalate solution the leaves already being exhaustively extracted with water.

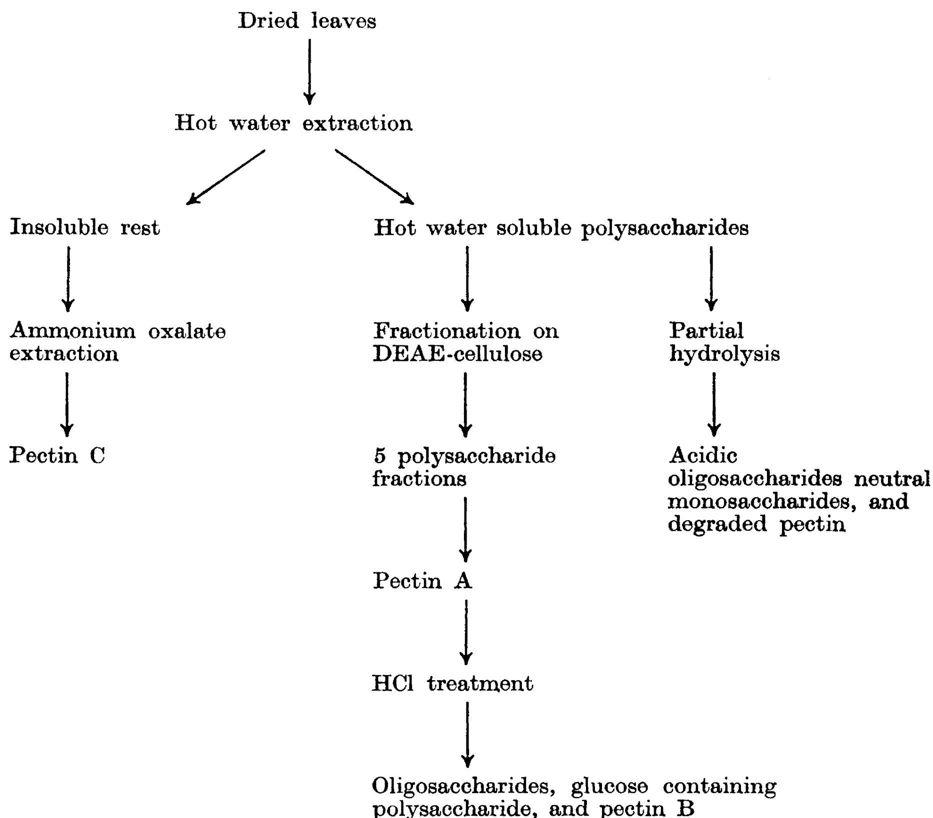
The water soluble pectin may, by extremely mild hydrolysis, be degraded to give, among other products, a polysaccharide (yield 5 %) consisting of D-glucose only.

Degradation of the water soluble pectin by more vigorous hydrolysis yielded among other products a series of acidic oligosaccharides which were separated on an anion exchange resin and by paper chromatography. The following acidic oligosaccharides were characterized:

1. *O*-(α-D-galactopyranosyluronic acid)-(1→4)-D-galacturonic acid;
2. *O*-(α-D-galactopyranosyluronic acid)-(1→4)-*O*-α-D-galactopyranosyluronic acid-(1→4)-D-galacturonic acid;
3. *O*-(α-D-galactopyranosyluronic acid)-(1→2)-L-rhamnose;
4. *O*-(α-D-galactopyranosyluronic acid)-(1→4)-D-galactose;
5. *O*-(β-D-galactopyranosyluronic acid)-(1→4)-D-galactose;
6. *O*-(β-D-glucopyranosyluronic acid)-(1→6)-D-galactose;
7. *O*-(β-D-glucopyranosyluronic acid)-(1→4)-L-fucose.

The main constituent of pectins is D-galacturonic acid, but they also contain neutral sugars, especially D-galactose, L-arabinose, and L-rhamnose. Polysaccharides of this type have been examined from several sources.[1–4] From the leaves of *Tussilago farfara* L. we have isolated from hot water extract five polysaccharide fractions, and the main fraction proved to be a pectin (pectin A).[5] The present investigation was done to continue our studies on the polysaccharides in the leaves of *Tussilago farfara L.*

Dried leaves of *Tussilago farfara* L. were extracted successively with hot water and hot aqueous ammonium oxalate (Scheme 1). Extraction with hot water gave a mixture of polysaccharides which was contaminated with inorganic material.[5] The ammonium oxalate extract gave ammonium pectate



Scheme 1.

which was eluted as a single band when the polysaccharide was chromatographed on diethylaminoethyl cellulose by the procedure of Neukom *et al.*[6] The uronic anhydride content was 66 %, and hydrolysis gave galacturonic acid and the neutral monosaccharides galactose, glucose, arabinose, and rhamnose as the main components. This pectin (pectin C) was not further studied.

Franz [7] has shown by hydrolyzing a crude water extract of the leaves that some fructose is present in addition to the sugars usually found in pectins. In order to study this more detailed the water soluble polysaccharides were separated on diethylaminoethyl cellulose into five fractions. Each fraction was hydrolyzed under mild conditions with oxalic acid and examined. Fructose was only present in the first fraction eluted from the column. This fraction is probably a mixture of a fructan and a more complex polysaccharide consisting mainly of galactose, glucose, and arabinose.

The water-soluble pectin (pectin A)

The analytical composition of this main fraction of water soluble polysaccharides is given in our previous publication.[5] One of the methyl sugars, discovered in traces in the hydrolysate of Pectin A, has now been identified as 2-*O*-methyl-D-xylose.

Mild acid hydrolysis of a pectin may be able to split off part of the polysaccharide without disturbing the main chain.[8] Attempts were made to perform an extremely mild hydrolysis with hydrochloric acid (0.06 M), followed by precipitation with ethanol of high polymer material.

This material was fractionated on a column of DEAE cellulose in phosphate form. Phosphate buffer eluted a fraction (yield 5 %) which appeared to give exclusively D-glucose on hydrolysis, changing its specific rotation, $[\alpha]_{546}$ from + 60°, before hydrolysis to + 50°. It is probably a glucan. The main part of the original pectin (yield 85 %) was eluted from the column by sodium hydroxide. This pectin B gave by complete hydrolysis spots corresponding to D-galacturonic acid, D-galactose, D-glucose, L-arabinose, and traces of other sugars.

Acidic oligosaccharides from pectin A

Partial hydrolysis of the isolated water soluble pectin released a number of acidic oligosaccharides. The crude polysaccharide from the water extract was hydrolyzed in the same way, but did not give any other acidic oligosaccharides. After hydrolysis the mixture of oligosaccharides was partly separated on a De-Acedite formate column by elution with formic acid. Further separations were carried out by chromatography on thick filter paper. The following oligosaccharides were isolated in sufficient amounts for characterisation (Table 1).

Table 1.

1. α-D-GalpA-(1→4)-D-GalA
2. α-D-GalpA-(1→4)-D-GalA-(1→4)-D-GalA
3. α-D-GalpA-(1→2)-L-Rha
4. α-D-GalpA-(1→4)-D-Gal
5. β-D-GalpA-(1→4)-D-Gal
6. β-D-GpA-(1→6)-D-Gal
7. β-D-GpA-(1→4)-L-fuc

These oligosaccharides were examined according to standard methods. Determination of the type of glycosic linkage was based upon optical rotation and in some cases upon the ability of α-galactosidase to hydrolyze the reduced methyl ester methyl glycosides.

Pectins have a backbone of α-D-galacturonic residues linked together by (1→4)-bonds.[2,3] From the partial hydrolysate, galacturonic acid, di- and trigalacturonic acid were isolated, and the plot of the degree of polymerization *versus* log $(1/R_F - 1)$ was linear, indicating that they were a homologous series.[9] The digalacturonic acid was proved to have the proposed structure which is typical of pectins. Also oligosaccharide 3 is often found by

hydrolysis of pectins.[1-3] It was not stained with triphenyltetrazolium chloride and hydrolysis gave galacturonic acid and rhamnose.

Glucuronic acid and fucose were not found in the hydrolysate of the crude polysaccharide 5 because of the small amounts present, but they were isolated as oligosaccharide 7. This oligosaccharide gave blue coloration with periodate – pararosaniline spray reagent,[10] and was not to be distinguished from *O*-(β-D-glucopyranosyluronic acid)-(1→4)-L-fucose. Glucuronic acid was also a constituent of oligosaccharide 6. This oligosaccharide has previously been found by Aspinall [1] in a lemon-peel pectin.

Oligosaccharides 4 and 5 contain both galacturonic acid and galactose. Methylation showed that both had (1→4), linkages but they had different mobilities in paper chromatography and electrophoresis. α-Galactosidase hydrolyzed only the reduced methyl ester methyl glycoside of oligosaccharide 4, and $[\alpha]_{546}$ was also different for the two sugars. Consequently oligosaccharide 4 is proposed to have an α-glycosidic linkage and oligosaccharide 5 to have a β-glycosidic linkage.

Galactose seems to play an important structural role. This is evident from the characterization of the acidic oligosaccharides 4, 5, and 6, which all contain galactose.

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 and 3 MM filter papers with the following solvent systems (v/v):

A. Butanol, pyridine, water, 5 : 3 : 2.
B. Ethyl acetate, acetic acid, formic acid, water, 18 : 3 : 1 : 4.
C. Ethyl acetate, pyridine, acetic acid, water, 5 : 5 : 1 : 3.
D. Butanol, ethanol, water, 5 : 1 : 4.

R_{GalA} values of the acid sugars refer to rates of movement relative to D-galacturonic acid. Thin layer chromatography (TLC) was carried out on 0.25 mm layers of Silica gel G with the following solvent systems (v/v):

E. Benzene, ethanol, 20 : 3.
F. Chloroform, ethanol, 25 : 2.
G. Benzene, ethanol, 4 : 1.

High volt electrophoresis was performed on Munktell No. 302 filter paper at *ca.* 40 V/cm in 0.05 M sodium tetraborate, pH 9.2, 0.05 M germante buffer, pH 10.7, and pyridine acetic acid buffer, pH 5.3. M_G values refer to the movements relative to glucose, and M_{GalA} to galacturonic acid. The optical rotation was measured by a Bendix-NPL automatic polarimeter with an interference filter for 546 nm (mercury green) at 20° in water.

Localization of the spots was obtained with diphenylamine-aniline-phosphoric acid and aniline phthalate for the reducing sugars and with periodate starch reagent for the sugar alcohols.

When nothing else is mentioned, acidic oligosaccharides were hydrolyzed in M sulphuric acid at 100° for 18 h, and neutral oligosaccharides were hydrolyzed in 0.5 M sulphuric acid at 100° for 18 h.

Extraction and fractionation of the polysaccharides

The isolation and separation of the hot water soluble polysaccharides are described earlier.[5] After being extracted with hot water 1 kg of the leaves was mixed with 6 l of 0.5 % ammonium oxalate and left at 80 – 90° for 3 h. The insoluble material was removed

by filtration, and 10 % calcium chloride was added until the precipitation of calcium pectate was complete. Calcium pectate was heated in 0.3 % ammonium oxalate solution for 0.5 h at 90°, calcium oxalate was removed by centrifugation, and the ammonium pectate was precipitated with 2 vol. acetone. 0.3 g ammonium pectate was dissolved in 0.025 M phosphate buffer, pH 6.0, and poured on a column of DEAE-cellulose (phosphate form) (2 × 30 cm). The column was eluted with 0.025 M, 0.05 M, 0.1 M, 0.25 M phosphate buffer, pH = 6,0, and a gradient of sodium hydroxide (0.01 – 0.3 M). Fractions (*ca.* 5 ml) were collected, and the eluted sugars were registered by the automatic polarimeter.

The water soluble polysaccharides were also separated on a DEAE-cellulose column, and the five fractions which were obtained were heated in 1 % oxalic acid at 100° for 2 h. After neutralization with calcium carbonate, paper chromatography and TLC, fructose was only found in the first fraction eluted from the DEAE-cellulose column.

2-*O*-Methyl-xylose was isolated from pectin A after hydrolysis. The sugar (15 mg), $[\alpha]_{546} + 35°$ (*c* 0.7), had R_{Rhamnose} 1.30 and 1.24 in B and D, and M_G 0.29 in borate buffer. The sugar did not move in germanate buffer, and was not stained with triphenyltetrazolium chloride. The methyl sugar was further not oxidized by silver carbonate on Celite in methanol after boiling for 15 min. This indicates a 2-*O*-substituted sugar.[12] The methoxyl content was determined gravimetrically after Zeisel to be 19.0 % (calc. for $C_6H_{12}O_5$, 19.0 %), and demethylation with boron trichloride [13] gave xylose.

Partial hydrolysis of pectin A

1. *Degradation to pectin B.* 100 mg of pectin A was dissolved in 50 ml 0.05 N sodium hydroxide, 50 ml 0.1 N hydrochloric acid was added, and the solution was left for 45 h at 37°. The polysaccharides were precipitated with 2 vol. of ethanol and washed with 70 % ethanol. The supernatant and washings were evaporated to a sirup and hydrolyzed with 1 N trifluoracetic acid for 2 h at 100°. Trifluoracetic acid was removed *in vacuo.* Paper chromatograms of the hydrolysate showed spots corresponding to D-glucose (main component), D-galactose, D-galacturonic acid, and L-arabinose, together with traces of other sugars.

The polysaccharides were dissolved in 0.025 M phosphate buffer, pH 6.0, and poured on a DEAE cellulose column (phosphate form) (1.8 × 20 cm). A polysaccharide was eluted with 0.025 M phosphate buffer, and pectin B was eluted with sodium hydroxide. The polysaccharide which is cut off from pectin A seems to consist of glucose only; $[\alpha]_{546} + 60°$ decreased to + 50° in *ca.* 1 h by dissolving the polysaccharide in 3 N H_2SO_4. The pectin having resisted degradation (85 %) gave by hydrolysis D-galacturonic acid (main component), D-galactose, D-glucose, and L-arabinose, together with traces of other sugars.

2. *Isolation of acid oligosaccharides.* 20 g of the crude water soluble polysaccharide was hydrolyzed with 1 N sulphuric acid (1 l) at 100° for 4 h. After cooling, the degraded polysaccharide was precipitated with 2 vol. of ethanol and was hydrolyzed again as done before. This was repeated until no sugar was released by hydrolysis. The supernatants were neutralized with barium hydroxide and barium carbonate, the precipitated barium salts were removed by centrifugation and washed several times with water. The combined centrifugate and washings were concentrated, treated with Amberlite resin IR-120 (H^+) to remove barium ions, and further concentrated to a sirup. The sirup was dissolved in water and added to a column of De-Acedite FF-IP, 100 – 200 mesh in formate form (4.5 × 60 cm). The column was eluted with water until no more sugar was registered by the polarimeter. The acidic oligosaccharides were then eluted in four fractions with 0.5 M, 1.0 M, and 2 M formic acid. The acidic oligosaccharides were not separated completely, and further separation was carried out by paper chromatography in solvents B and C. Galacturonic acid and seven acidic oligosaccharides enumerated in Table 1, were isolated in sufficient amount for further studies.

3. *Characterization of the acidic oligosaccharides.* The acidic oligosaccharides were examined by following reactions:

(1) After hydrolysis the hydrolysate was neutralized with the calculated amount of barium carbonate and treated with Amberlite IR 120 (H^+). Paper chromatography showed the components.

(2) The sugar (2 – 3 mg) was dissolved in water (5 ml), sodium borohydride (10 mg)

was added, and the solution left for 18 h. The solution was neutralized with Amberlite IR 120 (H^+) and evaporated to dryness. The reduced sugar was washed several times with methanol and hydrolyzed.

(3) The carboxyl group of the uronic acid in oligosaccharide was reduced after dissolving the sugar in methanol, containing Dowex W (H^+), and keeping it at 67° for 24 h. The methyl ester methyl glycoside was then reduced with sodium borohydride.

(4) 2-*O*-Substituted sugars were present when they were not stained with a freshly prepared solution of 2,3,5-triphenyltetrazolium chloride, 1 % in 1 M sodium hydroxide.

(5) The reduced methyl ester methyl glycosides were methylated as described by Hakomori.[14] 3 – 4 mg was dissolved in dimethyl sulfoxide (2 ml), and sodium hydride was added. After 5 min methyl iodide (1 ml) was added under cooling, and the reaction mixture was shaken for 2 h at room temperature. The addition of sodium hydride and methyl iodide was repeated, and after another 2 h shaking, the mixture was poured into water (4 ml) and extracted successively with chloroform. The chloroform extracts were washed with small amounts of water and evaporated at reduced pressure. The methylated oligosaccharides were hydrolyzed and studied on thin layer chromatography.

(6) The reduced methyl ester methyl glycosides were dissolved in water, α-galactosidase was added, and the mixture left for 20 h at 37°. The enzyme activity was stopped by heating the mixture for a few minutes in boiling water, and the coagulated enzyme was removed by membrane filtration. Oligosaccharides with galacturonic acid and an α-configuration were by this method hydrolyzed, and galactose was liberated.

Galacturonic acid (200 mg), $[\alpha]_{546}$ +59°, could be distinguished neither by paper chromatography nor by electrophoresis from D-galacturonic acid. Hydrolysis of the reduced methyl ester methyl glycoside yielded galactose.

Oligosaccharide 1. The sugar, $[\alpha]_{546}$ +125° (*c* 1.0), had R_{GalA} 0.22 in solvent B and M_G 0.95 in borate buffer. Hydrolysis yielded only galacturonic acid. The methyl ester methyl glycoside was reduced, and galactose was liberated on hydrolysis and by incubation with α-galactosidase. The sugar was stained with triphenyltetrazolium chloride. Methylation of the reduced methyl ester methyl glycoside gave a product which on hydrolysis and subsequent TLC in solvents B and F had mobilities of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose.

Oligosaccharide 2. The sugar (80 mg) had R_{GalA} 0.05 in solvent B and gave galacturonic acid only on hydrolysis. Galacturonic acid, oligosaccharides 1 and 2, all fell on a straight line when log $(1/R_F - 1)$ was plotted against n.

Oligosaccharide 3. The sugar (20 mg), $[\alpha]_{546}$ +110° (*c* 1.0), had R_{GalA} 0.80 in solvent B and M_G 0.55 in borate buffer. Reduction and hydrolysis gave galacturonic acid and rhamnitol, and hydrolysis of the reduced methyl ester methyl glycoside gave galactose and rhamnose. The oligosaccharide was not stained with triphenyltetrazolium chloride, and incubation of the reduced methyl ester methyl glycoside with α-galactosidase liberated galactose.

Oligosaccharide 4. The sugar (18 mg), $[\alpha]_{546}$ +80 (*c* 0.9), had R_{GalA} 0.20 in solvent B, M_G 0.90 in borate buffer, M_{GalA} 0.73 in pyridine acetate buffer, and hydrolysis gave galacturonic acid and galactose. The sugar was reduced, and hydrolysis gave galacturonic acid and galactitol. The reduced methyl ester methyl glycoside gave on hydrolysis only galactose, and galactose was also liberated when it was incubated with α-galactosidase. The sugar was stained with triphenyltetrazolium chloride, and methylation of the reduced methyl ester methyl glycoside, followed by hydrolysis, gave on TLC spots corresponding to 2,3,4,6-tetra-*O*-methyl-galactose and 2,3,6-tri-*O*-methylgalactose.

Oligosaccharide 5. The sugar (24 mg), $[\alpha]_{546}$ −8° (*c* 1.2), had R_{GalA} 0.50 in solvent B and M_G 0.78 in borate buffer, and hydrolysis have galacturonic acid and galactose. The reduced sugar gave galacturonic acid and galactitol, and the reduced methyl ester methyl glycoside gave galactose on acid hydrolysis. The sugar was stained with triphenyltetrazolium chloride, and methylation of the reduced ester methyl ester methyl glycoside followed by hydrolysis gave on TLC spots corresponding to 2,3,4,6-tetra-*O*-methylgalactose and 2,3,6-tri-*O*-methylgalactose.

Oligosaccharide 6. The sugar (8 mg), $[\alpha]_{546}$ +10° (*c* 0.4), had R_{GalA} 0.22 in solvent, M_G 0.95 in borate buffer, and gave glucuronic acid, glucuronolactone, and galactose on hydrolysis. Reduction followed by hydrolysis gave glucuronic acid, and glucuronolactone as the only reducing sugars. The reduced methyl ester methyl glycoside gave glucose and galactose on hydrolysis. The sugar was stained with triphenyltetrazolium chloride, and

hydrolysis of the methylated methyl ester methyl glycoside gave 2,3,4,6-tetra-*O*-methylglucose and 2,3,4-tri-*O*-methylgalactose.

Oligosaccharide 7. The sugar (15 mg), $[\alpha]_{546}$ −70 (*c* 0.75), had R_{GalA} 0.48 and 1.05 in solvents B and C, M_{G} 0.60 in borate buffer, M_{GalA} 0.80 in pyridine-acetic acid buffer, and gave glucuronic acid, glucuronolactone, and fucose on hydrolysis. The oligosaccharide was chromatographically and ionophoretically indistinguishable from *O*-(β-D-glucopyranosyluronic acid)-(1→4)-L-fucose, and after reduction and hydrolysis, glucuronic acid and glucuronolactone were the only reducing sugars. Hydrolysis of the reduced methyl ester methyl glycoside gave glucose and fucose. The sugar was stained with triphenyltetrazolium chloride and gave a blue coloration with periodate-pararosaniline reagent.[10] The absence of a yellow coloration indicated that malondialdehyde was not formed on periodate oxidation because of the 4-*O*-substituted fucose residue.[15]

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REFERENCES

1. Aspinall, G. O., Craig, J. W. T. and Whyte, J. L. *Carbohyd. Res.* **7** (1968) 442.
2. Battacharjee, S. S. and Timell, T. E. *Can. J. Chem.* **43** (1965) 758.
3. Aspinall, G. O. and Fanshawe, R. S. *J. Chem. Soc.* **1961** 4215.
4. Barret, A. J. and Northcote, D. H. *Biochem. J.* **94** (1965) 617.
5. Haaland, E. *Acta Chem. Scand.* **23** (1969) 2546.
6. Neukom, H., Deuel, H., Heri, W. J. and Kündig, W. *Helv. Chim. Acta* **43** (1960) 64.
7. Franz, G. *Planta Medica* **17** (1969) 218.
8. Heri, W. J., Neukom, H. and Deuel, H. *Helv. Chim. Acta* **44** (1961) 1940.
9. French, D. and Wild, G. M. *J. Am. Chem. Soc.* **75** (1953) 2614.
10. Hardy, F. E. and Buchanan, J. G. *J. Chem. Soc.* **1963** 5881.
11. McComb, E. A. and McCready, R. *Anal. Chem.* **24** (1952) 1630.
12. Morgenlie, S. *Acta Chem. Scand.* **25** (1971) 2773.
13. Bonner, T. G., Bourne, E. J. and McMally, S. *J. Chem. Soc.* **1960** 2929.
14. Hakomori, S. *J. Biochem. (Tokyo)* **55** (1964) 205.
15. Aspinall, G. O., Cottrell, W. J., Egan, S. V., Morrison, J. M. and Whyte, J. N. C. *J. Chem. Soc.* **C 1967** 1071.

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