Carbohydrate Polymers **18** (1992) 209–216

Enzymic degradation of pectic substances isolated from the inner bark of *Ulmus glabra* Huds.

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(Received 28 March 1991; revised version received 18 June 1991; accepted 9 October 1991)

Pectic substances were extracted from inner bark of *Ulmus glabra* Huds. using water at 100 °C. The water extract was fractionated into three polymer fractions using a combination of two different ion exchange chromatography columns (DEAE-Sepharose fast flow and PBE™ 94 (polybuffer exchanger)). The isolated pectic polymer fractions were degraded with pectolyase (endopolygalacturonase and endopectin lyase) after first having been deesterified by pectinesterase. Degradation products were fractionated by gel permeation chromatography (Bio-Gel P-2). The carbohydrate composition of the degraded products were determined by gas chromatography analysis of the trimethylsilyl methyl-glycoside derivatives and the glycosyl-linkages were established by methylation analysis before and after carboxyl reduction. The degradation products were also subjected to arabinogalactan-Yariv reagent crossed electrophoresis. The results show that the three pectic polymer fractions have similarities in the carbohydrate content and in the type of glycosyl-linkages present; however they react differently towards enzyme degradation and the Yariv reagent.

INTRODUCTION

The inner bark of the common elm, *Ulmus glabra* Huds., was in former days used for medicinal and nutritional purposes (Reichborn-Kjennerud, 1922; Nordhagen, 1954; Høeg, 1974). The inner bark contains special mucilage-containing sacs, and the powdered inner bark has gelling and thickening properties. The hot water extract of the inner bark of common elm consists of pectic substances. These pectic substances can be separated into at least nine different polymers (Barsett & Smestad Paulsen, 1985); three of these have been isolated and characterized (Barsett & Smestad Paulsen, 1991). Pectic substances have been extensively studied and the methods of investigation described (Pilnik & Voragen, 1970; Aspinall, 1982; O'Neill *et al.*, 1990; Selvendran & Ryden, 1990). Pectic substances are composed of a rhamnogalacturonan backbone in which (1 → 2) linked rhamnosyl residues are interspersed in chains of α(1 → 4) linked galacturonic acids partly esterified by methanol. Rhamnosyl residues are the main branching points of the chains. Side chain sugars are predominantly galactose and arabinose, but xylose and rare sugars such as apiose, fucose, methyl-fucose or methyl-xylose have been identified in some pectic substances. One of the pectic polymers isolated from hot water extract of inner bark of *Ulmus glabra*, Huds., has side chains consisting of 3-*O*-methyl-galactose besides galactose and arabinose. 3-*O*-methyl-galactose is (1 → 4) linked, and is shown to be covalently attached to the C-4 of rhamnose residues (Barsett & Smestad Paulsen, 1991). The inner bark of slippery elm, *Ulmus fulva*, also contains 3-*O*-methyl-galactose (Hirst *et al.*, 1951; Beveridge *et al.*, 1971) and it was suggested that this was attached to the C-4 of rhamnose residues in a rhamnogalacturonan backbone. Enzyme degradation of pectic substances has been performed by Thibault (1983) and Saulnier and Thibault (1987); these have shown that pectic substances isolated from both grape berries and cherry fruit are composed of 'smooth' homo-galacturonan areas, interspersed with very densely branched 'hairy' regions where neutral sugar side chains are located. The authors now report enzyme degradation and structural elucidation of three different pectic polymers isolated from the hot water extract of the inner bark of *Ulmus*

Carbohydrate Polymers 0144-8617/92/$05.00

glabra Huds. The polymers were degraded by pectolyase (endopolygalacturonase EC 3.2.1.15 and endopectin lyase EC 4.2.2.10, isolated from *Aspergillus japonicus*), after first having been subjected to deesterification by pectinesterase (EC 3.1.1.11).

METHODS

General methods

The water used was always distilled. Solutions were concentrated under diminished pressure at 40 °C in a Büchi Rotavapor®. All dialyses were performed using tubing with a nominal molecular weight cutoff of 12 000 or 3500, and all filtrations were performed through Whatman GF/A glass fibre filter. Centrifugation was performed in a Sorvall® RC2-B automatic super-speed refrigerated centrifuge at 2500 *g* and 10 °C for 20 min, or a MSE benchtop centrifuge at 1000 *g* for 5 min. Absorbance at 487 nm was measured using an LKB Novaspec 4049 spectrophotometer. Absorbance at 230 nm was measured using a Shimadzu UV-160A UV-visible recording spectrophotometer. Determination of methoxyl contents, quantitative determination of the carbohydrate composition, per-iodate oxidation, reduction of uronic acids in polymers and oligomers, methylation analysis and ethylation analysis were performed as described previously (Barsett & Smestad Paulsen, 1991).

Preparation of the elm bark extract for chromatography

The inner bark of the common elm (*Ulmus glabra* Huds.) (120 g) was extracted with 4 litres of ethanol-water (3 : 1) at 80 °C for 2 h to remove low molecular weight material and coloured matter. The extraction was repeated four times. The carbohydrate polymer was then extracted with 2 × 6 litres of water at 100 °C, dialysed and lyophilized. The crude extract thus obtained (7 g) was further purified after redissolution at 100 °C for 4 h, centrifuged at 1800 *g* and filtered through Whatman GF/A glass fibre filter to obtain a purified crude extract.

Ion exchange chromatography of the purified crude extract

The purified crude extract (0·8 g) was applied to a DEAE-Sepharose fast flow column (60 cm × 5 cm) converted into the chloride form. The column was coupled to a P-3 peristaltic pump (Pharmacia), and 11 ml fractions were collected using an Ultrorac 7000 fraction collector (LKB). The column was eluted at 1·57 ml/min, first with water (500 ml), then by gradient elution using 0–1 M sodium chloride for elution of the acidic polymers (Fig. 1). The fractions were tested for carbohydrate content colorimetrically (487 nm) by the phenol-sulphuric acid methods (Dubois *et al.*, 1956). Fractions 78–106 were pooled, dialysed, freeze-dried and designated Ug I (0·36 g), and fractions 107–125 were pooled, dialysed, freeze-dried and designated Ug II (0·1 g).

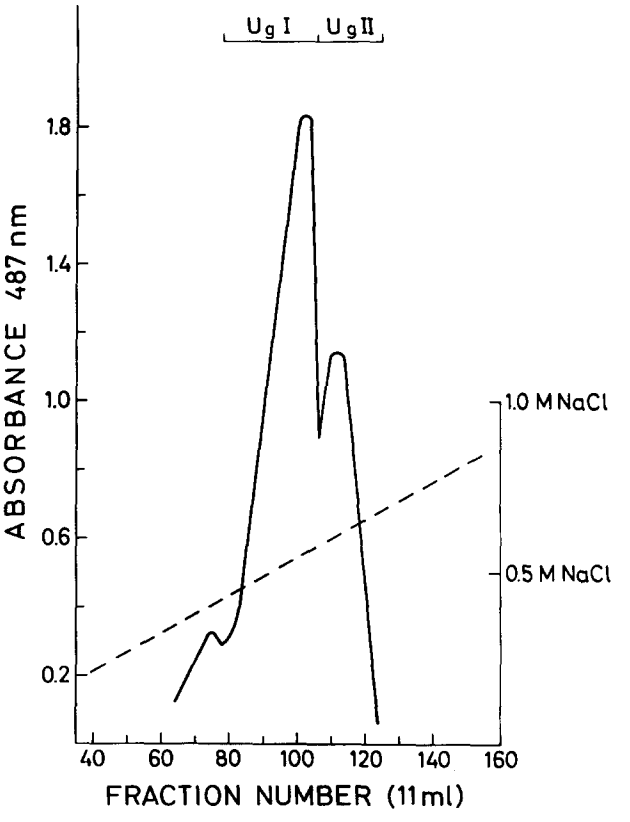

Fig. 1. Ion exchange chromatography of the hot water extract from the bark of common elm, on DEAE-Sepharose fast flow. (——) Carbohydrate profile; (- - - -) salt gradient (0–1 M sodium chloride).

Further purification of Ug I and separation of Ug II on a PBE (polybuffer exchanger)™ 94 column

Fraction Ug I was further purified by application on the PBE™ 94 column (40 cm × 2·5 cm) converted into the chloride form. Fractionation of Ug II was performed on the same column. For both separations, the column was coupled to a P-3 peristaltic pump (Pharmacia), and 65 ml fractions were collected using an Ultrorac 7000 fraction collector (LKB). The column was in both cases eluted at 1 ml/min, first with water (200 ml), then by gradient elution using 0–1 M sodium chloride. The fractions were tested for carbohydrate content as above. The elution profile of Ug I gave one fraction, and this further purified fraction was designated PB I. Fraction Ug II was separated into two polymers designated PB II and PB III (Fig. 2).

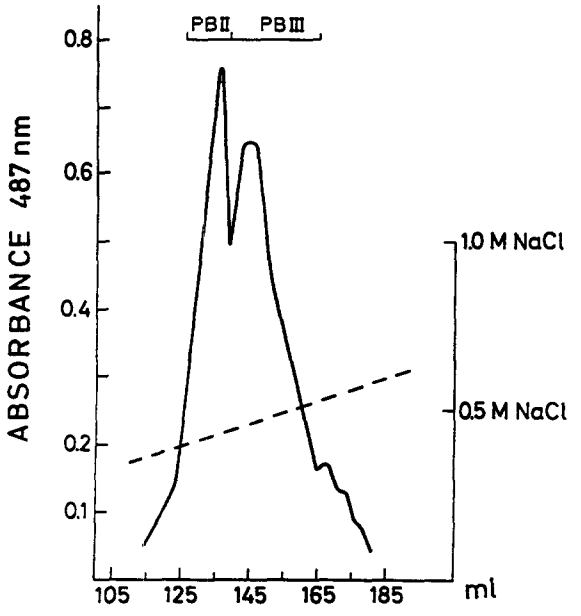

Fig. 2. Fractionation of Ug II on a PBE™ 94 column. (——) Carbohydrate profile; (- - - -) salt gradient (0–1 M sodium chloride).

Molecular weight estimation of PB I, PB II and PB III on Superose™ 12 HR 10/30

The gelfiltration column Superose 12 HR 10/30, was fitted to the FPLC system (Pharmacia) previously described (Barsett & Smestad Paulsen, 1985). A 1–2 mg sample in 500 µl was injected, and the column was eluted at 0·4 ml/min, using 0·01 M sodium chloride. Fractions of 0·3 ml were collected and tested for carbohydrate content as above.

Enzymes and degradation conditions

Prior to degradation, a 200 mg polysaccharide sample was deesterified with 5 mg pectinesterase (EC 3.1.1.11) from orange peel (Sigma Chemical Co.) in phosphate buffer (0·1 M, pH 7·5) at 30 °C for 7·5 h. The sample was then subjected to degradation with 7 mg pectolyase (endopolygalacturonase EC 3.2.1.15 and endopectin lyase EC 4.2.2.10, isolated from *Aspergillus japonicus*) (Sigma Chemical Co.) in citrate-phosphate buffer (0·1 M, pH 5·5) under magnetic stirring at 25 °C for 14 h. The solution was filtrated and concentrated *in vacuo* to 4 ml. All enzyme treatment was terminated with rapid heating to 100 °C, before cooling to room temperature.

Isolation of oligosaccharides by gel permeation chromatography on Bio-Gel P-2

The enzyme degraded polysaccharide (4 ml) was applied to a Bio-Gel P-2 column (60 cm × 2·5 cm). The column was coupled to a P-3 peristaltic pump (Pharmacia), and 2 ml fractions were collected using an Ultrorac 7000 fraction collector (LKB). The column was eluted at 10 ml/h with 2% butanol (anti-microbial agent) in water. The fractions were tested for carbohydrate content as above.

Glucose, raffinose, stachyose and maltohexaose were used as standards.

The enzyme degraded polysaccharide fraction PB I was separated into five fractions designated PB $I_{1\text{-}5}$. The corresponding five fractions isolated from PB II were designated PB $II_{1\text{-}5}$, and from PB III the isolated fractions were designated PB $III_{1\text{-}5}$ (Fig. 3).

Mild acid hydrolysis

To remove carbohydrate existing as furanose units, 20 mg of fraction PB I_1 was subjected to mild acid

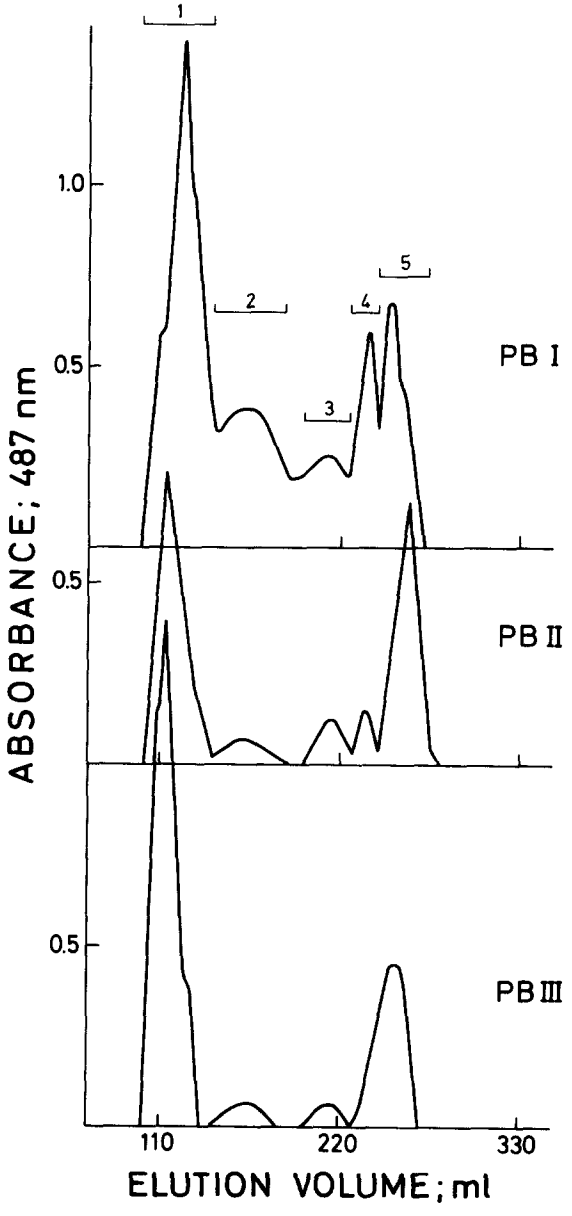

Fig. 3. Elution profiles after chromatography on Bio-Gel P-2, of enzyme degraded PB I, PB II and PB III.

hydrolysis with 0·005 M sulphuric acid (10 ml) at 100 °C for 3 h (Smestad *et al.*, 1975). The hydrolysate was neutralized with sodium bicarbonate (0·25 M) and fractionated on a Bio-Gel P-2 column, as described above. The higher molecular weight fraction corresponding to the main core, was designated PB I_1A. The low molecular weight fraction corresponding to the released furanose units, was designated PB I_5A.

Thin-layer chromatography (TLC) of the isolated oligosaccharides

TLC was carried out on Kiselgel 60 F 254 (20 cm × 20 cm) in isobutanol : formic acid : water (6 : 9 : 4) as a development solvent. Detection was carried out by spraying with 4% anilin in acetone : 4% diphenylamin in acetone : 85% phosphoric acid (5 : 5 : 1) followed by heating at 110 °C for 5 min (Bailey & Bourne, 1960). Galacturonic acid, digalacturonic acid and trigalacturonic acid (kindly provided by Dr Bertil Nilsson, Swedish Sugar Co., Arløv, Sweden) were used as standards.

Arabinogalactan-Yariv reagent crossed electrophoresis

Arabinogalactan-Yariv reagent crossed electrophoresis was performed essentially as described by Van Holst and Clarke (1986). A solution of 1% (w/v) agarose in 0·025 M tris, 0·2 M glycine (pH 8·3) was heated to boiling. A sample (15·5 ml) was poured on to a preheated, level glass plate (10 cm × 10 cm). Nine wells (1·5 mm in diameter) were made in the gel. The top well was filled with 15 µl of bromophenol blue (0·2 mg/ml) in 50 mM tris-HCl (pH 8·0) and the other wells were filled with 15 µl sample mixed with 0·8 µl of bromophenol blue (3 mg/ml). The first-dimension gel was run at 5 V/cm for about 55 min, or until the dye front had moved 4 cm. The running buffer was 0·025 M tris, 0·2 M glycine. After electrophoresis, the slice of a sample lane gel (1 cm × 5 cm) was moved to a Gel Bond film (Pharmacia) (6 cm × 5 cm) where the second gel (1 mm thick) was made of 1% (w/v) agarose in 0·025 M tris, 0·2 M glycine and 30 µg/ml β-glucosyl-Yariv reagent (the last component was added after boiling). Electrophoresis in the second dimension was performed at 10 V/cm for about 5 h. The non-precipitated Yariv reagent was removed by washing with 1% (w/v) NaCl followed by rinsing with distilled H_2O and the gels were dried in a warm air stream (Fig. 4).

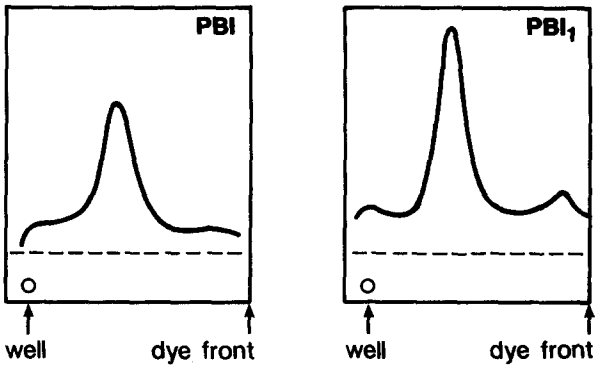

Fig. 4. Arabinogalactan-Yariv reagent crossed electrophoresis of PB I, 87 µg and PB I_1, 42·5 µg. The lowest part of the gel (1 cm × 5 cm) is the first-dimension gel and the dye front is bromophenol blue. The second-dimension gel contains 30 µg/ml Yariv reagent.

RESULTS AND DISCUSSION

Pectic substances were isolated from the inner bark of *Ulmus glabra* Huds. by extraction with water at 100 °C. This gave polymers with a higher content of arabinose and a higher relative content of galacturonic acid than the polymers previously described (Barsett & Smestad Paulsen, 1991). A partly purified extract was applied on a DEAE-Sepharose fast flow column, and the acidic polymers were eluted from the column by a salt gradient elution. The fractions marked Ug I and Ug II on Fig. 1 were pooled, dialysed and freeze-dried. Nothing was eluted in the first 40 fractions. For further fractionation of Ug I and Ug II, a PBE™ 94 column was chosen because this has the same ligand as the Mono P column which has been shown to be capable of separating polysaccharides of this type (Barsett & Smestad Paulsen, 1991). The PBE™ 94 column is preferred for preparative separation of the polysaccharide fractions, while the Mono P column in the FPLC system is preferred for analytical purposes. The acidic polymers were eluted with a salt gradient elution. The fraction Ug I was not separated further by this method or any other tried. The material isolated from PBE™ 94, was designated PB I. Fraction Ug II did separate into two new fractions, and the fractions marked PB II and PB III in Fig. 2 were pooled, dialysed and freeze-dried.

The relative carbohydrate composition, before and after per-iodate oxidation of the polysaccharide fractions isolated are given in Table 1. The fractions are quite similar with respect to the carbohydrate composition. Of the Ug II fractions, only one of them, fraction PB III, contained 3-*O*-methyl-galactose. The degree of esterification was determined by headspace gas analysis by gas chromatography. Due to the great amount needed for this determination, the degree of esterification of just fraction Ug I (31·8%) and Ug II (22·2%) was determined. In accordance with previous separation and characterization of acidic polysaccharides from *Ulmus glabra*, the elution profile of ion exchange chromatography has a clear connection with the amount of free COOH-groups. The molecular weight estimation of PB I, PB II and PB III on Superose™ 12 HR 10/30 in the FPLC system showed that all three fractions had an apparent molecular weight higher

Table 1. Relative carbohydrate composition (% wt), before and after per-iodate oxidation, of polysaccharide fractions isolated from the inner bark of *Ulmus glabra*

Monosaccharides	Ug I		Ug II		PB I		PB II		PB III[a]	
	Native	Peri. ox[b]	Native	Peri. ox	Native	Peri. ox	Native	Peri. ox	Native	Peri. ox
Ara	8	—	17	—	13	—	13	—	9	—
Rha	8	5	24	4	13	6	22	3	18	6
3-*O*-Me-Gal	—	—	5	5	—	—	—	—	5	5
Gal	15	4	6	1	8	2	10	2	7	—
GalA	69	19	48	11	66	17	55	14	61	10

[a]Glucuronic acid was present in trace amounts.
[b]Peri. ox, Per-iodate oxidation.

Table 2. Methylation analysis of various native and carboxyl-reduced polysaccharide fractions

Glycosyl residue	Position of *O*-methyl groups	Methylated products (% mol)					
		Rel.[a] R_t	Ug I	Ug II	PB I	PB II	PB III
Ara	2,3,5	0·62	4·0	10·0	6·4	7·9	5·6
	2,3,4	0·68	0·3	1·1	0·6		
	2,3	0·87	6·4	10·2	10·1	8·6	6·2
Rha	2,3,4	0·69	0·3		0·6		
	3,4	0·88	5·1	15·7	8·2	21·6	9·0
	2,4,5,6	0·91				tr.	
	2	1·07				tr.	
	4	1·13	1·7	3·9	2·7	3·6	1·8
	3	1·15	2·7	7·8	4·1	0·4	10·8
Gal	2,3,4,6	1·06	5·1	2·1	2·6	2·4	3·2
	2,3,6	1·33	5·3	4·2	2·9	7·6	6·8
	2,4,6	1·38	2·1	1·5	1·1		0·3
	2,3,4	1·59	1·1	0·2	0·5		
	3,6	1·71		1·1		0·5	0·6
	2,3	1·93		1·6			1·5
	2,4	2·09	3·1		1·6		
GalA[b]	2,3,6	1·33	62·8	40·6	58·6	47·4	54·2
GlcA[b]	2,3,4,6	1·00					tr.

[a]Relative to 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl-glucitol.
[b]Determined as the increase of methylated glycosyl residues after carboxyl-reduction.
tr., Trace.

than 300 000 with reference to standard dextrans. Fraction PB I had a lower molecular weight than PB II, and PB III had the highest molecular weight of the three fractions. The isolated fractions PB I, PB II and PB III seem to have much data in common with the isolated fractions A_{FPLC}, B_{FPLC} and C_{FPLC} previously described (Barsett & Smestad Paulsen, 1991). The polysaccharide fractions were methylated both before and after reduction of the carboxyl-groups, and the results are given in Table 2. All the fractions contain terminal arabinose in furanose form and (1 → 4/5) linked arabinose. Of the PBE™ 94 isolated fractions, only fraction PB I contains some terminal arabinose in the pyranose form. In all the fractions, rhamnose is (1 → 2) linked, and the branch point rhamnoses are either linked (1 → 3) or (1 → 4). In the fraction PB III which contains 3-*O*-methyl-galactose, (1 → 2) linked rhamnose with branch points on the C-4 position is responsible for the major part of the branch point rhamnoses present. This is the same as for the FPLC-isolated fraction C_{FPLC} previously described (Barsett & Smestad Paulsen, 1991); likewise, both these fractions contain glucuronic acid. When it comes to galactose, all the fractions isolated contain terminal galactose and (1 → 4) linked galactose. This include the 3-*O*-methyl-galactose. Of the PBE™ 94 isolated fractions, PB I also contain (1 → 3) linked galactose, some (1 → 6) linked galactose and branch point galactose linked both (1 → 3) and (1 → 6). PB II containing some branch point galactose linked both (1 → 4) and (1 → 2),

while PB II contain some (1 → 3) linked galactose and branch point galactose linked either both (1 → 4) and (1 → 2), or both (1 → 4) and (1 → 6). All the fractions contain (1 → 4) linked galacturonic acid, and the PB III fractions also contain trace amounts of terminal glucuronic acid. Even though galacturonic acid was not completely oxidized by per-iodate, methylation results showed no branch point galacturonic acid. This underoxidation may be due to formation of hemiacetals between neighbouring hexuronic acid residues during the per-iodate oxidation, as described by Painter and Larsen (1970).

In order to obtain further details of the polysaccharides isolated, the fractions PB I, PB II and PB III were subjected to enzyme degradation with pectolyase after deesterification with pectinesterase. Various pectic degrading enzymes and combinations of them gave the result that the method described gave the best yield of oligomers. The enzyme degraded polysaccharides were separated into five fractions each on a Bio-Gel P-2 column, and the fractions were designated PB I_{1-5}, PB II_{1-5} and PB III_{1-5}, respectively, as described in Fig. 3. The relative carbohydrate composition of the fractions isolated on the Bio-Gel P-2 column are given in Table 3. The separation conditions used were those which among various tested gave the best separation of the products obtained by the enzyme degradation. Fraction PB I_1 was subjected to mild acid hydrolysis for removal of carbohydrate existing in furanose form, and the resulting polymer is designated PB I_1A. The carbohydrate composition after this treatment is also given in Table 3. Most of the arabinose in fraction PB I_1 exists as furanose units. Thin-layer chromatography of the isolated oligosaccharides showed that the PB I, PB II and PB III fractions 4 and 5 contained just monosaccharides. The PB I_3, PB II_3 and PB III_3 contained disaccharides and the PB I_2, PB II_2 and PB III_2 contained trisaccharides. The PB I_1, PB II_1 and PB III_1 are heterogenous oligosaccharide fractions. The monomer fractions PB I_4, PB II_4 and PB III_4 consist of just galacturonic acid, while the monomer fractions PB I_5, PB II_5 and PB III_5 consist of both galacturonic acid and arabinose. The arabinose monomers must be due to some hydrolysis caused by the enzyme degradation conditions used. The disaccharides isolated are digalacturonic acid, and the trisaccharides isolated are trigalacturonic acid. This includes disaccharides and trisaccharides where the terminal galacturonic acid is unsaturated due to β-elimination. Spectrophotometric analysis at 230 nm of all the Bio-Gel P-2 isolated fractions showed very high absorbance in fractions 2 and 3, respectively, which refer to 4,5-unsaturated galactosyl uronic acid. This may be due to incomplete deesterification. TMS-derivatives (trimethylsilylation) of unsaturated galacturonic acid were identified by GC and GC/MS. These methanolysis results, together with the methylation results, showed that the 2 and 3 fractions isolated after enzyme degradation of PB II and PB III contained more unsaturated galacturonic acid due to β-elimination than the 2 and 3 fractions isolated after enzyme degradation of PB I. The relative carbohydrate composition of the enzyme degraded polysaccharide fractions showed that a higher proportion of galacturonic acid in the PB I fraction is degraded than in the two other fractions PB II and PB III. This may be due to longer sections of homo-galacturonic acid in the PB I polysaccharide than in the PB II and PB III polysaccharides. The pectolyase used for degradation of the polymers consists of both endopolygalacturonase (EC 3.2.1.15) and endopectin lyase (EC 4.2.2.10) isolated from *Aspergillus japonicus*. The endopolygalacturonase, which hydrolyzes the glycosidic linkages between two galacturonic acids, is hampered by rhamnosyl residues and neutral sugar side chains (Saulnier & Thibault, 1987). The endopectin lyase, which catalyzes the β-elimination reaction between methylesterified galacturonic acid, is blocked by steric factors and requires a certain sequence of methylesterified galacturonosyl residues (Rexova-Benkova & Markovic, 1976; Pilnik & Rombouts, 1981). Thus, when rhamnose is often interspersed in the backbone, the result will be low degradation with pectolyase. A rhamnogalacturonan with a high content of rhamnose in the backbone will have a different configuration to a rhamnogalacturonan which has longer regions of

Table 3. Relative carbohydrate composition of the fractions isolated on a Bio-Gel P-2 column, after enzyme degradation of PB I, PB II and PB III

Monosaccharides	PB I_1	PB I_1A[a]	PB II_1	PB III_1
Ara	19	3	6	tr.
Rha	25	30	23	23
3-*O*-Me-Gal				10
Gal	14	16	12	11
GalA	42	51	59	52
GlcA				4

[a] PBI_1A is obtained after mild acid hydrolysis of PB I_1.
tr., Trace.

homogalacturonic acid. This can explain why it seems to be much easier to deesterify the PB I polysaccharide which has longer regions of homo-galacturonic acid than the PB II and PB III polysaccharides, and why the heterogenic oligomers PB II_1 and PB III_1 contain a higher amount of galacturonic acid. The enzyme degradation products are methylated before and after carboxyl reduction, and the results are given in Tables 4 and 5. The methylated products of PB I_1 subjected to weak acid hydrolysis (PB I_1A) are also given in Table 4. Methylation of the heterogenous oligosaccharide fractions PB I_1, PB II_1 and PB III_1 showed that these products contain the same types of glycosyl-linkages as their parent polymers, and that the three polysaccharide fractions have structural differences. The PB I_1 oligomer fraction contains relatively less galacturonic acid than the two other oligomer fractions, and a higher part of the rhamnose units are branched. The side chains consist of arabinogalactan, and previous work (Barsett & Smestad Paulsen, 1991) has shown that galactose is linked to the rhamnose unit. PB II_1 is the fraction which contains the least amount of branch point rhamnoses. In this fraction, side chains of galactose are linked mostly to C-3 of the rhamnose. The PB III_1 fraction has side chains consisting of 3-*O*-methyl-galactose and galactose, linked mainly to C-4 of the rhamnose unit. 3-*O*-methyl-galactose is previously shown to be linked (1 → 4) to the rhamnose unit (Barsett & Smestad Paulsen, 1991), and methylation with CD_3I and ethylation of PB III_1 showed that 3-*O*-methyl-galactose is (1 → 4) linked. Pectic substances have been found to contain side chains of homo-arabinans, galactans and/or arabinogalactans (Albersheim *et al.*, 1973; Talmade *et al.*, 1973;Darvill *et al.*, 1980; Saulnier *et al.*, 1988).

To obtain further information on the composition of the side chains, the polysaccharides and their enzyme degradation products were subjected to arabinogalactan-Yariv reagent crossed electrophoresis. As shown in Fig. 4, only PB I and PB I_1 gave precipitates with Yariv reagent. PB II, PB II_1, PB III and PB III_1 gave a straight line with the Yariv reagent. These results show that PB I and PB I_1 have arabinogalactan side chains. The results also show that, although the three isolated polymers PB I, PB II and PB III contain monosaccharides linked with identical glycosidic linkages, the side chains must be of a different character. On the basis of these results, it is obvious that the isolated polymers PB I, PB II and PB III have structural differences which by the methods used in this study can only be detected with the Yariv reagent. The PB I polymer seems to have areas with homo-galacturonan interspersed with regions more densely branched, and this polymer contains arabinogalactan side chains. In the PB II and PB III polymers the rhamnose unit is probably more randomly interspersed in the backbone, and PB III polymer has a higher content of side chains than the PB II polymer. The side chains in PB II consist of galactose and arabinose linked mostly to C-3 of the rhamnose unit, while the side chains in PB III consist

Table 4. Methylation analysis of native and carboxyl-reduced fractions, isolated after enzyme degradation of PB I and PB II

Glycosyl residues	Position of *O*-methyl groups	Methylated products (% mol)							
		Rel.[a] R_t	PB I_1	PB I_1A[b]	PB I_2	PB I_3	PB II_1	PB II_2	PB II_3
Ara	2,3,5	0·62	9·8	3·2			2·6		
	2,3,4	0·68	0·5				tr.		
	2,3	0·87	12·9	0·6			5·3		
Rha	2,3,4	0·69	5·5	0·7			2·0		
	3,4	0·88	11·1	16·8			20·4		
	2,4	0·91					0·4		
	2	1·07					0·4		
	4	1·13	4·4	7·6			3·6		
	3	1·15	6·7	9·9			0·3		
Gal	2,3,4,6	1·06	7·7	6·7			3·1		
	2,3,6	1·33	2·6	4·8			9·0		
	2,4,6	1·38	1·3	2·3					
	2,3,4	1·59	1·2	1·9					
	3,6	1·71					1·0		
	2,4	2·09	1·5	1·4					
GalA[c]	2,3,4,6	1·06		10·2	37·5	55·6		tr.	tr.
	2,3,6	1·33	34·8	33·9	62·5	44·4	51·9	100	100

[a] Relative to 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl-glucitol.
[b] PB I_1A is obtained after mild acid hydrolysis of PB I_1.
[c] Determined as the increase of methylated glycosyl residues after carboxyl-reduction.
tr., Trace.

Table 5. Methylation/ethylation analysis of native and carboxyl-reduced fractions isolated after enzyme degradation of PB III

Glycosyl residue	Position of *O*-methyl/ *O*-ethyl groups	Methylated products (% mol)					Ethylated products (% mol)	
		with CH_3I				with CD_3I[a]		
		Rel.[b] R_t	PB III_1	PB III_2	PB III_3	PB III_1	Rel.[b] R_t	PB III_1
Ara	2,3,5	0·62	tr.					
Rha	3,4	0·88	17·4			34·7		
	4	1·13	1·4			6·9	1·23	15·8
	3	1·15	8·7			13·8	1·25	39·6
Gal	2,3,4,6	1·06	1·9			5·6	1·50	5·6
	2,3,6	1·33	19·1			16·8	1·77	18·5
	2,6	1·63	0·2			tr.		
	3,6	1·71	0·4			tr.		
	2,3	1·93	0·6			1·7		
3-*O*-Me-Gal	2,6	1·33				20·5	1·68	20·5
GalA[c]	2,3,4,6	1·06	0·9	32·6	tr.			
	2,3,6	1·33	45·3	67·4	100			
GlcA[c]	2,3,4,6	1·00	4·1					

[a] Ethylation and methylation with CD_3I were performed on the native PB III_1 fraction only.
[b] Relative to 1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methyl-glucitol.
[c] Determined as the increase of methylated glycosyl residues after carboxyl-reduction.
tr., Trace.

of 3-*O*-methyl-galactose, galactose and arabinose mostly linked to C-4 of the rhamnose unit. The results also show that ion exchange chromatography is affected by the degree of methylesterification of the galacturonic acid, the number of and composition of the side chains, and the shape of the molecule.

ACKNOWLEDGEMENT

The authors are indebted to F. Tønnesen for recording all the GC/MS data.

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