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# The CDTA-Soluble Pectic Substances from Soybean Meal are Composed of Rhamnogalacturonan and Xylogalacturonan but not Homogalacturonan

***Abstract:*** *Structural characteristics of pectic substances extracted from soybean meal cell walls (water unextractable solids) with a chelating agent-containing buffer (0.05*M *1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) and 0.05*M *$NH_4$-oxalate in 0.05*M *NaOAc buffer) were studied. The arabinogalactans present as side chains to the rhamnogalacturonan backbone were largely removed by enzymatic hydrolysis using* endo-*galactanase,* exo-*galactanase,* endo-*arabinanase, and arabinofuranosidase B. The remaining pectic backbone appeared to be resistant to enzymatic degradation by pectolytic enzymes. After partial acid hydrolysis of the isolated pectic backbone, one oligomeric and two polymeric populations were obtained by size-exclusion chromatography. Monosaccharide and linkage analyses, enzymatic degradation, and NMR spectroscopy of these populations showed that the pectic substances in the original extract contain both rhamnogalacturonan and xylogalacturonan regions, while homogalacturonan is absent.* © 2001 John Wiley & Sons, Inc. Biopoly 58: 279–294, 2001

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## INTRODUCTION

Pectic substances are a major group of polysaccharides in the primary cell wall of dicotyledonous plants. These pectic substances consist of a number of structurally different regions. Pectins from apple consist of highly methyl esterified linear homogalacturonan regions that alternate with "hairy" regions that comprise highly branched rhamnogalacturonan.[1] In addition, these hairy regions appeared to contain xylogalacturonan.[2] This concept of the appearance of pectic substances proved to be applicable to pectic substances from other sources—for example, cotton suspension-cultured cell walls, watermelon,[3] and pea hulls.[4,5] However, differences in the relative amounts of the subunits may exist.

Another type of pectic polysaccharide in the plant cell wall is RG-II. RG-II is a low-molecular weight, structurally well-defined, complex pectic polysaccharide. It can be released from the cell wall by endopolygalacturonase treatment. It consists of a homogalacturonan backbone to which side chains are attached. The side chains contain several rare "diagnostic" monosaccharides and three out of four side chains contain rhamnose.[6]

Studies on the structure of soybean pectic substances go back to 1967. An acidic polysaccharide complex was extracted from soybean meal,[7,8] which was shown to possess a highly branched structure composed of galacturonic acid, galactose, arabinose, xylose, fucose, and rhamnose. The interior chains were found to comprise 4-linked galactosyluronic acid and 2-linked rhamnosyl residues, and exterior chains were composed mainly of neutral sugar residues. Some of the rhamnose residues were branched at C4, and some galacturonic acid residues were branched with xylose residues through C3. Most of the fucose and a substantial proportion of the xylose residues were present as nonreducing end groups. These results indicated that soybean pectic substances contain both rhamnogalacturonan and xylogalacturonan regions. However, only the formulation of partial structures for the soybean acidic polysaccharide complex was permitted, because the data were obtained after partial acid hydrolysis. This is an aspecific way of hydrolysis, and resulted in rather small fragments ($DP \leq 6$).

In the preceding paper,[9] we showed that the pectic backbone from CDTA-extractable pectins (CDTA: 1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid) could be isolated after enzymatic removal of the arabinogalactan side chains. In this study, we report on both the enzymatic and acid hydrolysis of the remaining pectic backbone. Furthermore, structural analyses of this backbone and fractions thereof will be described.

## EXPERIMENTAL

### Materials

Water unextractable solids (WUS) were isolated from solvent-extracted, untoasted soybean meal. Soybean WUS (20 g) was extracted with 0.05*M* CDTA and 0.05*M* $NH_4$-oxalate in 0.05*M* NaOAc buffer, pH 5.2 (eight times 600 mL) at 70°C for 1 h, and subsequently washed with distilled water (two times 600 mL). After each extraction solubilized polymers were separated from the insoluble residue by centrifugation (19,000× *g*; 30 min). The combined supernatants were dialyzed against 0.1*M* $NH_4OAc$ buffer (pH 5.2) before dialyzing against distilled water. The extract was freeze dried to obtain the chelating agent soluble solids (ChSS) fraction.[10] The arabinogalactan side chains from the pectins in the ChSS extract were removed by enzymatic degradation and the remaining polymer was isolated and designated Fraction P.[9]

### Acid Hydrolysis of Fraction P

A 8 mg/mL solution of Fraction P was hydrolyzed in 0.1*M* HCl at 80°C. During hydrolysis, samples were taken after various time intervals, and the reaction was stopped by neutralization of the solution with an equal amount of 0.1*M* NaOH. The hydrolysates were analyzed with high-performance size-exclusion chromatography (HPSEC) and high-performance anion-exchange chromatography (HPAEC).

### Enzymatic Degradation

Solutions [0.25% (w/w)] of Fraction P in 50 m*M* NaOAc buffer (pH 5.0) containing 0.01% $NaN_3$ were incubated with a number of purified enzymes at 30°C, rotating "head over tail" for 24 h. The purified enzymes used were *Aspergillus aculeatus endo*-galactanase[11] (EC 3.2.1.89, 0.34 $\mu$g protein/mL substrate solution), *Aspergillus niger exo*-galactanase[12] (0.52 $\mu$g protein/mL substrate solution), *Kluyveromyces fragilis* polygalacturonase[13] (PG; E.C. 3.2.1.15, 0.49 $\mu$g protein/mL substrate solution), *Aspergillus niger* pectin lyase[14] (PL; E.C. 4.2.2.10, 9.76 $\mu$g protein/mL substrate solution), *Aspergillus aculeatus* rhamnogalacturonan hydrolase[15,16] (RG hydrolase; 0.53 $\mu$g protein/mL substrate solution), and *Aspergillus aculeatus exo*-galacturonase[17] (1.03 $\mu$g protein/mL substrate solution).

Solutions [0.5% (w/w)] of Fraction P were also incubated with technical multienzyme preparations under the conditions described above. The technical enzyme preparations used were Pectinex Ultra-SP-L derived from *Aspergillus aculeatus*, Viscozyme derived from *Aspergillus aculeatus* [Novo-Nordisk Ferment (Switzerland) Ltd., Dittingen, Switzerland], Rapidase liq+ derived from *Aspergillus niger/Trichoderma* sp. (Gist-brocades, Delft, The Netherlands), and Driselase derived from *Irpex lacteus* (Sigma, St.

Louis, MO, USA). The enzyme preparations were dialyzed against 50 m*M* NaOAc buffer (pH 5.0) and diluted 100 times. Fifty microliters of the enzyme solution was added to 0.5 mL of the substrate solution.

Solutions [0.1% (w/w)] in 0.05*M* NaOAc buffer (pH 5.0) containing 0.01% $NaN_3$ of the populations obtained after fractionation of the acid hydrolysate of Fraction P (PI, PII, and PIIIA) were incubated with PG (2.4 $\mu$g protein/mL substrate solution), RG hydrolase (0.18 $\mu$g protein/mL substrate solution), and *exo*-galacturonase (1.03 $\mu$g protein/mL substrate solution). In addition, these substrates were also incubated with xylogalacturonan hydrolase (XGH; 16 $\mu$g protein/mL substrate solution) for 1 h at 30°C. XGH was purified from the culture filtrate of *Aspergillus tubigensis* cDNA library expression cloned in *Kluyveromyces lactis.*[18]

All enzymes were inactivated by heating at 100°C for 10 min. Polysaccharide-degrading activities were determined by HPSEC and HPAEC analyses of the digests.

## Neutral Sugar Composition

Neutral sugar composition was determined by methanolysis combined with trifluoroacetic acid (TFA) hydrolysis.[19] Samples were first dissolved in distilled water (1 mg/mL). An aliquot of 20 $\mu$L of this solution was dried by a stream of air followed by methanolysis with 0.5 mL anhydrous methanol containing 2*M* HCl for 16 h at 80°C. After cooling, the liquid was evaporated by a stream of air and 0.5 mL of 2*M* TFA solution was added and heated for 1 h at 121°C. The samples were dried and 100 $\mu$L of distilled water was added. Analysis of the liberated products was performed using HPAEC.

## Uronic Acid Content

The uronic acid content was determined by the automated colorimetric *m*-hydroxydiphenyl assay[20,21] using an autoanalyzer (Skalar Analytical BV, Breda, The Netherlands). Corrections were made for interference by neutral sugars present in the sample as measured by the orcinol–sulfuric acid method.[22]

## Absolute Configuration Determination

Absolute configuration determination of the monosaccharides was performed for ChSS only,[23,24] since all other polymers were obtained from this material. The trimethylsilylated 2-butyl glycosides were analyzed by gas-liquid chromatography (GLC) on a Chrompack CP9002 gas chromatograph, equipped with a CP-Sil 5 CB DFc.25 (Chrompack) capillary column (25 m × 0.32 mm), using a temperature program of 140–240°C at 4°C/min.

## Methylation Analysis

The methylation analysis was carried out essentially as described.[25] To describe the process briefly: freshly ground NaOH pellets (250 mg) were added to solutions of samples in $Me_2SO$ (200 $\mu$L). After the material had dissolved, samples were cooled to 0°C and MeI (0.5 mL) was added, followed by sonication at room temperature. The reaction was stopped after 45 min by adding aq $Na_2S_2O_3$ (1 mL, 100 mg/mL) and $CHCl_3$ (1 mL). The chloroform layer was washed with water (3 × 0.5 mL), then concentrated. After hydrolysis of the residues with 2*M* TFA (0.3 mL; 120°C, 1 h), samples were dissolved in 0.5*M* $NH_4OH$ (250 $\mu$L) containing $NaBD_4$ (10 mg/mL) and kept for 1 h, then neutralized with aq 99% HOAc and concentrated. Boric acid was removed by repetitive coevaporation with 9:1 MeOH-HOAc and MeOH. After acetylation with $Ac_2O$ (0.5 mL; 120°C, 3 h), quenching with water (0.5 mL), and neutralization with $NaHCO_3$, the mixtures of partially methylated alditol acetates were extracted with $CH_2Cl_2$ (3 × 0.5 mL). The solutions were concentrated to about 20 $\mu$L ($N_2$), and analyzed by GLC (see above) and gas-liquid chromatography-electron impacs mass spectrometry (GLC-EIMS). GLC-EIMS analyses were carried out on a Fisons MD800/8060 system (electron energy, 70 eV; carrier gas, He) equipped with a DB-1 fused-silica capillary column (30 m × 0.32 mm, J&W Scientific). Samples were injected using a split injector (split flow 1/10), and a temperature program of 140–240°C at 4°C/min.

For the determination of the substitution pattern of galacturonic acid, the permethylated polysaccharide was reduced with superdeuteride (Aldrich Chemicals, 0.5 mL, 3 h). After quenching with 1:1 MeOH-$H_2O$, desalting (Dowex $H^+$, 2 mL),[26] and concentration, the sample was hydrolyzed, reduced, acetylated, and analyzed as described above.

## NMR Spectroscopy

NMR was performed on samples that were deuterium exchanged twice in $D_2O$ (99.9 atom % D, Cambridge Isotope Laboratories, USA) preceding NMR analyses and then dissolved in $D_2O$ (99.96 atom % D, Isotec, USA). If necessary, the pD of the NMR sample was adjusted to 6.5–7.5. One-dimensional/two-dimensional (1D/2D) high-resolution NMR spectra were recorded on a Bruker AMX-500 or a AMX-600 spectrometer (Bijvoet Center, Utrecht University), or a Bruker DRX-600 instrument (NSR Center, University of Nijmegen) at a probe temperature of 300, 333, or 353 K. Chemical shifts ($\delta$) are expressed in ppm relative to external glucose ($\delta$ Glc*p* H1$\alpha$ 5.227 and C1$\alpha$ 92.9, in $D_2O$ at all temperatures). 2D NMR experiments were performed essentially as described.[27]

## Size-Exclusion Chromatography

The acid hydrolysate of Fraction P (40 mg) was applied onto a Sephacryl S-500 HR column (110 × 0.5 cm), which was initially equilibrated in 0.05*M* NaOAc buffer pH 5.0, using a Hiload System (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Elution was carried out using the same buffer and the elution rate was 0.4 mL/min. Fractions (1.2 mL) were collected and assayed by automated methods for neutral sugar content and uronic acid content. The appro-

priate fractions were pooled, concentrated, dialyzed, freeze dried, and analyzed for neutral sugar composition and uronic acid content.

The low molecular mass material (population PIII) was fractionated further on a Bio-Gel P-2 column (100 × 2.6 cm; Bio-Rad Labs., Richmond, CA, USA), which was initially equilibrated in water at 60°C using a Hiload System. Elution was carried out using water and the elution rate was 0.5 mL/min. Fractions (7.5 mL) were collected and assayed as described above for the Sephacryl S-500 HR fractions.

## High-Performance Size-Exclusion Chromatography

HPSEC was performed on a SP8800 HPLC (ThermoQuest Corporation, San José, CA, USA) equipped with three columns (each 300 × 7.5 mm) of Bio-Gel TSK in series (40XL, 30XL, and 20XL; Bio-Rad Labs., Richmond, CA, USA) in combination with a TSK guard column (40 × 6 mm) and elution at 30°C with 0.4*M* NaOAc buffer pH 3.0 at 0.8 mL/min. Calibration was performed using pectins in the range 10–82 kDa. The eluate was monitored using a Shodex SE-61 Refractive Index detector (Showa Denko K.K., Tokyo, Japan).

## High-Performance Anion-Exchange Chromatography

HPAEC was performed on a Dionex Bio-LC system (Sunnyvale, CA, USA)[28] using a (4 × 250 mm) CarboPac PA1 column (Dionex). Twenty microliter aliquots were injected and the gradient was obtained by mixing solutions of 100 m*M* NaOH, 1*M* NaOAc in 0.1*M* NaOH, and distilled water, at a flow rate of 1 mL/min. Different gradients were used for the sugar composition after methanolysis combined with TFA hydrolysis, the release of monomers during acid hydrolysis,[19] rhamnogalacturonan oligomers,[29] and xylogalacsuronan hydrolase XGH digests.[18]

For the determination of galacturonan oligomers, the column was equilibrated with 0.2*M* NaOAc in 0.1*M* NaOH. Elution was performed with a linear gradient to 0.6*M* NaOAc in 0.1*M* NaOH in 35 min, and a linear gradient to 1*M* NaOAc in 0.1*M* NaOH in 5 min. The column was washed for 5 min with 1*M* NaOAc in 0.1*M* NaOH, and equilibrated again for 15 min with 0.2*M* NaOAc in 0.1*M* NaOH.

*Exo*-galacturonase digests were analyzed by equilibrating the column with 0.1*M* NaOH. Elution was performed with a linear gradient to 0.31*M* NaOAc in 0.1*M* NaOH in 25 min, and a linear gradient to 1*M* NaOAc in 0.1*M* NaOH in 5 min. The column was washed for 5 min with 1*M* NaOAc in 0.1*M* NaOH, and equilibrated again for 15 min with 0.1*M* NaOH.

# RESULTS AND DISCUSSION

Fraction P is the polymeric residue remaining after enzymatic removal of the arabinose and galactose-containing side chains from soybean ChSS. The sugar composition of both ChSS and Fraction P are presented in Table I. Determination of the absolute configuration of the starting material ChSS reveals the presence of L-arabinose, L-fucose, D-galactose, D-galacturonic acid, L-rhamnose, and D-xylose. Fraction P yields 30% of the polysaccharides present in the ChSS extract, and represents 12% of the polysaccharides present in soybean WUS. The residual amount of arabinose plus galactose in Fraction P represents 8% of the amount in the ChSS extract. Thus, the side chains containing arabinose and galactose are largely removed. Based on the general structural features of pectic substances, the sugar composition of Fraction P indicated that it contains very highly substituted pectic structures, among which are rhamnogalacturonan and presumably xylogalacturonan regions.[9] Further characterization of Fraction P is performed by enzymatic degradation studies.

**Table I Monosaccharide Composition of Soybean Polysaccharide Fractions Expressed as mol %[a]**

| Fraction | Rha | Fuc | Ara | Xyl | Gal | GalA |
|---|---|---|---|---|---|---|
| ChSS | 4 | 4 | 25 | 7 | 40 | 21 |
| P | 11 | 9 | 7 | 18 | 12 | 43 |
| PI | 9 | tr | 2 | 26 | 6 | 58 |
| PII | 16 | 3 | 0 | 20 | 9 | 55 |
| PIIA | 24 | 3 | 2 | 12 | 13 | 46 |

[a] tr: Trace amount.

## Enzymatic Degradation of Fraction P

The galactose content in Fraction P (12 mol %, Table I) is high and represents 12% of the galactose present in the ChSS extract before degradation of the pectic arabinogalactan side chains. Reincubation of Fraction P with *endo*-galactanase and *exo*-galactanase did not show a further release of galactose.

Galacturonic acid represents 43 mol % of the constituent sugars of Fraction P. Both PG and PL could not bring about changes in the molecular mass distribution of the polymers in this fraction, and galacturonic acid oligomers could not be detected in the HPAEC elution patterns. Saponification of the methyl esters with 0.1*M* NaOH did not enable the degradation by PG.

The presence of rhamnose residues (11 mol %) in Fraction P suggests the presence of rhamnogalacturonan regions. The enzyme RG hydrolase is able to cleave galactopyranosyluronic acid–rhamnopyranosyl linkages within the rhamnogalacturonan backbone when acetyl esters on C2 and/or C3 on the galactu-

**Table II Linkage Analysis of Soybean Polysaccharide Fractions**[a]

| Glycosyl Residue[b] | ChSS | P | PI | PII | PIIIA |
|---|---|---|---|---|---|
| t-Ara*f* | 13 | 4 | 17 | — | tr |
| 5-Ara*f* | 11 | 6 | — | — | — |
| 2,5-Ara*f* | 2 | tr | — | — | — |
| 3,5-Ara*f* | 3 | tr | — | — | — |
| t-Fuc*p* | 4 | 12 | tr | — | tr |
| t-Gal*p* | 4 | 19 | 1 | 12 | 34 |
| 4-Gal*p* | 49 | 7 | 5 | 6 | 17 |
| 4,6-Gal*p* | 1 | tr | — | — | — |
| 2,3,4,6-Gal*p* | 1 | 9 | 65 | 12 | tr |
| t-Rha*p* | — | — | — | — | 8 |
| 2-Rha*p* | 1 | 10 | 1 | 32 | 8 |
| 2,4-Rha*p* | 2 | 6 | 1 | 12 | 17 |
| t-Xyl*p* | 3 | 13 | 10 | 26 | 8 |
| 2-Xyl*p* | 4 | 10 | — | — | 8 |
| 4-Xyl*p* | 1 | 1 | tr | — | — |
| 2,3-Xyl*p* | 1 | 3 | — | — | — |

[a] Expressed as relative peak areas of corresponding partially methylated alditol acetates. Galacturonic acid is determined in separate experiments, which revealed the presence of t-Gal*p*A in ChSS, P, and PI, 4-Gal*p*A in every polysaccharide fraction, 3,4-Gal*p*A in ChSS, P, PI, and PII; tr: trace amount; —, not detected.

[b] Numbers preceding residues indicate positions of attachment of other glycosyl residues in the intact polysaccharide (e.g., 5-Ara*f* = 5-substituted arabinofuranose); t, nonreducing terminal residue.

ronic acid residues are absent, and the rhamnose residue is unbranched or substituted through C4 with a single galactose residue.[28] Only after saponification of Fraction P, does the HPAEC elution pattern of the digest show the release of small amounts of rhamnogalacturonan oligomers. HPSEC, however, does not show changes in the elution pattern in the polymeric region, irrespective of saponification. Thus, acetyl-containing rhamnogalacturonan regions occur in the extremities of Fraction P and become susceptible for RG hydrolase after saponification.

The high xylose content (18 mol %) suggests the presence of xylogalacturonan regions in Fraction P. Linkage analysis shows that part of the galacturonic acid residues is branched at C3, and that about 50% of the xylose is nonreducing terminal xylose (Table II). Single unit side chains of xylose do not hinder *exo*-galacturonase and this enzyme should be able to release galacturonic acid residues and xylosyl galacturonic acid dimers [$\beta$-Xyl*p*-(1 $\rightarrow$ 3)-Gal*p*A] from the polymer. The HPAEC elution pattern shows the release of small amounts of galacturonic acid and the characteristic xylosyl galacturonic acid dimer by *exo*-galacturonase, particularly after alkaline saponification. However, the HPSEC elution pattern does not show changes. This is explained by the fact that *exo*-galacturonase is an *exo*-enzyme and therefore not able to change the hydrodynamic volume of polymers to a large extent. This is in agreement with Beldman et al.,[17] who were also able to release the xylosyl galacturonic acid dimer [$\beta$-Xyl*p*-(1 $\rightarrow$ 3)-Gal*p*A] from a soluble pectic polysaccharide from soy[30] using *exo*-galacturonase. Approximately 0.7% of the galacturonic acid present in Fraction P is released from the nonreducing chain end by *exo*-galacturonase. After saponification, there was an increase of the amount of galacturonic acid released ($\times$4.5) to 3.6%.

None of the purified enzymes used in these experiments were able to cause a large shift in the molecular mass distribution of the pectic structures in Fraction P. To check if there are any enzymes at all which are able to degrade Fraction P, some technical multienzyme preparations were tested. None of these enzyme preparations fragmented the pectic backbone.

## Acid Hydrolysis of Fraction P

Since enzymatic degradation of Fraction P was not possible with the available enzymes, the polymers were degraded by acid hydrolysis in 0.1*M* HCl at 80°C. The differences in susceptibility of the glycosidic linkages to acid hydrolysis[31,32] were used to remove the (neutral) side chains as much as possible without seriously degrading the pectic backbone. The hydrolysis of Fraction P with HCl was followed in time by analysis of the hydrolysates using HPSEC and HPAEC.

The HPAEC elution patterns show a rapid release of arabinose, fucose, galactose, and xylose from Fraction P. An 8 h treatment with 0.1*M* HCl at 80°C removed most of the neutral side chains.

Line a in Figure 1 represents two populations containing high molecular mass polymers. The first population probably originates from aggregation, which results in accumulation of molecules in the void volume. These aggregates are not observed at low concentrations of P. The increase of the total RI area after hydrolysis was caused by the solubilization of polymers that were not completely soluble in 0.1*M* HCl without heat treatment. The intensity of the peak of high molecular mass material decreases during acid hydrolysis and the formation of degradation products can be observed in the HPSEC elution patterns. The largest shift of the molecular mass distribution occurs between 8 and 24 h of hydrolysis (Figure 1, lines e and f). Prolonged acid hydrolysis causes almost complete degradation of the high molecular mass polymers, including degradation of the pectic backbone (Figure 1, line f).

It appears that the pectic backbone is hardly affected during the first 8 h of acid hydrolysis. The

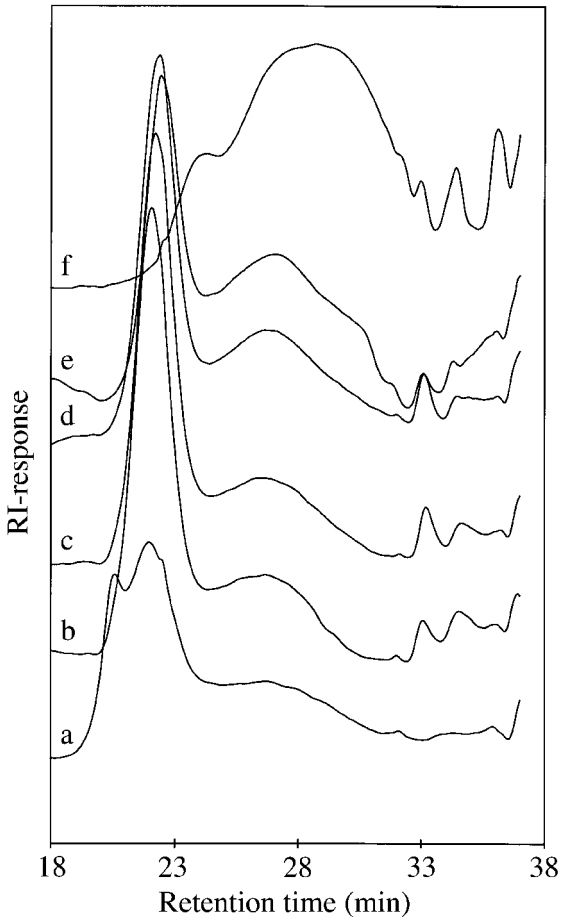



**FIGURE 1** HPSEC elution patterns of Fraction P during acid hydrolysis. (a) Blank; after (b) 1 h, (c) 2 h, (d) 4 h, (e) 8 h, and (f) 24 h of acid hydrolysis.

elution pattern of this hydrolysate still shows a peak with high molecular mass (retention time 22.5 min; molecular mass $>$ 82 kDa), a peak representing material with an intermediate molecular mass (retention time 27.1 min; molecular mass $\approx$ 11 kDa) and some oligomers (Figure 1, line e). The ratio of the areas under these peaks is 4:6:1. Characterization of the polymeric residue after 8 h of hydrolysis can provide information about the structure of Fraction P, and consequently about the structure of the CDTA-extractable pectins.

## Fractionation of the Acid Hydrolysate of Fraction P

Fraction P was hydrolyzed with 0.1*M* HCl for 8h on a large scale and the hydrolysate fractionated by size-exclusion chromatography. The elution profile (Figure 2) shows three populations. In the first two populations galacturonic acid prevails, whereas in the third population, containing low molecular mass degradation products, neutral sugars predominate. The recovery of the hydrolysate after fractionation on Sephacryl S-500 HR is almost 100%.

The desalting and further fractionation of the third population (PIII) was performed by Bio-Gel P-2 chromatography. The elution profile (not shown) shows a peak eluting in the void volume of the column (PIIIA), containing oligomeric pectic material with a ratio of neutral sugars to galacturonic acid of 1:2.1. The second population (PIIIB) contains neutral sugars only.

## Characterization of the Populations of the Acid Hydrolysate

The sugar compositions of the parental fraction and the populations obtained after enzymatic and acid hydrolysis are shown in Table I. The ratio neutral sugars to galacturonic acid in the ChSS extract is relatively high (3.8:1), because all the arabinogalactan side chains of the pectins are still present. Fraction P still contains a large amount of neutral sugars (ratio is 1.4:1), although the major part of the arabinose and galactose residues was removed by enzymatic digestion. Acid hydrolysis causes a further decrease of the relative amounts of neutral sugars in PI, PII and PIIIA.

The high xylose content in Fraction PI (26 mol %), which is terminally linked (Table II), suggests the presence of xylogalacturonan regions. The PI population has the highest ratio of xylose to galacturonic acid (1:2.2), similar to the value determined in Fraction P (1:2.3), in spite of the fact that part of the xylose is removed during acid hydrolysis. Rhamnogalacturonan regions are indicated by the presence of rhamnose (9 mol %), which is both 2- and 2,4-linked (Table II). The presence of these two structural units is also suggested in the PII population by the high

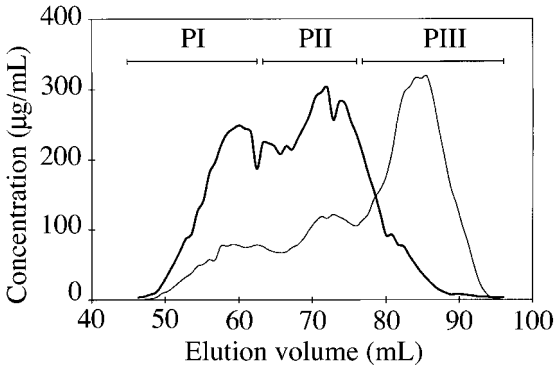



**FIGURE 2** Elution profile of the acid hydrolysate of Fraction P on Sephacryl S-500 HR. Uronic acid concentration (thick line) and neutral sugar concentration (thin line).

xylose and rhamnose contents (20 and 16 mol %, respectively), and the occurrence of the same linkages (Table II) as in the PI population.

Population PIIIA contains only small pectic fragments, as can be concluded from the elution behavior on size-exclusion chromatography. The high rhamnose content in Fraction PIIIA (24 mol %) and the galacturonic acid content (46 mol %) suggest that this population is very rich in rhamnogalacturonan structures, substituted with (arabino)galactan side chains. These structures exceed 63% of this population. The ratio of arabinose plus galactose to rhamnose is 1:1.6; therefore, not all the rhamnose residues are substituted with neutral sugar residues. This population may contain some xylogalacturonan sequences, as indicated by the presence of 12 mol % xylose.

## NMR Analyses of the Populations of the Acid Hydrolysate

Generally, NMR analysis of small fragments is easier than of polymers. Due to the presence of a dominating noncarbohydrate contaminant in the minor Fraction PIIIA, being available in low amounts only, this fraction was not analyzed by NMR spectroscopy. As the signals in the 1D $^1$H NMR spectrum of PI are broader than in that of PII, the latter will be described first. The acid form of PII (PII-H) showed a better resolution in the NMR spectra (data not shown) and was used for further analyses.

The anomeric region ($\delta$ 4.5–5.5) in the 1D $^1$H NMR spectrum of PII-H (Figure 3A) shows several H1$\alpha$ and H1$\beta$ signals. Using total correlated spectroscopy (TOCSY; (Figure 3B) measurements, and heteronuclear single quantum correlation (HSQC; Figure 3C) and heteronuclear multiple bond correlation (HMBC) experiments, most of the $^1$H and $^{13}$C signals could be assigned (Tables III and IV, respectively). The low-intensity downfield H1 signal for residue A was assigned to $\alpha$-fucose H1; its TOCSY track (Figure 3B) showed a clear $\alpha$-fucose H1,2,3,4 spin system.[33]

The H1 TOCSY tracks for residues B and C show complete spin systems up to signals in the methyl region at $\delta$ 1.276 and 1.217, respectively, thereby indicating that B and C are 6-deoxysugars. Based on the monosaccharide analysis data and the typical NMR positions for the B/C H2 protons ($\delta \sim$ 4.10, O2-substituted $\alpha$-*manno*-hexoses[34]), both residues were assigned as 2-substituted $\alpha$-rhamnose residues. Moreover, inspection of the spin systems of residues B and C suggests an additional substitution at O4 of rhamnose B, whereby the ratio of 2- to 2,4-substituted rhamnose as deduced from the H6 signals in the 1D $^1$H NMR spectrum is 2.6:1. This is confirmed by linkage analysis data (Table II), although linkage analysis results in qualitative rather than quantitative information. It should be noted that a $\alpha$-rhamnose residue usually does not give a TOCSY signal between H1 and H2, as a result of the equatorial H atom at C2. Therefore, the observed transfer of magnetization is probably caused by spin diffusion. A further support for the 2- or 2,4-substitution of rhamnose are the $\delta$ values of the rhamnose B/C C2 and C4 atoms in the $^{13}$C NMR spectra (Table IV; methyl $\alpha$-L-rhamnopyranoside: $\delta_{C2}$, 71.0; $\delta_{C4}$, 73.1[35]). The $^1J_{C1,H1}$ coupling constant value of 173 Hz for both rhamnose residues point to a $\alpha$-configuration.[36]

The H1 signals of the residues D—H in the $\alpha$-anomeric region were all correlated with $\alpha$-galacturonic acid residues ($^1J_{C1,H1} \sim$ 173 Hz). Making use of the HSQC and HMBC spectra, in nearly all cases the typical spin systems H1,2,3,4,5 and C1,2,3,4,5 for a *galacto*-hexose could be derived (Tables III and IV, respectively). The downfield chemical shift value of H5 ($\delta \sim$ 4.5–4.6) is indicative for the differentiation between galacturonic acid and galactose. Presumably, spin diffusion is responsible for the observation of the H5 cross peaks on the H1 TOCSY tracks. According to their $^1$H and $^{13}$C chemical shifts, at least two different substitution patterns for $\alpha$-galacturonic acid exist. Residues G and H represent 4-substituted galacturonic acid residues and D, E, and F 3,4-substituted ones[2] ($\alpha$-D-galactopyranosyluronic acid: $\delta_{C3}$, 69.5; $\delta_{C4}$, 70.9[35]); the assignment of residue F as 3,4-substituted galacturonic acid is ambiguous. The deduced substitution patterns fit the linkage analysis data.

Monosaccharide and methylation analysis combined with NMR spectroscopy demonstrated the presence of terminal galactopyranose (I) and 4-substituted galactopyranose (J) in the molar ratio 2.2:1. Both TOCSY H1$\beta$ ($\delta$ 4.59 and 4.60/4.61, respectively; Table III) tracks show the typical cross-peak pattern of H1,2,3,4 of a *galacto*-hexose. Downfield shifts of H4 and C4 of residue J prove a 4-substitution. The $\beta$ configurations of both galactose residues are supported by $^1J_{C1,H1}$ values of 163 Hz, as determined from a HSQC experiment (Figure 3C). The presence of 2,3,4,6-substituted galactopyranose (Table II) could not be confirmed by NMR spectroscopy and is likely to be the result of undermethylation.

Nuclear Overhauser effect (NOE) cross peaks were observed for galacturonic acid G/H H1,rhamnose B/C H2 (strong), galacturonic acid G/H H1,rhamnose B/C H1 (strong), galacturonic acid G/H H1,rhamnose B/C H3 (weak), rhamnose B/C H1,galacturonic acid G/H H4 (strong), and rhamnose B/C H1,galacturonic acid G/H H5 (weak). These data imply the presence of rhamnogalacturonan regions. The NMR spectra do

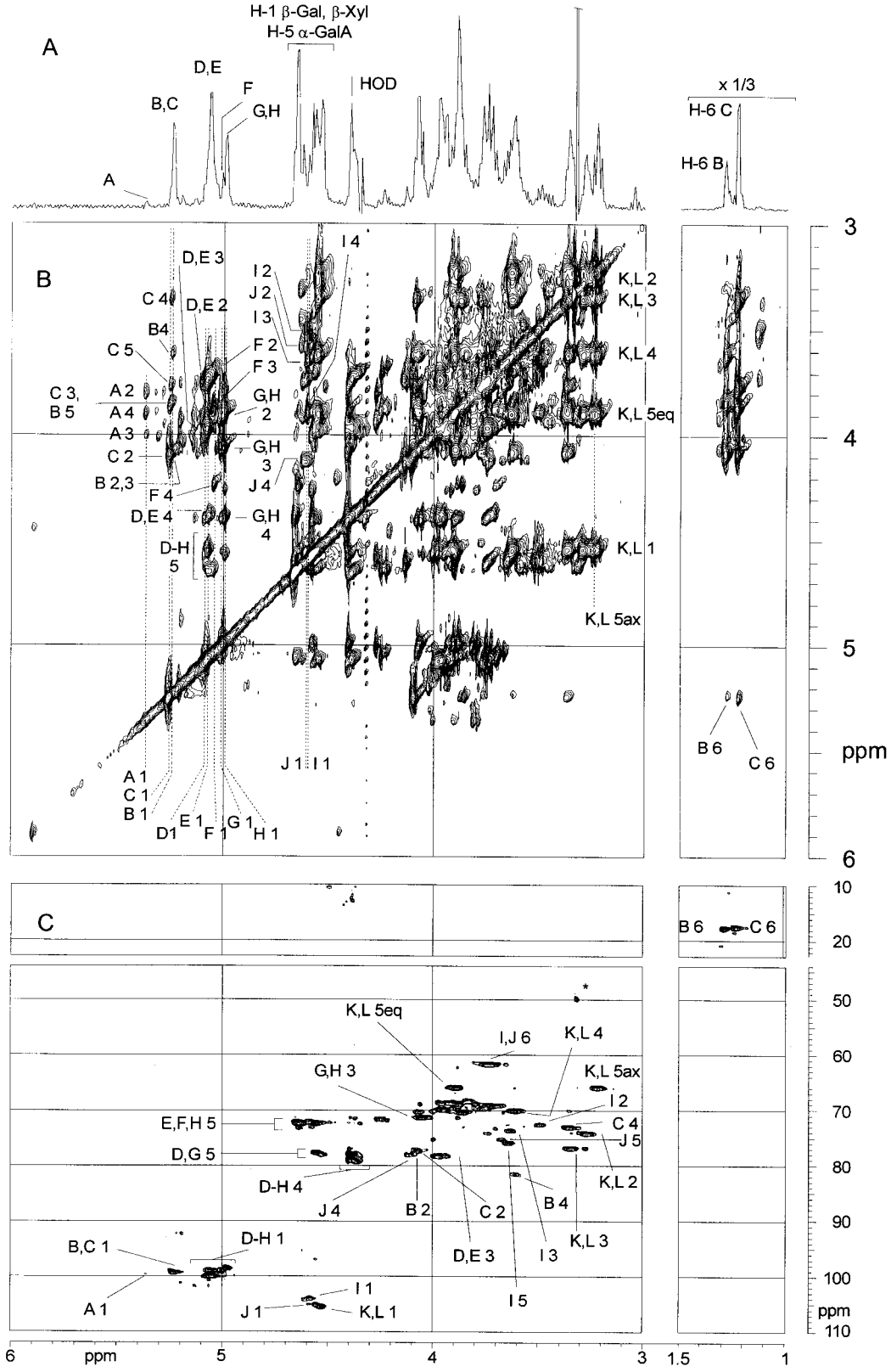



**FIGURE 3** 1D $^{1}$H NMR spectrum (A); TOCSY spectrum, mixing time 124 ms (B), and HSQC spectrum (C) of PII-H, recorded at 600 MHz and 333 K. Peak labels of A represent different residues, which are explained in the text. For an overview, see Tables V and VI. B 2 means a cross peak between H-2 and C-2 of residue B. Xylose H-5 equatorial and axial are indicated with eq and ax, respectively. Asterisk: not assigned to a methyl ester as the chemical shift is too low ($\delta$ 50 instead of 53.4).

**Table III** **$^{1}$H Chemical Shifts of PII-H, Recorded at 333 K**

| Residue | Type | H-1 | H-2 | H-3 | H-4 | H-5(ax) | H-5eq/H-6a/H-6b |
|---|---|---|---|---|---|---|---|
| Fuc | | | | | | | |
| A | α-Fuc-(1 → | 5.37 | 3.80[a] | 4.00[a] | 3.90[a] | —[b] | — |
| Rha | | | | | | | |
| B | → 2,4)-α-Rha-(1 → | 5.235 | 4.10 | 4.06 | 3.62 | 3.84 | 1.276 |
| C | → 2)-α-Rha-(1 → | 5.24 | 4.08 | 3.86 | 3.36 | 3.77 | 1.217 |
| GalA | | | | | | | |
| D | → 3,4)-α-GalA-(1 → | 5.07 | 3.70 | 3.98 | 4.38 | 4.54 | |
| E | → 3,4)-α-GalA-(1 → | 5.06 | 3.73 | 3.98 | 4.38 | 4.65 | |
| F | | 5.02 | 3.67 | 3.85 | 4.24 | 4.64 | |
| G | → 4)-α-GalA-(1 → | 5.01 | 3.89–3.87 | 4.06 | 4.38 | 4.56 | |
| H | → 4)-α-GalA-(1 → | 4.99 | 3.89–3.87 | 4.03 | 4.38 | 4.56 | |
| Gal | | | | | | | |
| I | β-Gal-(1 → | 4.59 | 3.49 | 3.63 | 3.89 | 3.65 | 3.76/3.71 |
| J | →4)-β-Gal-(1 → | 4.60/4.61 | 3.53/3.57 | 3.72/3.75 | 4.13 | 3.68 | 3.76/3.71 |
| Xyl | | | | | | | |
| K | β-Xyl-(1 → | 4.54 | 3.27 | 3.35 | 3.61 | 3.22 | 3.92 |
| L | β-Xyl-(1 → | 4.54 | 3.27 | 3.35 | 3.61 | 3.22 | 3.90 |

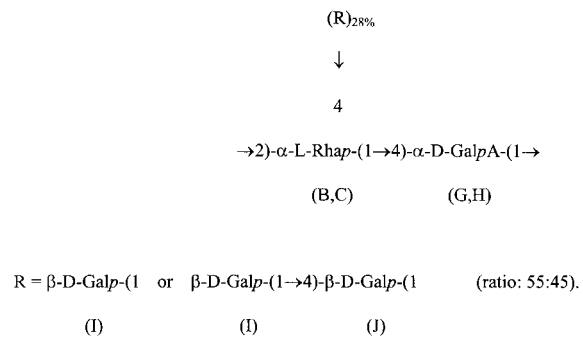
[a] Values may have to be interchanged.
[b] —, Not determined.

not give any indication for an irregular distribution of 2- (residue C) and 2,4-substituted (residue B) rhamnose. Therefore, it was concluded that these residues are distributed regularly in the rhamnogalacturonan chain. An additional cross peak between galactose I/J H1 and rhamnose B H4 connects residues I/J with the rhamnogalacturonan backbone. Elongation of the galactose residue J at O4 is evidenced by a cross peak between galactose I H1 and galactose J H4. In summary this building block can be depicted as follows:

**Table IV** **$^{13}$C Chemical Shifts of PII-H, Recorded at 333 K**

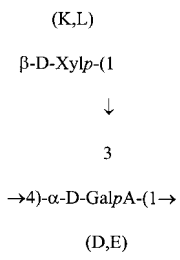
| Residue | Type | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|---|
| Fuc | | | | | | | |
| A | α-Fuc-(1 → | 99.7 | 68.8[a] | 75.4[a] | 69 | —[b] | 20.8 |
| Rha | | | | | | | |
| B | → 2,4-α-Rha-(1 → | 99.4 | 77.9 | 70.3 | 81.57 | 68.4 | 17.45 |
| C | → 2-α-Rha-(1 → | 99.2 | 77.4 | 70.47 | 73.1 | 69.7 | 17.69 |
| GalA | | | | | | | |
| D | → 3,4)-α-GalA-(1 → | 98.97 | 69.14 | 78.3 | 77.0/77.7[c] | 78.1 | 176.3[d] |
| E | → 3,4)-α-GalA-(1 → | 99.94 | 69.14 | 78.3 | 77.9[c] | 72.3 | 176.1[d] |
| F | | 99.46 | — | — | 78.5[c] | 72.5 | 175.9[d] |
| G | → 4)-α-GalA-(1 → | 98.9 | 68.84 | 71.28 | 79.0[c] | 77.8 | 175.4[d] |
| H | → 4)-α-GalA-(1 → | 98.5 | 68.84 | 71.28 | 79.4[c] | 72.3 | |
| Gal | | | | | | | |
| I | β-Gal-(1 → | 104.2 | 72.6 | 73.7 | 69.6 | 76.0 | 61.7 |
| J | → 4)-β-Gal-(1 → | 104.3/105.1 | 72.9/74.2 | 73.3/74.2 | 78.1 | 75.4 | 61.4 |
| Xyl | | | | | | | |
| K/L | β-Xyl-(1 → | 105.53 | 74.22 | 76.92 | 70.11 | 65.89 | |

[a] Values may have to be interchanged.
[b] —, Not determined.
[c] Values for GalA C-4 may have to be interchanged.
[d] Values for GalA C-6 may have to be interchanged.




Clear NMR indications for xylose were found in the HSQC spectrum of PII-H (Figure 3C), showing cross peaks between C5 and H5ax and between C5 and H5eq. Further assignments followed from TOCSY and correlated spectroscopy experiments, as well as from literature NMR data for terminal $\beta$-xylose residues[2,37] ($^1J_{C1,H1}$ 163 Hz). Based on the presence of two different $\delta$ values for xylose H5eq ($\delta$ 3.92 and 3.90), two xylose residues, K and L, were established.

As the NOE spectroscopy spectrum shows, a weak interresidual xylose K/L H1,galacturonic acid D/E H3 cross peak, a $\beta$-Xyl*p*-(1 → 3)-$\alpha$-Gal*p*A D/E element is indicated. For galacturonic acid D/E H1, several NOE cross peaks are observed, namely with galacturonic acid D/E H2,3,4,5. Taking into account the (1 → 3) linkage between xylose and galacturonic acid, the NOE cross-peak galacturonic acid D/E H1,galacturonic acid D/E H4 can be interpreted as an interresidual cross-peak reflecting 1 → 4 linkages between neighboring galacturonic acid D/E residues. In this reasoning the cross-peak galacturonic acid D/E H1,galacturonic acid D/E H3 can be explained as a second interresidual and/or an intraresidual (spin diffusion) cross peak. It should be noted that the cross-peaks galacturonic acid G/H H1,galacturonic acid G/H H2,3 (rhamnogalacturonan backbone, see above) can only be interpreted as intraresidual cross peaks, whereby the galacturonic acid G/H H1,galacturonic acid G/H H3 cross peak is probably caused by spin diffusion, which stops at H3. In summary, this building block can be depicted as follows:




For the assignment of the NMR spectra of PII, the same rationale was used as described for PII-H. Similar $^1$H and $^{13}$C chemical shifts were found for the different residues in Fraction PII. Galacturonic acid appeared to be methyl-esterified ($\delta$ 3.85/3.83 and 35.4 for $^1$H and $^{13}$C, respectively). In the xylogalacturonan part of PII approximately 85% of the galacturonic acid residues is methyl-esterified. This was concluded from the intensities of the xylose H1,H2 cross peaks in a COSY spectrum of PII, as the chemical shift of xylose H2 strongly depends on galacturonic acid being methyl-esterified or not (xylose H2, $\delta$3.056/3.038 and 3.280/3.272 for methyl-esterified and nonesterified galacturonic acid, respectively).[2]

Like for PII-H, several spin systems were identified for Fraction PI using TOCSY and HSQC (Figure 4) spectra (Table V). The presence of xylogalacturonan was indicated by the chemical shift values of xylose, assigned in a similar way as described for Fraction PII-H. Rhamnogalacturonan was identified by the spin system of rhamnose (Figure 4). The ratio of (1,2)-linked rhamnose to (1,2,4)-linked rhamnose in Fraction PI is 1.4:1 (H6 rhamnose $\delta$ 1.24 and 1.30, respectively). The HSQC spectrum (Figure 4) contains a clear signal for a methyl ester ($^1$H, 3.84–3.76; $^{13}$C, 53.4), indicating methyl esterification of galacturonic acid, and acetyl signals ($^1$H, 2.06; $^{13}$C, 21.2), presumable belonging to *O*-acetyl groups linked to galacturonic acid.

## Structural Features of the Populations of the Acid Hydrolysate

All three populations in the acid hydrolysate from Fraction P appear to contain xylogalacturonan, rhamnogalacturonan, and some remaining (arabino)galactan side chains. Assuming that the rhamnose to galacturonic acid ratio in the rhamnogalacturonan regions can vary from 1:1 to 1:20,[38] the amount of rhamnogalacturonan (including arabinogalactan substituents) is estimated to exceed 26% of the PI population (2% Ara + 6% Gal + 9% Rha + at least 9% GalA) and 41% of PII (9% Gal + 16% Rha + at least 16% GalA). Combination of the results described above shows that the average length of the remaining arabinogalactan side chains in the PI population is 2.1 residues. The average length of the remaining galactan side chains in Fraction PII is 1.4 residues. Based on the contents of xylose and galacturonic acid (26 and 58 mol %, respectively) and a xylose to galacturonic acid ratio in xylogalacturonan varying between 1:1 and 1:2, xylogalacturonan accounts for 52–78% of the PI fraction, and for 40–60% of the PII fraction. In addition, the polymeric populations (PI and PII) still contain methyl-esterified galacturonic acid residues. Acetylation of galacturonic acid residues occurs at position 2 or 3. The degree of acetylation decreases

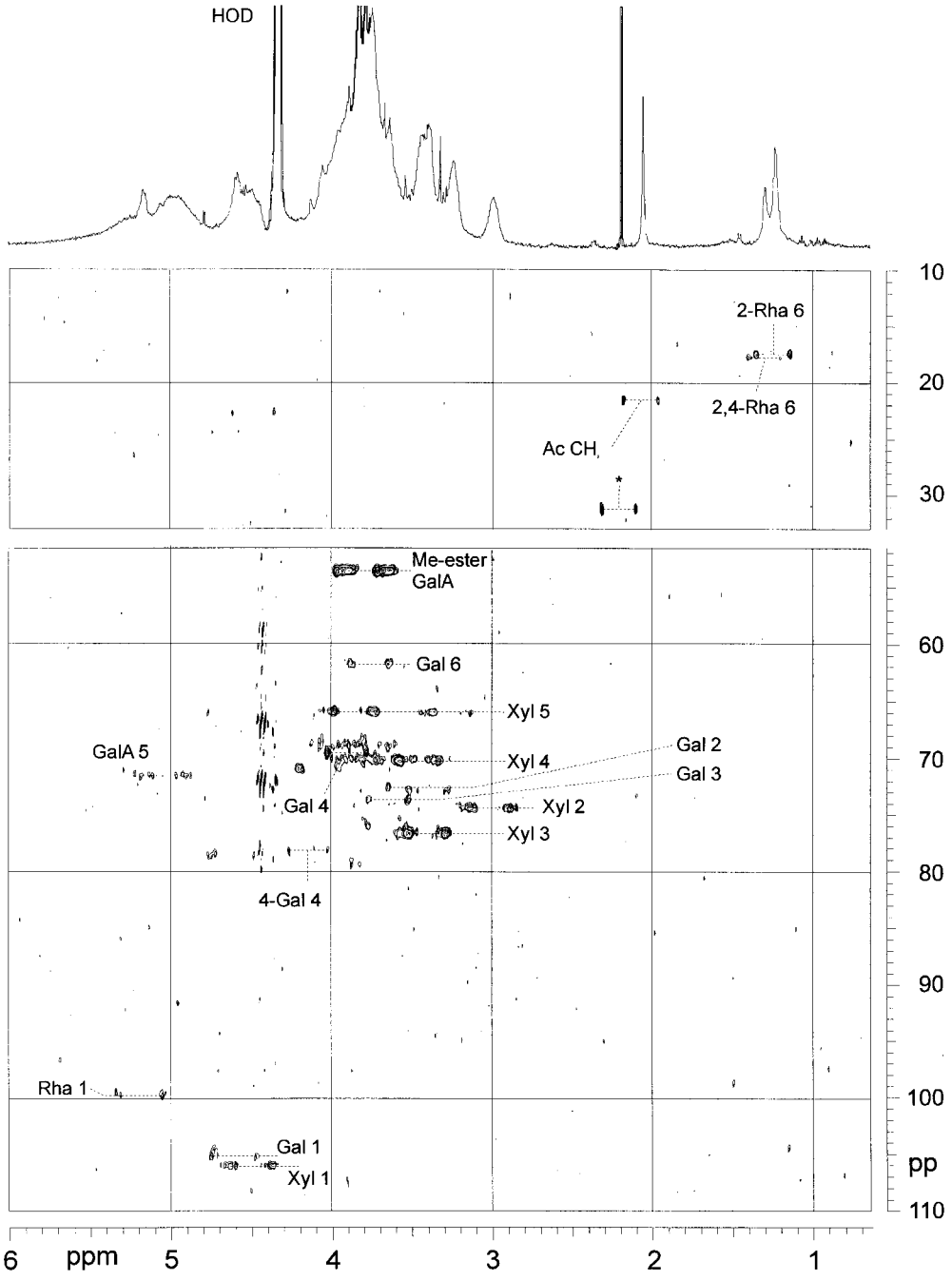



**FIGURE 4** HSQC spectrum of PI, recorded at 600 MHz and 333 K. Xyl 2 refers to a cross peak between H-2 and C-2 of Xyl. Asterisk: not assigned to an acetyl group as the $^{13}C$ chemical shift is too high ($\delta$ 30.8 instead of 21.1).

from 47% in Fraction P to 16% in Fraction PI, as determined by NMR spectroscopy. Removal of acetyl groups must have occurred during acid hydrolysis. Labile groups such as ester-linked components are likely to be removed under acidic conditions.[39]

The presence of homogalacturonan in populations PI and PII is not necessary to explain the high galacturonic acid content; it can be accounted for by the rhamnogalacturonan and xylogalacturonan regions in these populations. The amount of galacturonic acid in the rhamnogalacturonan regions in populations PI and PII will exceed 9 and 16 mol %, respectively. In addition, at most 52 mol % of galacturonic acid in population PI and 40 mol % of galacturonic acid in population PII can be present in xylogalacturonan regions. The NMR spectra of PII-H confirms the

**Table V $^{13}$C Chemical Shifts of PI Recorded at 333 K**

| Residue | Type | Me/Ac | C-1 | C-2 | C-3 | C-4 | C-5 | C-6 |
|---|---|---|---|---|---|---|---|---|
| Fuc | α-Fuc-(1 → | | —[a] | — | — | — | — | — |
| Rha | → 2,4)-α-Rha-(1 → | | 99.6 | — | — | — | — | 17.5 |
| | → 2)-α-Rha-(1 → | | 99.6 | — | — | — | — | 17.3 |
| GalA | | | — | — | — | — | 71.4 | — |
| GalA6Me | | 53.4 | | | | | | |
| Acetyl | | 21.2 | | | | | | |
| Gal | β-Gal-(1 → | | 105.0 | 72 | 73.4 | 69.4 | — | 61.5 |
| | → 4)-β-Gal-(1 → | | 105.0 | 72 | | 78.1 | — | 61.5 |
| Xyl | β-Xyl-(1 → | | 105.8 | 74.3 | 76.4 | 70.0 | 65.7 | |

[a] —, Not determined.

absence of a (α1 → 4)-linked homogalacturonan. The absence of homogalacturonan regions in pectic substances has never been reported before.

## Enzymatic Degradation of the Populations from the Acid Hydrolysate of Fraction P

To obtain additional information about the structure of the pectic backbone in soybean meal, the populations PI, PII, and PIIIA were incubated with PG, RG hydrolase, *exo*-galacturonase, and XGH. The digests were analyzed with both HPSEC (Figure 5) and HPAEC (Figure 6).

Although PI is obtained after fractionation on Sephacryl S-500 HR, it contains two polymeric populations on HPSEC analysis (Figure 5Aa). Both PG (Figure 5Ab and 6Aa) and XGH (Figure 5Ad and 6Ca) cause only slight changes in the elution pattern of Fraction PI, and are unable to release oligomeric degradation products from it. The HPAEC elution pattern of the *exo*-galacturonase digest (Figure 6Da) shows the release of both galacturonic acid and xylosyl galacturonic acid dimer, while the HPSEC elution pattern (Figure 5Ae) hardly changed. RG hydrolase, on the other hand, causes the HPSEC elution pattern to change. While the molecular mass of the first peak remains unchanged, and the molecular mass of the second peak decreases only slightly (Figure 5Ac), the amount of polymeric material in both populations decreases and a shoulder with lower molecular mass arises (as indicated by the arrow). RG hydrolase released very small amounts of characteristic RG hydrolase oligomers[28] (Figure 6Ba).

The PII population shows one polymeric population on HPSEC analysis (Figure 5Ba). Only incubation with RG hydrolase causes a considerable change of the HPSEC elution pattern (Figure 5Bc). The oligomers released by RG hydrolase (Figure 6Bb) were assigned by comparison with the rhamnogalacturonan oligomers from apple modified hairy regions (MHR).[28] Both XGH (Figure 6Cb) and exogalacturonase (Figure 6Db) are able to release oligosaccharides from Fraction PII. XGH releases small amounts of xylosyl galacturonic acid dimer in addition to very small amounts of unknown oligomers, and exogalacturonase releases galacturonic acid and xylosyl galacturonic acid dimer. PG was not active on Fraction PII, and removal of the methyl-ester groups from PII (PII-H) did not increase its susceptibility for PG.

The amounts of galacturonic acid and the xylosyl galacturonic acid dimer released from PII (Figure 6Db) are higher than the amounts released from PI. *Exo*-galacturonase released 8.0% of the galacturonic acids present in PI and 10.5% of the galacturonic acids present in PII. The content of the dimer cannot be quantified properly, because a standard is absent. When the response factor of galacturonic acid is used to quantify the amount of dimer in the digests, 3.8% of the galacturonic acid present in PI and 3.2% of the galacturonic acid present in PII is released as the xylosyl galacturonic acid dimer. This indicates that the degree of substitution of galacturonic acid with xylose (remaining after weak acid hydrolysis) in the extremities of the pectic substances in PI is higher than in PII. The total amount of material released from PII is higher than from PI, which can be explained by the number of potential degradation sites for *exo*-galacturonase. Starting with the same substrate concentration, the number of molecules (and accordingly the number of nonreducing chain ends) in the PII solution is higher than in the PI solution.

The PIIIA blank contains predominantly oligomeric material in addition to a low amount of polymeric material (Figure 5Ca). PG is not able to degrade Fraction PIIIA. Both RG hydrolase and XGH decrease the small amount of polymeric material but do not release characteristic oligosaccharides. *Exo*-galac-

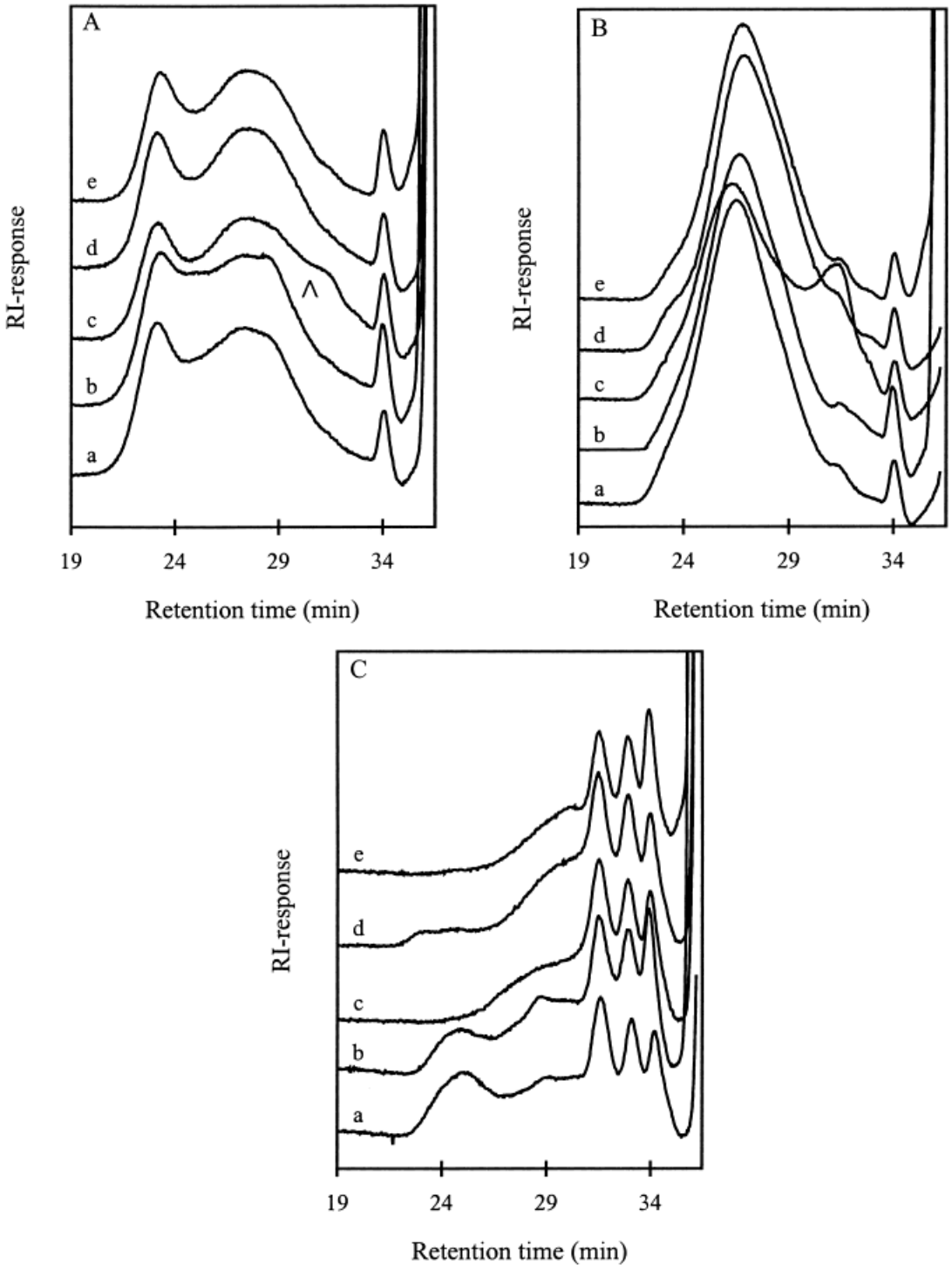



**FIGURE 5** HPSEC elution patterns of the digests of PI (A), PII (B), and PIIIA (C) before (a) and after enzymatic degradation with PG (b), RGase (c), XGH (d), and *exo*-galacturonase (e).

turonase degrades the peak of polymeric material, and changes the relative amounts of the oligomers present in this fraction (Figure 5Ce) and releases monomeric galacturonic acid and the xylosyl galacturonic acid dimer (Figure 6Dc).

The high galacturonic acid content of PI and PII could give the impression that a homogalacturonan is present in these populations. However, enzymatic degradation with PG confirms our earlier statement that none of the fractions contains homogalacturonan.

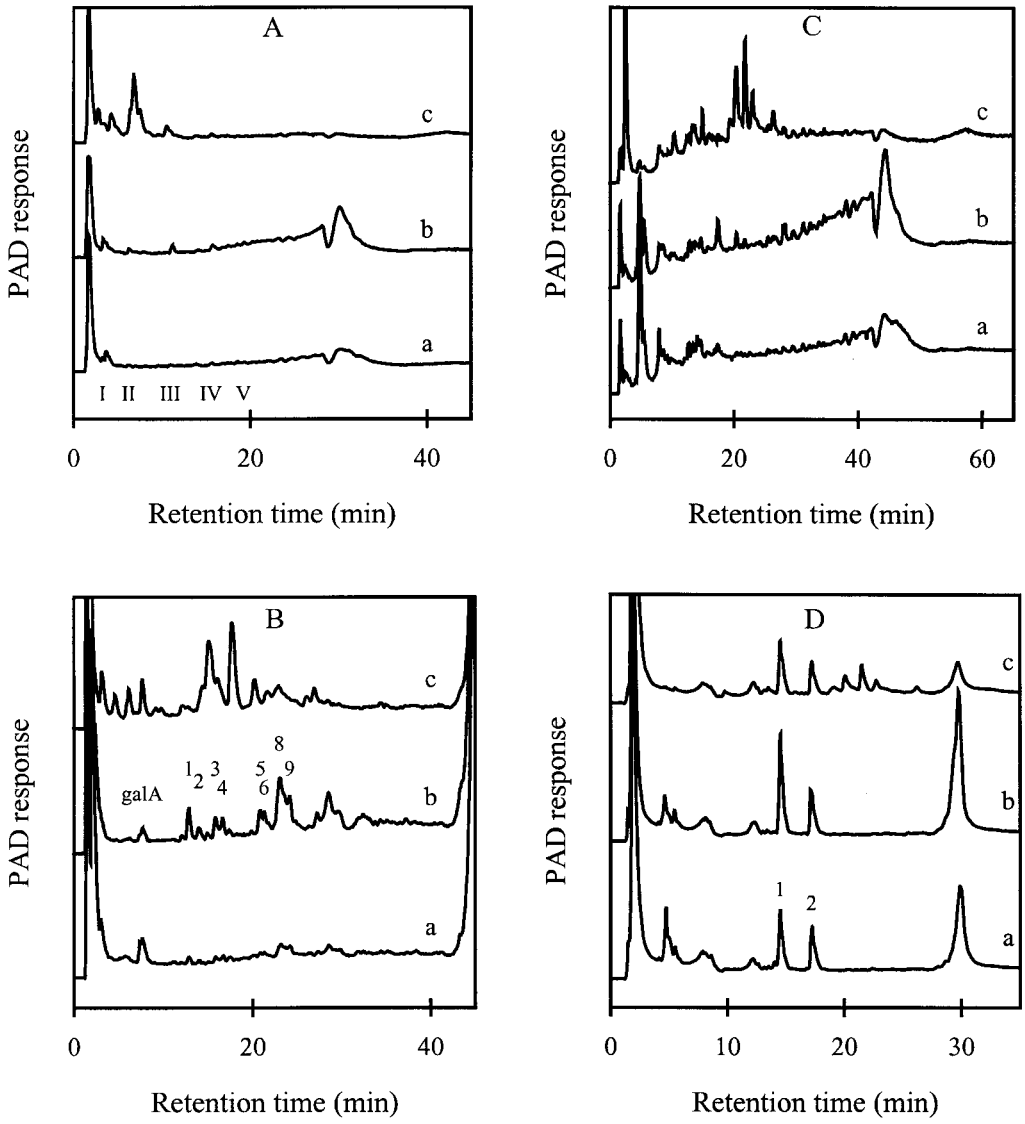



**FIGURE 6** HPAEC elution patterns of the digests of PI (a), PII (b), and PIIIA (c) after enzymatic degradation with PG (A), Rgase (B), XGH (C), and *exo*-galacturonase (D). In A, the elution times of galacturonic acid and galacturonan oligomers are marked with a Roman numeral corresponding to their degree of polymerization. In B, the elution times of galacturonic acid and structures 1–6, 8, and 9 as described by Schols et al.[28] are marked. In D, the elution times of galacturonic acid and the xylosyl galacturonic acid dimer are marked with 1 and 2.

Most of the rhamnogalacturonan oligomers released from apple MHR[28] can also be released from PI and PII, but not from PIIIA. The PII fraction appears to contain a relatively large amount of rhamnogalacturonan. The ratio of rhamnose to galacturonic acid in Fraction PIIIA is relatively high (0.53), but a suitable ratio of rhamnose to galacturonic acid is no guarantee for degradation by RG hydrolase.[40] Fraction PIIIA presumably contains some rhamnogalacturonan oligomers, which are too small[41] or contain too many substituents to be further degraded.

Xylogalacturonan appears to be present in all populations from Fraction P, as shown by the release of galacturonic acid and the xylosyl galacturonic acid dimer. However, *exo*-galacturonase is much more effective in the degradation of soybean xylogalacturonan than XGH. The latter might be hindered by the degree of substitution or the distribution of the xylose residues over the main chain.

## CONCLUSIONS

A large part of the arabinogalactan side chains can be removed from CDTA-soluble pectins from soybean

meal by the combined action of *endo*-galactanase, *exo*-galactanase, *endo*-arabinanase, and arabinofuranosidase B. It appears that the remaining polymeric structure (Fraction P) cannot be degraded by the purified enzymes tested here. Moreover, even crude commercial multienzyme preparations—containing a wide range of pectin-degrading enzymes—were not able to degrade the pectic backbone present in Fraction P. Therefore, resort had to be taken to weak acid hydrolysis, which is less specific. Monitoring the release of sugar residues and the molecular mass distribution in time showed that the pectic backbone was hardly affected during the first 8h of hydrolysis. Prolonged incubation resulted in the release of galacturonic acid residues and disappearance of the high molecular mass material.

The acid hydrolysate of Fraction P was fractionated into two polymeric populations (PI and PII), one oligomeric fraction (PIIIA), and monomeric sugars (PIIIB). The oligomeric fraction (PIIIA) cannot be degraded further and probably contains rhamnogalacturonan oligomers and some xylogalacturonan oligomers. This study showed that PI and PII contain xylogalacturonan and rhamnogalacturonan regions, and all analyses agreed on the absence of homogalacturonan regions. The NMR analyses of PII-H clearly demonstrate that this technique is suitable for the characterization of complex plant polysaccharides.

The absence of homogalacturonan in population PI and PII indicates the absence of homogalacturonan in Fraction P, because it is unlikely that these regions are degraded by acid hydrolysis. This is in agreement with the undegradability of Fraction P by PG (after saponification) and PL. Fraction P was obtained from the ChSS fraction by enzymatic removal of a large part of the arabinogalactan side chains, not changing the pectic backbone. So, the CDTA-extractable pectin from soybean is composed of both xylogalacturonan and rhamnogalacturonan (hairy regions), and homogalacturonan is absent. It should be mentioned that although these pectic substances do not contain homogalacturonan, they are still extracted by CDTA. It has been suggested that pectins extractable with hot chelating agents originate from the middle lamella, where they are presumed to be present in the form of calcium pectate gels.[42] This gelation is due to the formation of intermolecular junction zones between homogalacturonan regions of different chains. Since homogalacturonan appears to be absent in the ChSS extract from soybean meal, the presence of a calcium pectate gel must be excluded. It was verified that only a small part of these CDTA-extractable pectic substances could be extracted from the WUS with a (hot) buffer solution. Probably an unknown specific effect of CDTA, other than the chelating effects, can solubilize pectic substances. Renard and Thibault[43] suggested this earlier.

The enzymatic degradation of Fraction P showed that CDTA-extractable pectic polysaccharides from soybean are different from pectic polysaccharides extracted from both other leguminous sources, like pea,[5] and nonseed tissues, like apple,[29,44,45] carrot,[46] kiwifruit,[47,48] onion,[49] pear,[50] potato,[51,52] suspension-cultured sycamore cells,[26,53,54] and sugar beet.[55] All these pectins contain homogalacturonan and rhamnogalacturonan regions, which can be degraded (possibly after saponification) with polygalacturonase and rhamnogalacturonase, respectively. CDTA-extractable pectins from soybean meal could not be degraded by these enzymes. Acid hydrolysis improves the susceptibility of the remaining polymers for RG hydrolase and *exo*-galacturonase.

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