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Pectic substances isolated from apple cellulosic residue: structural characterisation of a new type of rhamnogalacturonan I

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Abstract

The cellulosic residue (CR) of ripe apples (variety 'Glockenapfel') was analysed. Uronic acid in the CR indicated the presence of pectic substances. The objectives of the work were to isolate and characterise the CR associated pectic substances and to find possible cross-links between the two cell wall polymers.

Prior to characterisation enzymatic degradation of the cellulose was necessary. The degradation products were removed by ultrafiltration and the enzyme resistant material submitted to linkage analysis to obtain information about the structure of the CR-pectin. A model of the CR-pectin consisting of five units was developed: a highly ramified rhamnogalacturonan I backbone containing an equal number of galacturonic acid (1,4-GalA*p*) and rhamnose (sum of 1,2-Rha*p* and 1,2,4-Rha*p*) residues was found. About 80% of the rhamnose residues was origin of side chains. Attached to the backbone was a xylogalacturonan side chain, containing 1,4-GalA*p*, 1,3,4-Gal*p*, 1,4-Xyl*p* and T-Xyl*p*. Highly ramified arabinans, with a 1,3-linked backbone, made up more than half of the neutral sugar residues. Two galactose containing units were found: a 1,4-linked galactan with few 1,3- and 1,6-linked short side chains (located near to the backbone) and a short strictly linear 1,4-galactooligomer. The postulated model was confirmed by further degradation of the CR with pectin specific enzymes. An interaction between cellulose and CR-pectin is suggested to occur through the galactan side chains.

Keywords: Apple pectic substances; Cellulose; Rhamnogalacturonan I; Characterisation

1. Introduction

The plant cell wall represents a complex structure of different macromolecules linked together from a more or less rigid assembly. It encases the cell, protecting and giving it form, yet at the same time allowing it to expand during growth. Several layers form the cell wall; the most external one is the middle lamella (McCann et al., 1995). It is primarily composed of pectic substances and has a decisive influence on the texture of fruit tissue. The inner layer, called the primary cell wall, is deposited while the cell is growing. The rather thin primary cell wall shows a higher degree of organisation than the middle lamella and consists of polysaccharides (cellulose, hemicelluloses and pectin) with a relatively small proportion of protein and phenolic compounds (Carpita & Gibeaut, 1993).

Pectins are generally considered to feature homogalacturonan regions, (1 → 4 linked α-D-galacturonic acid units) which are interrupted by ramified rhamnogalacturonan I regions with a backbone built up by the → 4)-α-D-GalA*p*-(1 → 2)-α-L-Rha*p*-(1 → dimer. Neutral sugar side chains such as arabinans, galactans or arabinogalactans are attached to most rhamnose residues at the O-4 (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996). The more complex rhamnogalacturonan II has been identified in the primary cell walls of many plants as well (Vidal et al., 2000).

While the fine structure of extractable pectic substances is well known, almost no information is available on pectins which are associated to hemicelluloses and cellulose within the cell wall. The main focus of this investigation was therefore laid on a pectin-containing fraction obtained by harsher extraction conditions yielding a residue rich in cellulose, but containing a substantial amount of uronic acid as an indication for the presence of pectic substances. Selvendran and Ryden (1990) claimed the existence of cellulose associated pectic substances in cell wall preparations of potatoes.

The existence of pectic substances in the cellulosic

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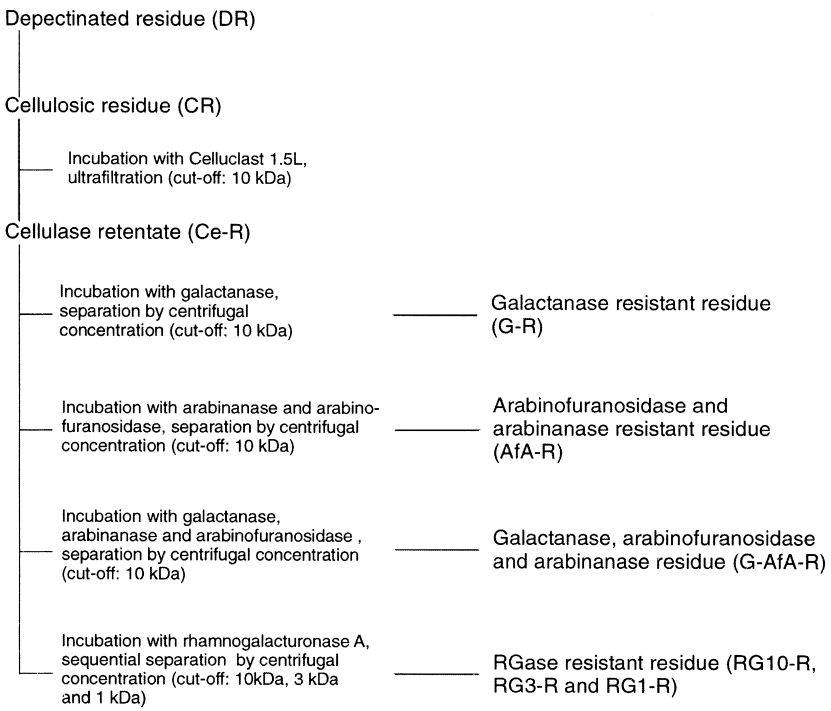

Fig. 1. Overall scheme of the degradation experiments carried out on the ultrafiltration-retentate of the cellulase degraded cellulosic residue (Ce-R).

residue should still be proved and information about its structural elements provided. The cellulosic residue of apples was obtained after extraction of the depectinated residue with strong alkali. Prior to structural characterisation an enrichment of the pectic substances was necessary. This was achieved by enzymatic degradation of the cellulose. Elucidation of the structural features of the pectic polymers associated with cellulose was based on methylation analyses and degradation experiments with pectin specific enzymes. It is assumed that the cellulose associated pectic polymers are somehow linked to the non-pectic cell wall polymers (McCann, Wells, & Roberts, 1990). Indication for a possible connection between the two cell wall polymers was hoped to be found. The results are expected to enlarge the knowledge on the fine structure of pectic substances and the cohesion of the wall.

2. Experimental

2.1. Material

Ripe apples (variety 'Glockenapfel') were investigated. Sampling was performed by Fischer (1993) in her doctoral thesis. The ripe apples were picked at optimum maturity, 14 weeks after the end of the cell division phase (June drop) and stored for one week prior to analyses.

2.2. Preparation of the depectinated residue

Extraction of pectic substances and hemicelluloses was carried out according to the method developed by Selvendran, Stevens, and O'Neill (1985). The AIR was extracted by CDTA followed by two extraction steps with Na_2CO_3 under different conditions as described by Fischer and Amadò (1994) and Fischer, Arrigoni, and Amadò (1994).

2.3. Preparation of the cellulosic residue

The depectinated residue (DR) was further extracted with NaOH at different concentrations to yield the fractions 1 M HC and 4 M HC rich in hemicelluloses, and the cellulosic residue (CR). About 3 g of DR was stirred with 500 ml of a solution of 1 M NaOH and 26 mM $NaBH_4$ at 20–22 °C for 8 h. The mixture was then filtered on a G3 sintered glass filter funnel and the residue washed with distilled water. The filtrate and the washings were brought to pH 4.6 with conc. HCl, dialysed against deionised water (Servapor 44146, Serva & Co, Heidelberg, Germany), concentrated and freeze-dried. This yielded fraction 1 M HC. The residue was stirred with 500 ml of a solution of 4 M NaOH and 26 mM $NaBH_4$ at 20–22 °C for 16 h. The mixture was filtered as above and the residue was washed with water, dialysed against deionised water and freeze-dried. This residue was called cellulosic residue (CR). The filtrate and washings were brought to pH 4.6 with conc. HCl, dialysed against deionised water, concentrated and freeze-dried. This yielded fraction 4 M HC.

2.4. Enzymatic degradation with cellulase

The degradation of cellulose in the CR was performed with Celluclast 1.5L (Novo Nordisk A/S, Bagsvaerd, Denmark). A sample material of 500 mg was suspended in 40 ml 0.05 M acetate buffer (pH 4.5, containing 0.01% NaN_3) and incubated with 200 μl Celluclast 1.5L-solution (5% (v/v)) at 55 °C for 15 h. The enzyme was inactivated at 100 °C for 10 min and after cooling down to room temperature (RT), the reaction mixture was subjected to ultrafiltration (UF). Using the reducing sugar assay (according to Nelson (1944), Somogyj (1952) and Spiro (1966)) Celluclast 1.5L was tested on its activities on different substrates as well as its efficiency to degrade native cellulose.

2.5. Ultrafiltration

Removal of the oligomeric degradation products was performed by UF. The reaction mixture was ultrafiltrated directly after enzymatic degradation in a 250 ml stirred cell (Amicon Inc, Beverley, USA) under nitrogen pressure (4.5 bar) using a polyethersulfone membrane with a cut-off of 10 kDa (Millipore Corporation, Bedford, USA). The UF-retentate (Ce-R) was freeze-dried and stored at − 28 °C until analysed.

2.6. Centrifugal separation

Centrifugal separation was carried out in a Sorvall RC-5B centrifuge (Kendro Laboratory Products, Newtown, USA) at 5000 g using Macrosep centrifugal concentrators (Pall Filtron, Northborough, USA) with molecular mass cut-offs of 10, 3 and 1 kDa. Retentates and filtrates were freeze-dried and stored at − 28 °C until analysed.

2.7. Enzymic degradation by pectin specific enzymes

The procedure for extraction, degradation and fractionation of the pectic substances from the DR of apple (variety 'Glockenapfel') is summarised in Fig. 1.

2.8. Degradation with endo-β-(1 → 4)-D-galactanase

Galactans were degraded with an endo-β-(1 → 4)-D-galactanase (EC No. 3.2.1.89) from *Aspergillus niger* (Megazyme International Ireland Ltd, Wicklow, Ireland). A sample material of 50 mg was dissolved in 10 ml 0.02 M sodium acetate buffer (pH 4.5) and incubated with 100 μl enzyme solution (approximately 80 IU) at 45 °C for 5 h. The enzyme was inactivated at 100 °C for 10 min and after cooling down to RT, the reaction mixture was subjected to centrifugal separation to give the enzyme resistant retentate G-R.

2.9. Degradation with a mixture of endo-α-(1 → 5)-L-arabinanase and α-L-arabinofuranosidase

Extensive degradation of the arabinans was reached by simultaneous incubation with an *endo*-α-(1 → 5)-L-arabinanase (EC No. 3.2.1.55) from *A. niger* (gift from Novo Nordisk A/S) and an α-L-arabinofuranosidase (EC No. 3.2.1.99) from *A. niger* (gift from Novo Nordisk A/S). A sample material of 50 mg was dissolved in 10 ml 0.02 M sodium acetate buffer (pH 4.5) and incubated with 100 μl enzyme mixture (approximately 75 IU arabinofuranosidase and approximately 100 IU arabinanase) at 40 °C for 48 h. The enzymes were inactivated at 100 °C for 10 min and after cooling down to RT, the reaction mixture was subjected to centrifugal separation yielding the enzyme resistant retentate AfA-R.

2.10. Sequential degradation with endo-β-(1 → 4)-D-galactanase and a mixture of endo-α-(1 → 5)-L-arabinanase and α-L-arabinofuranosidase

Removal of the side chains was achieved by stepwise incubation with an endo-β-(1 → 4)-D-galactanase followed by degradation with a mixture of endo-α-(1 → 5)-L-arabinanase and α-L-arabinofuranosidase. A sample material of 50 mg was dissolved in 10 ml 0.02 M sodium acetate buffer (pH 4.5) and incubated with 100 μl galactanase solution (approximately 80 IU) at 45 °C for 5 h. The enzyme was inactivated at 100 °C for 10 min and after cooling down to RT, 100 μl enzyme mixture (approximately 75 IU arabinofuranosidase and approximately 100 IU arabinanase) was added and incubated for 48 h at 40 °C. The enzymes were inactivated at 100 °C for 10 min and after cooling down to RT, the reaction mixture was subjected to centrifugal separation yielding the enzyme resistant retentate G-AfA-R.

2.11. Degradation with rhamnogalacturonase A

Degradation of the backbone of RG I was performed with a rhamnogalacturonase A from *A. aculeatus* (gift from Novo Nordisk A/S). A sample material of 50 mg was dissolved in 10 ml 0.02 M sodium acetate buffer (pH 3.5) and incubated with 50 μl RGase A solution at 30 °C for 7 h. The enzyme was inactivated at 100 °C for 10 min and after cooling down to RT, the reaction mixture was subjected to centrifugal separation yielding the three fractions RG10-R, RG3-R and RG1-R.

2.12. Analytical methods

Neutral sugar and uronic acid contents were determined by GC as alditol-acetates (Blakeney, Harris, Henry, & Stone, 1983) and photometrically by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973), respectively. Analysis of the glycosidic linkages in

Table 1
Percent distribution of the fractions obtained by the extraction of the depectinated residue (DR) of ripe apples, variety 'Glockenapfel', with alkali

Fraction	% of DR	% of AIR
1 M Hemicelluloses (1 M HC)	18.1	10.4
4 M Hemicelluloses (4 M HC)	24.0	13.8
Cellulosic residue (CR)	43.2	24.8
Total amount	85.3	

polysaccharides was performed by methylation analysis after carbodiimide-activated reduction with $NaBD_4$. Carbodiimide-activated reduction was carried out by a method developed by Kim and Carpita (1992) and modified by Wechsler (1997). Methylation analysis was performed based on Harris, Henry, Blakeney, and Stone (1984) and Kvernheim (1987), as modified by Wechsler (1997).

2.13. Determination of the molecular weight distribution

A solution of 4 mg sample material in 2 ml sodium acetate buffer (0.02 M or 0.05 M, pH 4.5) was prepared. Insoluble material was removed by filtration (Titan Syringe Filters, PVDF-membrane, pore size 0.45 mm, Scientific Resources Inc, Eatonwon, USA). High performance size-exclusion chromatography (HPSEC) was performed on a Merck Lachrom HPLC (Merck Ltd, Darmstadt, Germany) equipped with a Ultrahydrogel Linear column (Waters Corporation, Milford, USA). Elution was carried out at 35 °C with 0.05 M sodium acetate buffer (pH 4.5) at 1.0 ml/min. The eluate was monitored using a refractive index detector (RI) or a diode array detector (240–260 nm). Pullulans (Shodex Standard P-82, Showa Denko K.K., Kawasaki, Japan) dissolved (0.2% (w/v)) in 0.05 M acetate buffer (pH 4.5) were used as standards for molecular weight calibration. The sample Ce-R was additionally analysed using a Viscotek Triple Detection System TDA 302 (Viscotek, Houston, USA).

Table 2
Neutral sugar, uronic acid and protein contents of the cellulosic residue of ripe apples, variety 'Glockenapfel' (% dm of CR, mv ± sd; $n = 3$)

Components	Content
Rhamnose	0.5 ± 0.04
Fucose	0.05 ± 0.01
Arabinose	9.9 ± 0.3
Xylose	3.3 ± 0.1
Mannose	1.2 ± 0.05
Galactose	7.6 ± 0.3
Glucose[a]	59.5 ± 0.3
Uronic acid	6.7 ± 0.3
Protein[a]	0.14 ± 0.1

[a] $n = 2$.

3. Results and discussion

3.1. Extraction, yield and characterisation of the CR

The classic extraction procedure for cell wall material proposed by Selvendran and O'Neill (1987) leads from the alcohol insoluble residue (AIR) to three pectin-rich fractions and the DR. This residue was used as starting material for the present work. It is rich in hemicelluloses and cellulose and still contains pectic substances. The percentage of the fractions obtained by alkali extraction of the DR of the ripe 'Glockenapfel' are shown in Table 1. The hemicellulose fractions (1 M HC and 4 M HC) will only be briefly discussed here, as an ongoing project is focused on the pectic substances in these fractions (Lutz, Oechslin, & Amadò, 2002). The CR represents the bulk of cell wall material and corresponds to slightly more than 20% of the AIR. The yield of CR obtained was in the range given by Albersheim et al. (1996) for primary cell walls (20–40% cellulose).

The overall characterisation of the CR is shown in Table 2. The presence of pectic substances was confirmed by remarkable amounts (6.7%) of uronic acid found in CR. As expected the most abundant neutral sugar was glucose originating from cellulose. It made up approximately 60% of the dm of CR. High amounts of galactose and arabinose, and minor amounts of rhamnose and xylose, as well as traces of fucose were found, further supporting the presence of pectic substances. The low protein content of the CR practically excludes the possibility of cross-linking glycoproteins. The findings for the neutral sugar distribution and the uronic acid content of the CR in this investigation are in good agreement with literature data. Redgwell, Fischer, Kendal, and MacRae (1997) found considerable amounts of galactose and arabinose which were not extracted with 4 M KOH and remained associated with the residue of cell wall material of apples. Renard, Voragen, Thibault, and Pilnik (1991) found a similar sugar distribution in α-cellulose residue of apple cell walls: glucose which was enriched (53.5% dm), and also appreciable amounts of galacturonic acid (18.3% dm), arabinose (10.9% dm), galactose (7.9% dm), xylose (4.6% dm) and rhamnose (0.7% dm) were detected.

The amount of glucose found by neutral sugar analysis allowed an estimation of the cellulose content of the CR. Pectic substances contain practically no glucose. The only other source of glucose in the CR besides cellulose could be hemicelluloses, but it is assumed that they were completely removed by the alkaline extraction. Baba, Sone, Misaki, and Hayashi (1994) showed by antibody labelling that after extraction with strong alkali (4 M KOH) the residue was practically devoid of hemicelluloses, and only 1.1% of the dm was left.

The high cellulose amount of the CR hindered solubilisation and as a consequence methylation analysis was not possible. A partial removal of the cellulose by enzymatic

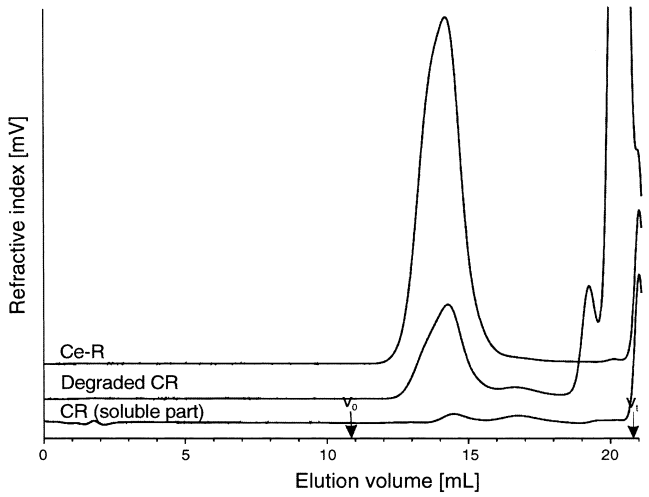

Fig. 2. HPSEC of the soluble part of the cellulosic residue (CR), the cellulase degraded CR and the ultrafiltration-retentate of the cellulase degraded cellulosic residue (Ce-R).

degradation was considered to be a promising approach to allow further analysis of the cellulose associated pectic substances.

3.2. Cellulose degradation with Celluclast 1.5L

Out of different cellulase preparations only Celluclast 1.5L (Novo Nordisk A/S) was active on native cellulose. In the reducing sugar assay the activity of Celluclast 1.5L and its efficiency to degrade native cellulose was tested and information about its side-acitvities was obtained. With respect to the aim of the enzymatic treatment of CR it can be summarised that Celluclast 1.5L exhibited activity towards native cellulose but neither the pectic backbone nor the neutral sugar side chains were attacked (data not shown).

To separate the oligo- and monomeric degradation products from the enzyme resistant part ultrafiltration (with a cut-off of 10 kDa) was chosen. It can be seen in Fig. 2 that this procedure allowed a complete removal of the degradation products. An average molecular weight of approximately 400 kDa was determined for the high molecular weight fraction by calibration with pullulan standards. The results of the low angle laser light scattering detection of Ce-R obtained with a Viscotek system allowed the calculation of the molecular weight of the pectic polymers as well. Using a d*n*/d*c* value of 0.074 (amylopectin in water) an average molecular weight ($\bar{M}_w$) of 497.7 kDa for the population with the higher molecular weight and 20 kDa for the smaller one was calculated. This value corresponds quite well with results obtained with HPSEC using pullulan standards.

The yield of the retentate (Ce-R) after cellulase degradation and ultrafiltration corresponded to 46.8% of the dm of CR. From the amount of glucose found in the Ce-R it can be deduced that the enzymatic cellulose degradation was effective. The weight loss by the enzyme degradation corresponds well with the loss of glucose in the Ce-R sample. A considerable enrichment of the other pectic sugar residues in the dry matter was observed.

Elucidation of the glycosidic linkage composition (Table 3) revealed a highly branched structure of the polymer

Table 3
Glycosyl-linkage composition of the ultrafiltration-retentate of the cellulase degraded cellulosic residue (Ce-R) of ripe apples, variety 'Glockenapfel'

Residues	mol%	%
∑ Neutral sugars		94.3
∑ Uronic acid		5.7
Ratio branched/terminal		1.1
∅ *L* (Average linearity)		2.5
Rhamnose		
T-Rha*p*	n.d.	n.d.
1,2-Rha*p*	0.7	14.6
1,2,4-Rha*p*	4.0	85.4
	4.7	100.0
Galacturonic acid		
T-GalA*p*	n.d.	n.d.
1,4-GalA*p*	5.2	91.2
1,3,4-GalA*p*	0.5	8.8
	5.7	100.0
Arabinose		
T-Ara*f*	14.5	29.2
T-Ara*p*	1.4	2.8
1,2-Ara*f*	0.1	0.3
1,3-Ara*f*	5.0	10.0
1,5-Ara*f*	12.3	24.8
1,2,5-Ara*f*	0.8	1.6
1,3,5-Ara*f*	11.0	22.2
1,2,3,5-Ara*f*	4.5	9.1
	49.6	100.0
Galactose		
T-Gal*p*	1.8	6.3
1,3-Gal*p*	0.9	3.1
1,4-Gal*p*	25.0	85.6
1,6-Gal*p*	0.2	0.7
1,3,4-Gal*p*	0.2	0.6
1,4,6-Gal*p*	1.1	3.7
	29.2	100.0
Xylose		
T-Xyl*p*	0.9	57.6
1,4-Xyl*p*	0.6	42.4
	1.5	100.0
Glucose		
T-Glc*p*	1.1	14.0
1,4-Glc*p*	6.3	77.5
1,3,4-Glc*p*	0.3	3.4
1,4,6-Glc*p*	0.4	5.1
	8.1	100.0
Fucose		
T-Fuc*p*	0.2	100.0
	0.2	100.0
Mannose		
1,4-Man*p*	0.6	100.0
	0.6	100.0

Table 4
Glycosyl-linkage composition of enzyme resistant residues obtained according to the fractionation scheme shown in Fig. 1

Residues	G-R		AfA-R		G-AfA-R	
	mol%	%	mol%	%	mol%	%
Rhamnose						
T-Rha*p*	0.1	0.3	0.3	1.0	0.2	0.6
1,2-Rha*p*	2.5	31.4	6.7	26.8	11.0	46.6
1,4-Rha*p*	n.d.	n.d.	0.3	1.3	0.1	0.5
1,2,3-Rha*p*	n.d.	n.d.	0.2	0.9	n.d.	n.d.
1,2,4-Rha*p*	5.5	68.3	17.5	70.0	12.4	52.3
	8.1	100.0	25.0	100.0	23.7	100.0
Galacturonic acid						
T-GalA*p*	n.d.	n.d.	n.d.	n.d.	0.3	1.4
1,4-GalA*p*	7.0	81.6	20.9	82.9	14.7	80.4
1,3,4-GalA*p*	1.6	18.4	4.3	17.1	3.3	18.2
	8.6	100.0	25.2	100.0	18.3	100.0
Arabinose						
T-Ara*f*	25.5	36.6	1.0	10.1	1.0	35.9
T-Ara*p*	1.1	1.6	0.5	4.4	0.3	11.3
1,2-Ara*f*	0.2	0.2	n.d.	n.d.	n.d.	n.d.
1,3-Ara*f*	7.4	10.6	6.4	63.2	0.4	12.9
1,5-Ara*f*	16.0	22.9	1.8	18.0	1.1	39.9
1,2,5-Ara*f*	0.8	1.2	n.d.	n.d.	n.d.	n.d.
1,3,5-Ara*f*	14.0	20.1	0.3	3.0	n.d.	n.d.
1,2,3,5-Ara*f*	4.8	6.8	0.1	1.3	n.d.	n.d.
	69.8	100.0	10.1	100.0	2.8	100.0
Galactose						
T-Gal*p*	4.1	58.6	8.6	42.8	5.0	55.5
1,3-Gal*p*	1.4	20.1	1.3	6.6	0.6	6.9
1,4-Gal*p*	1.2	16.9	8.9	44.3	2.7	30.0
1,6-Gal*p*	0.3	4.4	1.3	6.3	0.7	7.6
1,3,4-Gal*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1,4,6-Gal*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	7.0	100.0	20.1	100.0	9.0	100.0
Xylose						
T-Xyl*p*	3.5	88.2	8.4	87.2	6.8	88.3
1,4-Xyl*p*	0.5	11.8	1.3	12.8	0.9	11.7
	4.0	100.0	9.7	100.0	7.7	100.0
Glucose						
T-Glc*p*	0.4	18.3	0.9	6.4	2.0	5.7
1,4-Glc*p*	2.0	81.7	6.4	87.6	33.1	94.3
1,3,4-Glc*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1,4,6-Glc*p*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	2.4	100.0	7.3	100.0	35.1	100.0
Fucose						
T-Fuc*p*	0.2	100.0	0.6	100.0	0.4	100.0
	0.2	100.0	0.6	100.0	0.4	100.0
Mannose						
T-Man*p*	n.d.	n.d.	1.3	62.0	1.6	64.7
1,2-Man*p*	n.d.	n.d.	0.3	12.8	n.d.	n.d.
1,4-Man*p*	n.d.	n.d.	0.5	25.2	0.9	35.3
	n.d.	n.d.	2.1	100.0	2.5	100.0

n.d., not detected.

present in Ce-R. The ratio of 1.1 for terminal to branched residues indicated good methylation practice. The low average linearity strongly indicates the high degree of ramification of the pectic substances in Ce-R. Out of the distribution of the sugar residues and their linkage distribution a predominance of RG I like structures is suggested. Backbone structures as described for RG I by Lau, McNeil, Darvill, and Albersheim (1985) were found. Equal amounts of rhamnose (1,2-Rha*p* and 1,2,4-Rha*p*) and galacturonic acid (1,4-GalA*p*) residues were present. Neutral sugar side chains were attached to 85.4% of the rhamnose residues. Highly ramified arabinans represented approximately 50% of all sugar residues. The galactans were predominantly linear, indicated by the fact that 85.6% of all galactose residues were 1,4-linked. To elucidate their structural features further investigations were performed. Indication for the presence of a xylogalacturonan as described by Schols, Posthumus, and Voragen (1990) in modified hairy regions (MHR) of apple pectin exists, but needs further clarification.

3.3. Degradation with pectin specific enzymes

Degradation experiments (Fig. 1) with pectin specific enzymes were carried out to proof or deny the existence of a connection between cellulose and pectin. If a cross-link exists it should be possible to isolate fractions enriched with cellulosic and pectic residues. The degradation experiments should also provide more information about the structure of the pectic polymers associated with cellulose.

3.4. Degradation with endo-β-(1 → 4)-D-galactanase

It can be seen in Table 4 that the total galactose content decreased by a factor 4–7.0% after treatment of Ce-R with an *endo*-β-(1 → 4)-D-galactanase. The detected decrease of 1,2,4-Rha*p* can only be explained if the used galactanase-preparation possesses a β-galactosidase side activity. The dramatically reduced amount of 1,4-Gal*p* residues in G-R clearly indicated that most of the galactose of the Ce-R was 1,4-linked and linear. Linear long galactans were predominant in Ce-R. 1,3- and 1,6-linked galactose residues were enriched but no branched residues could be found anymore. These findings led to the suggestion that linear parts of the galactans had been degraded from the non-reducing end to the branching points of the short side chains (1,3,4- and 1,4,6-Gal*p* residues found in Ce-R). The increased amount of 1,3- and 1,6-linked residues suggested that the short side chains were located near to the branching point of the galactan with the rhamnogalacturonan backbone. In addition to the long linear galactan with its few short side chains, strictly linear and 1,4-linked galactooligomers are proposed to be present, which could be verified by enzymatic degradation of the rhamnogalacturonan backbone.

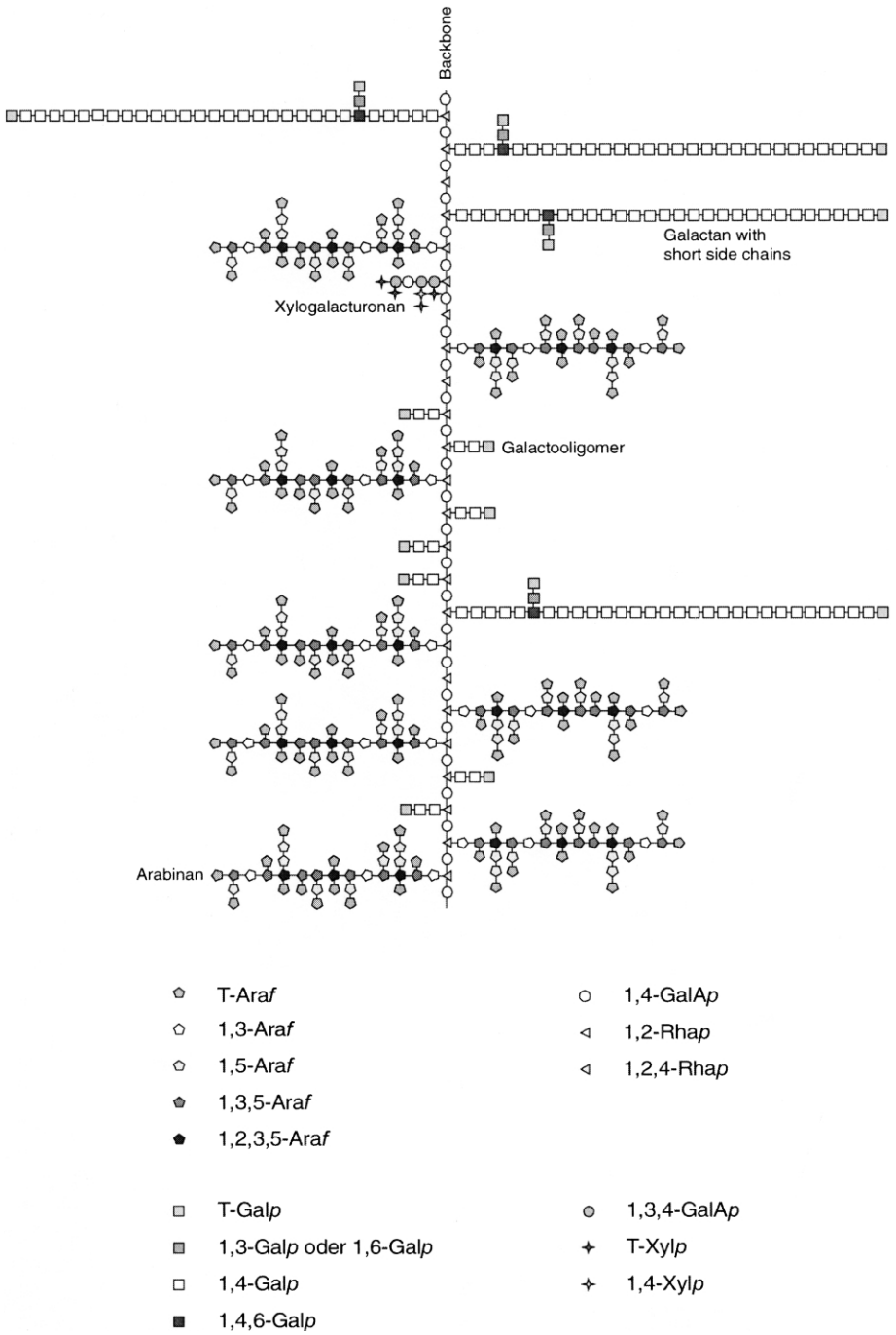

Fig. 3. Model for cellulose associated pectic substances of ripe apples (variety 'Glockenapfel').

3.5. Degradation with a mixture of endo-α-(1 → 5)-L-arabinanase and α-L-arabinofuranosidase

The combined action of an *endo*-α-(1 → 5)-L-arabinanase and an α-L-arabinofuranosidase was used to degrade the arabinans present in the Ce-R. Since the action of the *endo*-arabinanase can strongly be hindered by the presence of arabinofuranosyl side chains an arabinofuranosidase was used in combination with the *endo*-enzyme. Debranching has been shown to have a beneficial effect on the degradation of arabinans by *endo*-arabinanase (Beldman, Saerle-van Leeuwen, De Ruiter, Siliha, & Voragen, 1993).

Methylation analysis (Table 4) of the enzyme resistant fraction AfA-R revealed the removal of 80% of arabinose residues. More than 60% of the arabinose present in AfA-R was 1,3-linked. The fact that 1,3-Ara*f* was not removed led to the speculation that it might be directly connected to the pectic backbone. If 1,3-Ara*f* would have been part of the arabinan side chains, it would have been removed. In contrast to the arabinans hitherto described, the arabinan

backbone in the cellulose-associated pectin seems to contain 1,3-linked instead of 1,5-linked arabinose residues in its backbone. Taking into account the differently linked arabinose residues found in Ce-R, the arabinan present in the cellulosic residue is thought to be highly ramified. The size of the arabinan side chains may differ but further investigations are needed to clarify this point. The mannose residues present in AfA-R were shown to originate from the enzyme preparations used, which possibly are manno-glycoproteins.

3.6. Sequential degradation with endo-β-(1 → 4)-D-galactanase and a mixture of endo-α-(1 → 5)-L-arabinanase and α-L-arabinofuranosidase

To obtain more information on the fine structure of the backbone of the CR-pectin and about the presence and position of the xylogalacturonan present, a degradation experiment with all enzymes necessary to remove the neutral sugar side chains was performed. In the enzyme resistant residue not only backbone elements but also xylogalacturonan structures were enriched. Two different xylogalacturonan structures can be envisaged. The xylogalacturonan is either part of the pectic backbone, as proposed for MHR of apples by Schols et al. (1990), or occurs as a side chain. The surplus of T-Xyl*p* could be explained if xylogalacturonan was a side chain with 1,4- and 1,3,4-GalA*p* in the backbone and substituted with monmeric xylose or dimeric xylosyl-xylose residues. The xylogalacturonan side chain could be terminated with T-Xyl*p*.

HPSEC of the enzyme resistant fraction (G-AfA-R) showed the existence of two populations: a shoulder at a molecular weight of approximately 190 kDa and the main peak at 23 kDa. The population with the higher molecular weight could correspond to pectic polymers in which the neutral sugar side chains had only been partly removed. The population with an average molecular weight of 23 kDa could represent the backbone without neutral sugar side chains.

3.7. Degradation with rhamnogalacturonase A

To obtain additional information on the fine structure of the backbone, Ce-R was incubated with RGase A. The CR had been obtained by alkaline extraction of the DR, thus all acetyl ester groups had been saponified and no inhibition of the RGase A was expected. HPSEC of the RGase A incubated Ce-R showed three fractions which were separated by centrifugal separation. The glycosidic linkage analyses revealed differences between the sugar distributions of the three fractions. These results suggested a blockwise distribution of side chains along the rhamnogalacturonan backbone to occur.

4. Conclusion

4.1. Model for the pectic substances associated with cellulose

Based on the results obtained by the characterisation of the cellulose associated pectin and the degradation experiments with specific enzymes, a model of the pectin present in the cellulosic residue have been developed. Different structural features of these pectic polymers have been identified. CR-pectin contains a rhamnogalacturonan I backbone to which different side chains are attached. The presence of RG II can be excluded in the cellulose-associated pectin since no structural units of this polysaccharide were detected.

The model of the pectic substances associated with cellulose is displayed in Fig. 3. The backbone contains equal amounts of rhamnose (1,2-Rha*p* and 1,2,4-Rha*p*) and 1,4-GalA*p* which is typical for rhamnogalacturonan I. A xylogalacturonan is present, indicated by 1,3,4-GalA*p* and a surplus of 1,4-GalA*p* residues. All ramified galacturonic acid residues of the xylogalacturonan could be substituted either with T-Xyl*p* or a 1,4-Xyl*p*-Xyl*p* dimer. It seems most likely that xylogalacturonan occurs as a side chain with a xylopyranose at its non-reducing end. The general structure of L-arabinans described in the literature emerges as a 1,5-linked chain of α-L-arabinofuranosyl residues which are substituted in position O-3 and/or O-2 by short 1,3-linked α-L-arabinofuranosyl side chains (Carpita & Gibeaut, 1993). In contrast to the arabinans hitherto described, the arabinan in the cellulose-associated pectin contains 1,3-linked arabinose residues in its backbone. The differently linked arabinose residues present in the CR indicate the fine structure of the arabinans to be highly ramified. To detect if their seize differ, further investigations are needed.

About 90% of all galactose residues are present as 1,4-linked residues. This suggests that quite long linear galactans are predominant. The galactans have been shown to bear short side chains with 1,3- or 1,6-linked galactose residues. The short side chains are postulated to be located near to the branching point of the galactan with the rhamnogalacturonan backbone. A second type of galactose containing side chains are strictly linear 1,4-linked galactooligomers occurring in the neighbourhood of the highly ramified arabinans (they were found in the same RGase fractions). The galactooligomers are similar to those reported by Strasser and Amadò (2001) for CDTA-soluble pectin of red beet.

The side chains along the RG-backbone seem to be distributed blockwise as indicated by the degradation pattern of the backbone with RGase A. Fractions with different sugar compositions were obtained.

The model presented for the pectic substances associated to cellulose includes structural elements which have not been found in chelator-soluble pectic substances obtained from apple cell walls. Reasons for

these differences remain to be explained as well as the function of this pectin, which is yet unknown. Further investigations are necessary to understand the function of the pectic substances associated to the cellulose. To our knowledge up to now no structural features as presented for CR-pectins have been reported.

4.2. Connection between the cellulose and the pectic polymers

No final proposal for an interaction of the pectin with cellulose can be made. A speculative possibility would be an interaction between the galactan side chains and the cellulose. This hypothesis is supported by the fact that in fractions containing galactan side chains, high amounts of 1,4-linked glucose were always present as well. This finding contrasts with the results reported by Foster, Ablett, McCann, and Gidley (1996), who claimed that pectic material which resisted extraction with 4 M KOH was associated with cellulose via the backbone rather than via the side chain regions of the pectic polysaccharide. Cross-linking by phenolic compounds cannot be excluded in the present work. If such cross-links exist, only ether-linkages would be possible since alkali extraction is known to cleave ester-linkages.

Several explanations for the presence of pectic polysaccharides in the cellulosic residue can be discussed. Firstly, swelling of cellulose by the alkaline-extraction of hemicelluloses could be the cause for a collapse of the fibrils resulting in an embedding of the pectins. Secondly, a change in the solubility-properties and behaviour of the pectins after alkaline treatment could be a possibility. Re-extraction with aqueous solution could possibly liberate the pectin and leave a pectin free CR. Thirdly, a covalent cross-link between pectic polymers and cellulose could exist. The results presented here can neither exclude chemical cross-linking between pectin and cellulose nor embedding and/or solubility effects due to the alkaline treatment. Interweavement of the cellulose/hemicellulose network with the pectic network could have had an enforcing influence on the embedding caused by the swelling of the cellulose. A non-covalent interaction between cellulose and the long, mainly linear 1,4-linked galactan side chain of the pectin could also be the reason for the presence of pectins in the CR. Experimental evidence has been presented which supports this proposal. Fractions rich in glucose after treatment either with cellulase or pectin degrading enzymes contained large amounts of 1,4-linked galactose residues representing the long 1,4-linked galactans. Extraction of the CR has also to be considered since it represents an invasive technique in which no clear separation of the interwoven networks is possible and artefacts can be formed. Further investigations are needed to elucidate the nature of the connection between the two polymers cellulose and pectin.

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References

Albersheim, P., Darvill, A. G., O'Neill, M. A., Schols, H. A., & Voragen, A. G. J. (1996). An hypothesis: The same six polysaccharides are components of the primary cell walls of all higher plants. In J. Visser, & A. G. J. Voragen (Eds.), *Pectin and pectinases* (pp. 47–56). Amsterdam: Elsevier Science.

Baba, K., Sone, Y., Misaki, A., & Hayashi, T. (1994). Localisation of xyloglucan in the macromolecular complex composed of xyloglucan and cellulose in pea stems. *Plant Cell Physiology*, *35*, 439–444.

Beldman, G., Saerle-van Leeuwen, M. J. F., De Ruiter, G. A., Siliha, H. A., & Voragen, A. G. J. (1993). Degradation of arabinans by arabinases from *Aspergillus aculeatus* and *Aspergillus niger*. *Carbohydrate Polymers*, *20*, 159–168.

Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983). A simple and rapid preparation of alditol acteates for monosaccharide analysis. *Carbohydrate Research*, *113*, 291–299.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484–489.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: Consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal*, *3*, 1–30.

Fischer, M (1993). Changes in the pectic substances during the ripening of apples. Doctoral Thesis No. 10336, Swiss Federal Institute of Technology, Zurich.

Fischer, M., & Amadò, R. (1994). Changes in the pectic substances of apples during development and postharvest ripening. Part 1: Analysis of the alcohol-insoluble residue. *Carbohydrate Polymers*, *25*, 161–166.

Fischer, M., Arrigoni, E., & Amadò, R. (1994). Changes in the pectic substances of apples during development and postharvest ripening. Part 2: Analysis of the pectic fractions. *Carbohydrate Polymers*, *25*, 167–175.

Foster, T. J., Ablett, S., McCann, M. G., & Gidley, M. J. (1996). Mobility-resolved ^{13}C NMR spectroscopy of primary plant cell walls. *Biopolymers*, *39*, 51–66.

Harris, P. J., Henry, R. J., Blakeney, A. B., & Stone, B. A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydrate Research*, *127*, 59–73.

Kim, J.-B., & Carpita, N. C. (1992). Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiology*, *98*, 646–653.

Kvernheim, A. L. (1987). Methylation analysis of polysaccharides with buthyllithium in dimethyl sulfoxide. *Acta Chemica Scandinavica Series B*, *41*, 150–152.

Lau, J. M., McNeil, M., Darvill, A. G., & Albersheim, P. (1985). Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell walls of plants. *Carbohydrate Research*, *137*, 111–125.

Lutz, M. V., Oechslin, R., & Amadò, R. (2002). Structural features of pectic substances from hemicellulosic extracts of apples. In A. G. J. Voragen, H. Schols and R. Visser (Eds.), *Advances in pectin and pectinase research* (in press) Dordrecht: Kluwer.

McCann, M. C., Roberts, K., Wilson, R. H., Gidley, M. J., Gibeaut, D. M.,

Kim, J. B., & Carpita, N. C. (1995). Old and new ways to probe plant cell-wall architecture. *Canadian Journal of Botany*, *73*, 103–113.

McCann, M. C., Wells, B., & Roberts, K. (1990). Direct visualisation of cross-links in the primary cell wall. *Journal of Cell Science*, *96*, 323–334.

Nelson, N. (1944). A photometric adaption of the Somogyj method for determination of glucose. *Journal of Biological Chemistry*, *153*, 375–380.

Redgwell, R. J., Fischer, M., Kendal, E., & MacRae, E. A. (1997). Galactose loss and fruit ripening: High-molecular-weight arabinogalactans in the pectic polysaccharides of fruit cell walls. *Planta*, *203*, 174–181.

Renard, C. M. G. C., Voragen, A. G. J., Thibault, J.-F., & Pilnik, W. (1991). Studies on apple protopektin. IV: Apple xyloglucans and influence of pectin extraction treatments on their solubility. *Carbohydrate Polymers*, *15*, 387–403.

Schols, H. A., Posthumus, M. A., & Voragen, A. G. J. (1990). Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydrate Research*, *206*, 117–129.

Selvendran, R. R., & Ryden, P. (1990). Isolation and analysis of plant cell walls. In P. M. Dey (Ed.), *Methods in plant biochemistry* (pp. 549–578). London: Academic Press.

Selvendran, R. R., Stevens, B. J. H., & O'Neill, M. A. (1985). In C. T. Brett, & J. R. Hillman (Eds.), *Biochemistry of plant cell walls* (pp. 39–78). Cambridge: Cambridge University Press.

Selvendran, R. R., & O'Neill, M. A. (1987). Isolation and analysis of cell walls from plant material, In D. Glick (Ed.), *Methods of Biochemical Analysis, Vol. XXXII* (pp. 25–123). New York: John Wiley & Sons.

Somogyj, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry*, *195*, 19–23.

Spiro, R. G. (1966). Analysis of sugars found in glycoproteins. *Methods in Enzymology*, *8*, 3–26.

Strasser, G. R., & Amadò, R. (2001). Pectic substances from red beet (*Beta vulgaris conditiva*). Part I. Structural analysis of rhamnogalacturonan I using enzymic degradation and methlyation analysis. *Carbohydrate Polymers*, *44*, 63–70.

Vidal, S., Doco, T., Williams, P., Pellerin, P., York, W. S., O'Neill, M. A., Glushka, J., Darvill, A. G., & Albersheim, P. (2000). Structural characterisation of the pectic polysaccharide rhamnogalacturonan. II: Evidence for the backbone location of the aceric acid containing oligoglycosyl side-chain. *Carbohydrate Research*, *326*, 277–294.

Wechsler, D. E (1997). Charakterisierung der Struktur von Pektinen während der Reifung und Lagerung von Äpfeln. Doctoral Thesis No. 12 044, Swiss Federal Institute of Technology, Zurich.