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Some preliminary results on the action of rhamnogalacturonase on rhamnogalacturonan oligosaccharides from beet pulp

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Sugar-beet pulp was saponified and then hydrolysed with 0.1 M HCl at 80°C for 72 h, and a rhamnogalacturonan fraction was isolated by ion-exchange chromatography on AG 1X8 resin. Four individual oligomers, and a mixture of oligomers with higher degrees of polymerization, were obtained by chromatography on BioGel P-4. They all presented the α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1) $_n$ -2)-L-Rhap structure (with $n \geq 2$). The five fractions were submitted to hydrolysis with rhamnogalacturonase. The enzyme was active on oligomers with degrees of polymerization ≥ 10 , and gave as main products α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap(1 $\rightarrow$ 4)-D-GalAp and α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)-D-GalAp.

Keywords: rhamnogalacturonan; rhamnogalacturonase; sugar beet

Pectins are plant cell-wall polysaccharides which comprise two distinct structural features: 'smooth regions', composed of (1 $\rightarrow$ 4)-linked α -D-galacturonic acid residues, with degrees of polymerization $>100^1$, and 'hairy regions', where a rhamnogalacturonan backbone, composed to a large extent of alternating 2-linked α -L-rhamnose and 4-linked α -D-galacturonic acid residues, carries side chains (on the O4 position of rhamnose residues) composed essentially of galactose and arabinose residues^{2,3}. Rhamnogalacturonan regions typically contain about 5% of the galacturonic acid residues and may be isolated after degradation of pectins⁴ or cell walls² by pectolytic enzymes.

Rhamnogalacturonase is an enzyme which cleaves the main chain of pectins in the rhamnogalacturonan areas. It has been isolated from a commercial preparation of *Aspergillus aculeatus*⁵ and culture broth from *Trametes sanguinea*⁶, and its end products from isolated 'modified hairy regions' are oligomers with a basic structure of α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)-D-GalAp³.

Although rhamnogalacturonase is known to hydrolyse linkages within the rhamnogalacturonan backbone, and is even able to extract pectins from apple cell walls⁷, the exact mechanism is not completely understood. During acid hydrolysis of pectins¹, we identified a fraction representative of the rhamnogalacturonan backbone of pectins. We have now isolated and characterized a series of rhamnogalacturonan oligomers from an acid hydrolysate of sugar-beet pulp. Subsequently, we incubated these oligosaccharides with rhamnogalacturonase and studied the products liberated.

Experimental

Materials

Sugar-beet pulp from Sofalia (Ennezat, France) was de-esterified as described previously⁸; rhamnogalacturonase was isolated from a commercial preparation of *Aspergillus aculeatus* as described by Schols *et al.*⁵.

Acid hydrolysis

Eight grams of de-esterified beet pulp were hydrolysed in 0.1 M HCl at 80°C for 72 h¹. After hydrolysis, the slurry

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was filtered on a G4 sintered glass filter. The filtrate was neutralized to pH 5.9 with NaOH and filtered again before chromatography. The residue was dried by solvent exchange after washing.

Fractionation of the acid hydrolysate

Ion-exchange chromatography was performed on an AG 1X8 column (1.6 × 54 cm) (BioRad, St Louis, MO, USA) eluted with ammonium acetate buffer, pH 6, at a flow rate of 1.33 ml min⁻¹. The column was first washed with 150 ml of 0.05 M buffer, followed by a gradient up to 1.2 M in 600 ml and then to 2 M in 200 ml. Gel-permeation chromatography on a BioGel P-4 column (2.5 × 90 cm) (BioRad), eluted with 0.1 M acetate buffer, pH 3.6, was carried out according to the method described by Thibault⁹. In all cases, the collected tubes were assayed for uronic acid and neutral sugars. Fractions were desalted by filtration on a Sephadex G-10 column (5 × 60 cm) (Pharmacia, Uppsala, Sweden) eluted with water, and freeze-dried.

Enzyme assays

The oligomers (0.5 mg ml⁻¹ in 50 mM acetate buffer, pH 5) were incubated with 45 µg ml⁻¹ of rhamnogalacturonase at room temperature. The enzyme was inactivated by treatment for 5 min at 100°C. The incubation was followed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA100 column (4 × 250 mm) with guard column. The chromatographic system was a Dionex Bio-LC system (Sunnyvale, CA, USA), including a quaternary gradient pump, eluent de-gas (helium) module, and a pulsed electrochemical detector in the pulsed amperometric detection (PAD) mode. A Spectra Physics SP8800 autosampler (San José, CA, USA) and a Spectra Physics Winner data collection system were used. The column was eluted at a flow rate of 1 ml min⁻¹ by a gradient of NaOAc in 0.1 M NaOH: from 150 mM NaOAc to 600 mM NaOAc in 50 min then to 1 M NaOAc in 10 min, and re-equilibration with 150 mM NaOAc for 15 min.

Analytical methods

Galacturonic acid and total neutral sugar concentrations were measured by automated *meta*-hydroxy-

diphenyl¹⁰ and orcinol assays¹¹, respectively. Corrections were made for interference of uronic acids in the neutral sugars assay. Individual neutral sugars were analysed as their alditol acetate derivatives by gas chromatography¹² after 2 h hydrolysis by 2 M trifluoroacetic acid (TFA) at 120°C or 3 h hydrolysis by 1 M H₂SO₄ at 100°C¹³, preceded by 1 h pre-hydrolysis in 72% H₂SO₄ for de-esterified beet pulp and the residue from acid hydrolysis. Degrees of methylation (DM) and acetylation (DA) were calculated as molar ratios from the contents of methanol and acetic acid, respectively, determined according to the method described by Voragen *et al.*¹⁴, and of galacturonic acid.

Results

Isolation of rhamnogalacturonan oligosaccharides

De-esterified sugar-beet pulp, as reported previously⁸, was found to be rich in arabinose, glucose and galacturonic acid (234, 237 and 183 mg per g dry weight, respectively), with minor amounts of galactose and rhamnose (57 and 19 mg per g). Despite the de-esterification treatment, residual methanol (DM = 7) and acetic acid (DA = 5) could be detected. The residue from acid hydrolysis was enriched in glucose (533 mg per g), galacturonic acid (288 mg per g), xylose (22 mg per g) and mannose (26 mg per g), and almost devoid of rhamnose (3 mg per g), arabinose (1 mg per g) and galactose (0 mg per g). Enrichment in galacturonic acid of the residue is due to the insolubility of the homogalacturonic regions under acidic conditions¹. The hydrolysate (Table 1) was very rich in arabinose and rich in galactose and galacturonic acid. Sugar analysis without hydrolysis before derivatization showed that 82% of the arabinose and 50% of the galactose, but only 12% of the rhamnose, were present as monomers.

Ion-exchange chromatography of the acid hydrolysate (Figure 1a and Table 1) resulted in one major, non-retained fraction (fraction 1). This fraction contained the majority of all the neutral sugars, except rhamnose, which was mostly retained on the column. In fraction 1, rhamnose was mostly (85%) present in monomeric form. Six acidic peaks were obtained, of which five were analysed for their neutral sugar content, with the last

Table 1 Compositions (mol%) of the beet-pulp hydrolysate and its fractions. See Figure 1 for identification of the fractions

	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
Acid hydrolysate	5.6	0.4	65.4	1.7	0.2	12.7	0.6	13.4
Ion-exchange fractions								
1	1.0	0.5	79.0	2.0	0.2	14.8	0.9	1.7
2	7.2	0	0.8	6.1	0.5	33.3	1.1	51.1
3	33.2	2.8	0	2.3	1.1	10.7	1.3	48.6
4	49.0	0	0.5	0.3	1.3	0.9	0.5	47.6
5	18.4	0	0.3	0.3	0	0.8	1.2	79.0
6	11.2	0	0.2	0.3	0	0.4	1.2	86.7
Gel-permeation fractions								
4A	47.2	0	1.0	0.4	1.5	1.3	0.7	48.0
4B	53.5	0	0	0	1.1	0.6	1.6	43.3
4C	52.0	0	0.6	0	0.9	0.7	0	45.9
4D	48.5	0	0	0	1.3	0.6	0	49.7
4E	61.0	0	0.4	1.0	1.9	1.1	0.7	33.8

All values were obtained after TFA hydrolysis, except for the acid hydrolysate and fraction 1, for which the molar percentages were calculated from the concentrations found after TFA hydrolysis for rhamnose, mannose and glucose, and after H₂SO₄ hydrolysis for the other neutral sugars¹³

Figure 1 Fractionation of the acid hydrolysate from beet pulp: (a) on an AG 1X8 column (ion exchange); (b) on a BioGel P-4 column (gel permeation): (—) galacturonic acid; (-----) neutral sugars; (---) ammonium acetate buffer, pH 6

peak being almost devoid of neutral sugars. Rhamnose was the main neutral sugar of the retained fractions, except fraction 2 (galactose). Fractions 4 to 6 contained nearly only rhamnose and galacturonic acid, with an increasing proportion of galacturonic acid as the elution proceeded. Fraction 4, which was the main rhamnogalacturonic acid fraction and had a rhamnose:galacturonic acid ratio of ~ 1 , was further fractionated on BioGel P-4 (Figure 1b). Its gel-permeation pattern showed one purely acidic fraction, eluting at a K_{av} value of 0.75, and a series of rhamnogalacturonan oligosaccharides (Table 1). Fractions 4B to 4D were composed almost exclusively of rhamnose and galacturonic acid in $\sim 1:1$ molar ratio and were shown by HPAEC (Figure 2) to contain one major product. Fraction 4A contained a series of oligomers, as expected from the gel-permeation pattern. Fraction 4E, which had a higher rhamnose content, was composed of at least three oligomers. Except for fraction 4E, the BioGel P-4 and HPAEC patterns

Figure 2 HPAEC analysis of the rhamnogalacturonan oligosaccharides isolated from a sugar-beet pulp acid hydrolysate by chromatography on AG 1X8 columns (ion exchange) and on BioGel P-4 columns (gel permeation), and their reaction products with rhamnogalacturonase. A, B, C, D, E: patterns for fractions 4A, 4B, 4C, 4D and 4E, respectively, from Figure 1b. 0 h: fractions prior to incubation with rhamnogalacturonase. 1 h, 3 h, 6 h, 24 h: fractions after 1 h, 3 h, 6 h, 24 h incubation with rhamnogalacturonase. PAD: pulsed amperometric detection

point to the presence of a series of homologous rhamnogalacturonan oligosaccharides. These oligomers did not co-elute on the Carbo-Pak PA100 column with rhamnogalacturonan oligosaccharides generated

by rhamnogalacturonase treatment of 'modified hairy regions'¹⁵. Together with the known sensitivity to acid hydrolysis of glycosidic bonds¹⁶, this led us to propose the following structure: α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)-L-Rhap. Calibration of the column with oligogalacturonides indicated degrees of polymerization of 6 (4E), 8 (4D), 10 (4C) and 12 (4B) for the rhamnogalacturonan oligomers (i.e. 3, 4, 5 and 6 repeats, respectively, of a galacturonic acid-rhamnose repeating unit).

Action of rhamnogalacturonase on the rhamnogalacturonan oligosaccharides

The effect of rhamnogalacturonase on the rhamnogalacturonan oligosaccharides is shown in Figure 2. It was not active on oligomers 4E and 4D. Even after 24 h, fraction 4C showed only limited changes, which might actually be due to degradation of some contaminating material from fraction 4B. However, the rhamnogalacturonase had a marked effect on fractions 4B and 4A. The oligosaccharide from fraction 4B was cleaved to give three reaction products, two of which were identified by comparison with standards: peak 2 eluted at the same time as α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)-D-GalAp¹⁵, and peak 3 eluted at the same time as α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 4)-D-GalAp¹⁷. No standards were available for peak 1, but since an α -rhamnohydrolase¹⁷ could cleave it, releasing rhamnose, it probably represents the oligomer α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)-L-Rhap. The rhamnohydrolase¹⁷ also released rhamnose from peak 2, but was inactive with regard to peak 3. Reassembly of these oligosaccharides in a strictly alternating pattern of rhamnose and galacturonic acid gives the following order: 2-3-1, i.e. once again rhamnose was at the reducing end. Treatment of the mixture of oligomers in fraction 4A resulted in a limited number of reaction products, including those identified above and the higher homologue α -L-Rhap-(1 $\rightarrow$ 4)- α -D-GalAp-(1 $\rightarrow$ 2)- α -L-Rhap-(1 $\rightarrow$ 2)- α -D-GalAp (peak 4)¹⁵.

Conclusion

By controlled acid hydrolysis of de-esterified beet pulp, we obtained a series of rhamnogalacturonan oligosaccharides devoid of side chains. Rhamnogalacturonase was able to cleave the oligomers with a degree of polymerization ≥ 10 . Identification of the reaction products confirmed that the oligomers presented the strictly alternating structure which has already been

described for the rhamnogalacturonan regions of the backbone of pectins^{2,3}. Identification of the reaction products confirmed that these oligosaccharides presented inverted extremities compared to the oligomers produced by action of the rhamnogalacturonase on isolated 'modified hairy regions'¹⁵, i.e. they had a terminal galacturonic acid residue and a rhamnose residue at the reducing end. This was to be expected, as a neutral sugar to uronic acid bond is more sensitive to acid hydrolysis than a uronic acid to neutral sugar bond¹⁶. The time course studies, which showed proportional evolution of the peaks of all three reaction products for oligomer 4B (keeping in mind the widely varying response factors obtained during PAD detection), indicated that the enzyme cleaved the rhamnogalacturonan backbone simultaneously at two points. These results require confirmation by more detailed structural determinations and further kinetic studies.

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