Enzymatic degradation of cell walls of apples and characterization of solubilized products

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Partially depectinated apple cell walls were digested by pectin lyase or endoglucanase or a combination. By combining these commercial enzymes, a higher yield of 22.2% of carbohydrate material was obtained compared with only 13.9% and 5.7%, respectively, when using them singly. Only small amounts of carbohydrates were extracted by buffer (0.8%). The solubilized extracts were fractionated using a combination of ion-exchange chromatography and gel filtration. The individual subfractions were analysed for neutral sugar and uronic acid content. The results indicated the existence of a synergistic effect between pectin lyase and endoglucanase based on the percentage of material extracted.

Keywords: apple pectin; pectin lyase; endoglucanase

Plant cell walls are a complex of polymers such as polysaccharides, proteins and lignin mutually connected in a rigid structure. The chemical structure of the carbohydrate portion of cell walls is mostly established^{1,2}, but little is known of the cross-links that hold the cell wall polymers together³⁻⁶. Carbohydrate material can be extracted from the cell wall using non-degradative procedures (water, buffer etc.) and degradative procedures (acids, alkalis etc.)⁷. Over the last decade, more attention has been focused on polysaccharide-degrading enzymes (cellulases, hemicellulases or pectinases) because they specifically degrade given types of linkages and can provide more information about interconnections between individual cell wall polymers. Recently, synergism has been noticed in apple cell wall degradation using pectin lyase and endoglucanase⁸. This synergistic effect was mostly due to an increase in the extraction of 'hairy' regions of the pectin compounds. These results suggest the existence of a connection between pectins and xyloglucans, but detailed structural studies of the released carbohydrate material have not been performed. In addition, only the high-molecular-weight solubilized carbohydrate materials have been characterized. Therefore, we carried out new extractions in order to study the low-molecular-weight carbohydrate fractions arising from degradation of apple cell walls by pectin lyase or endoglucanase or a combination of them.

Experimental

Material

The apples (*Malus malus* L., var. Golden Delicious) were bought from a local supermarket in November 1993. 'Monocomponent' pectin lyase was purchased from Novo Nordisk (Denmark) and endoglucanase from Megazyme (Australia). Endoglucanase is a cellulase which can hydrolyse xyloglucan linkages. Both enzymes were assayed⁹ on polygalacturonic acid, arabinogalactan, galactan, arabinan and microcrystalline cellulose. In addition, pectin lyase was tested on citrus pectin and endoglucanase on xyloglucan and carboxymethylcellulose.

Preparation of partially depectinated cell wall material

Apples (2.5 kg) were peeled, cored and ground. The pulp (1800 g) was added to 2 vol 96% ethanol and boiled for 20 min. The cell wall material was collected by filtration, suspended in 70% ethanol (2 vol) and stirred at room temperature for 1 h. The procedure was repeated until the filtrate was sugar-free (five times).

Alcohol-insoluble solid was partially depectinated by stirring in 0.1 M sodium acetate buffer (4 litres), pH 4.5, for 1 h at room temperature. The process was repeated five times and the buffer-insoluble residue (BIR) was collected, washed with distilled water, then freeze-dried.

Enzymatic extractions

Enzymatic degradation of wall material (1% w/v) was carried out in 50 mM sodium acetate buffer (pH 4.5 containing 0.01% merthiolate) for 8 h at 40°C with

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mixing. The amounts of enzymes added were calculated based on the amount theoretically required to degrade the polymer present in 8 h under standard conditions. The suspensions were centrifuged for 10 min at 3000*g*. The pellets were washed with distilled water and freeze-dried. The supernatants were boiled for 10 min, concentrated, desalted on Sephadex G-10 and freeze-dried. A control experiment was performed under the same conditions but in the absence of enzyme.

Analytical methods

The isolated carbohydrates were analysed after prehydrolysis by 13 M sulfuric acid for 1 h followed by hydrolysis with 1 M sulfuric acid at 100°C for 3 h or with 2 M trifluoroacetic acid at 120°C for 1 h. The uronic acid and neutral sugar content was determined by automated meta-hydroxydiphenyl¹⁰ and orcinol assays¹¹, respectively. The quantitative determination of the neutral sugars was carried out in the form of their alditol acetates¹² after gas chromatography.

Chromatographic methods

Gel-filtration chromatography was performed on a column (1.5 × 95 cm) of Sephacryl S 500. The samples (5 mg in 0.5 ml) were loaded on the column and eluted ascendingly with 0.1 M sodium acetate buffer, pH 4.5, at a flow rate of 18 ml h⁻¹. Fractions of 3 ml were collected and analysed for uronic acid and neutral sugar content.

Ion-exchange chromatography was performed on a column (2.5 × 20 cm) of DEAE-Sepharose CL-6B in acetate form. The extracts (blank 8 mg, pectin lyase 150 mg, endoglucanase 50 mg and pectin lyase plus endoglucanase 150 mg) were applied to the column and eluted with water. The retained material was washed with linear gradients of water to 0.1 M sodium acetate buffer, pH 4.5, then 0.1 to 1 M buffer, and finally with 1 M buffer. Fractions of 10 ml were collected and assayed for uronic acid and neutral sugar content. Appropriate fractions were combined, desalted on Sephadex G-10 and freeze-dried.

Results and discussion

Yields and composition of the extracts

Pectin lyase showed only low side activities on arabinogalactan, galactan, arabinan and microcrystalline cellulose and no activity on polygalacturonic acid. Endoglucanase showed low side activities on arabinogalactan and microcrystalline cellulose and no activity on polygalacturonic acid, galactan and arabinan. The starting material for enzymatic degradation (buffer-insoluble residue) was rich mainly in glucose (40.7%) and galacturonic acid (22.4%). It also contained arabinose (13.8%), xylose (10.1%), galactose (7.1%) and small

amounts of rhamnose (2.4%), mannose (2.0%) and fucose (1.5%). The yields and sugar composition of the released carbohydrate extracts after treatment of apple cell walls with enzymes are shown in Table 1. By combining

Figure 1 Sephacryl S 500 chromatography of the extracted carbohydrates (eluted by 0.1 M sodium acetate buffer, pH 4.5): (■) galacturonic acid; (□) neutral sugars

Table 1 Yield (% BIR) and sugar composition (mol%) of the carbohydrates extracted from the buffer-insoluble residue (BIR) by enzymes

	Yield	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
BIR	—	2.4	1.5	13.8	10.1	2.0	7.1	40.7	22.4
Blank	0.8	4.7	0.0	22.9	4.1	0.0	5.4	3.6	59.3
Pectin lyase	13.9	4.3	0.4	28.1	4.9	trace	7.5	1.1	53.7
Endoglucanase	5.7	1.9	4.1	6.8	20.1	trace	8.0	29.6	29.5
Pectin lyase + endoglucanase	22.2	4.8	1.9	27.6	13.3	trace	10.2	12.1	30.1

Table 2 Yield (mg) and sugar composition (mol%) of Sephacryl S 500 fractions of the extracts

	Fraction	Yield	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
Blank			4.7	0.0	22.9	4.1	0.0	5.4	3.6	59.3
Pectin lyase	a	1.3	9.6	0.3	55.9	7.0	0.0	8.3	0.9	18.0
	b	3.5	1.6	0.3	3.8	0.7	0.3	4.0	1.0	88.3
Endoglucanase	a	1.6	2.1	0.6	8.9	2.6	0.3	3.5	3.8	78.2
	b	3.3	1.1	8.0	1.9	30.1	2.2	11.2	41.0	4.5
Pectin lyase + endoglucanase	a	1.7	9.9	0.5	53.3	9.2	0.0	9.9	1.3	15.9
	b	3.2	0.9	2.7	2.7	14.0	1.4	7.4	17.5	53.4

Figure 2 Ion-exchange chromatography on DEAE-Sephacryl S 500 of the extracted carbohydrates: (■) galacturonic acid; (□) neutral sugars; --- molarity of sodium acetate buffer (pH 4.5)

pectin lyase and endoglucanase, a yield of 22.2% of carbohydrate material was obtained compared with only 13.9% and 5.7% achieved by separate pectin lyase and endoglucanase treatments, respectively. Only 0.8% of carbohydrate material was solubilized when using the buffer only. The amounts of released carbohydrates were much lower than those reported by Renard *et al.*¹³, probably due to their use of purified enzymes. Material rich in galacturonic acid and arabinose was extracted using the buffer. The pectin lyase had a marked effect on the degradation of the cell wall. The released pectin material was rich in galacturonic acid, arabinose and galactose. Degradation of the cell wall by endoglucanase was lower than by pectin lyase. This extract contained mainly glucose, xylose and galacturonic acid. The combined pectin lyase and endoglucanase extract was rich in galacturonic acid, arabinose, xylose, glucose and galactose.

Gel-filtration chromatography

Sephacryl S 500 chromatography of the extracts showed the range of their molecular distribution (Figure 1). The blank contained mostly high-molecular-weight material. The endoglucanase extract was composed of two fractions. The high-molecular-weight material (a) was rich in galacturonic acid (Table 2) while the low-molecular-weight material (b) was rich in neutral sugars (glucose, xylose, galactose and fucose). The pectin lyase extract also contained two fractions. The high-molecular-weight material (a) was rich in neutral sugars while the low-molecular-weight material (b) was rich in galacturonic acid. In the combined pectin lyase and endoglucanase extract, the size and sugar composition of the high-molecular-weight fraction (a) were similar to that of the high-molecular-weight fraction of the pectin lyase extract (Figure 1). The second fraction (b) showed the low-molecular-weight products of the two enzymes. It contained mainly galacturonic acid, glucose, xylose and galactose.

Ion-exchange chromatography

Ion-exchange chromatography of the extracts showed their heterogeneous character (Figure 2). The blank was composed of two fractions. The non-retained fraction was very small and rich in neutral sugars while the retained fraction was rich in galacturonic acid (Table 3). The endoglucanase extract showed two main fractions. The non-retained peak (A) was composed of glucose, xylose, galactose and fucose. A very small retained fraction (B) contained mainly galacturonic acid, xylose, arabinose and rhamnose. The main retained peak (C) was acidic material that was poor in neutral sugars. The tail of that peak (D)

Table 3 Yield (mg) and sugar composition (mol%) of the ion-exchange fractions

	Fraction	Yield	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
Blank	A	0.3	1.5	2.1	43.0	12.1	4.2	10.5	23.3	3.4
	B	7.4	3.3	0.0	11.4	1.5	0.0	3.4	0.0	80.4
Pectin lyase	A1	5.1	0.0	1.8	26.9	9.3	0.0	44.9	13.6	3.4
	A2	29.7	0.2	0.0	1.7	0.5	0.2	4.6	0.8	92.0
	B	14.6	0.3	0.0	1.3	2.5	0.2	0.9	0.6	94.2
	C	21.8	1.5	0.0	12.0	2.4	0.0	2.6	0.4	81.1
	D	24.5	3.8	0.0	29.5	4.1	0.0	4.4	0.0	58.1
	E	52.7	7.6	0.0	30.4	3.7	0.0	5.1	0.0	53.3
Endoglucanase	A	32.0	0.0	6.3	2.4	32.1	2.5	10.9	45.8	0.0
	B	0.5	13.9	0.0	11.1	21.9	0.0	4.7	17.7	31.6
	C	12.9	4.3	0.0	7.5	3.8	0.0	1.9	0.0	80.0
	D	2.6	4.6	0.0	11.7	1.3	0.0	3.3	0.0	79.1
Pectin lyase + endoglucanase	A	45.5	1.8	4.4	12.1	25.5	2.0	14.9	32.3	6.9
	B	15.3	0.4	0.0	2.5	4.6	0.2	2.0	1.5	88.8
	C	22.1	4.6	0.0	35.2	7.4	0.0	7.1	0.0	45.7
	D	16.7	5.1	0.0	35.4	7.1	0.0	6.7	0.0	45.7
	E	14.0	6.8	0.0	36.2	6.5	0.0	6.2	0.0	44.3
	F	33.4	11.2	0.0	35.9	6.8	0.0	7.6	0.0	38.5

had a similar sugar composition. It seems that fraction (C) was similar to the retained material solubilized by buffer (B). The pectin lyase extract showed six peaks: two non-retained and four retained fractions. The first non-retained fraction (A1) was negligible and rich in neutral sugars. The main non-retained fraction (A2) contained almost exclusively galacturonic acid. Peaks B and C were composed of acidic material poor in neutral sugars. The last fractions (D and E) were rich in galacturonic acid and arabinose. The combined pectin lyase and endoglucanase extract showed an elution pattern similar to that obtained with pectin lyase, but the proportions of arabinose, rhamnose and galactose in the retained fractions B–E were higher. The non-retained fraction (A) was rich in neutral sugars such as galactose, xylose, glucose and arabinose. These elution patterns were different from those reported by Renard *et al.*¹³, because they had eliminated the low-molecular-weight material by dialysis prior to ion-exchange.

From these results, it is evident that the endoglucanase is not able to release pectin material from cell walls itself without using pectolytic enzymes. Combining the endoglucanase with pectin lyase increased the degradation of pectic compounds from the apple cell walls. These results suggest the possible existence of a connection between pectin and xyloglucan polymers in the cell wall. More light will be shed on this problem by detailed structural characterization of the released products, especially the low-molecular-weight products. This will be discussed in a subsequent paper.

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