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Studies on Extraction of Pectins from Citrus Peels, Apple Marks and Sugar-beet Pulps with Arabinanase and Galactanase

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ABSTRACT

Pectin was extracted from citrus, apple and sugar-beet pulps, with an enzyme preparation from Bacillus subtilis. *The preparation contained* endo-*arabinanase,* endo-*galactanase and residual* endo-*pectate lyase. Extraction conditions were 30°C and 0·03 M sodium acetate buffer, pH 5·0, so that pectate lyase activity was minimised. Under these conditions, an appreciable amount of pectin (4·9% by the buffer and 10·8% by the enzymes) could be extracted from citrus pulp, but very little from apple and sugar-beet pulps. The citrus pectin had a relatively low molecular weight (47000) compared to acid-extracted pectin (82000). The breakdown may be due to either enzymatic or chemical β-elimination. Significant amounts of pectin (12·9%) can also be extracted from citrus pulp with water and chelating agents. It was concluded that pectins cannot efficiently be extracted with these enzymes.*

INTRODUCTION

Pectins have properties such as gelation and stabilisation which make them useful in the food industry. They are commercially extracted from the wastes of the fruit juice industry such as citrus peels and apple marks.

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The extraction with acid is most often used: nitric acid or sulphuric acid is added to a heated slurry of the wastes until a pH of about 2·0 is reached. Pectins are then released and can be precipitated with alcohol after filtration. The yields are 5–10% of the initial product (Pilnik *et al.*, 1980).

This acid extraction has several disadvantages such as water pollution, corrosion and sometimes filtering problems due to the disintegration of the cell walls. Furthermore, the action of the acid may result in a breakdown of the polymer chains, which is undesirable, as the degree of polymerisation is one of the most important parameters of a commercial pectin. It is clear that specific extraction with enzymes could be advantageous and that some of the above-mentioned disadvantages could be solved by this approach. In earlier attempts to extract pectin from the cell walls with enzymes, 'protopectinase'-producing microorganisms, or *endo*-polygalacturonase have been used (Sakai & Okushima, 1978, 1980; Sakai & Yoshitake, 1984). The problem, however, is when to stop the enzyme, because a protracted reaction time will result in a pectin with a low degree of polymerisation.

According to the model of the cell wall proposed by Keegstra *et al.* (1973), hydrolysis of the galactan and/or arabino-galactan side-chains will result in the release of the rhamnogalacturonan. By using specific enzymes, namely *endo*-arabinanases and *endo*-galactanases, which must be free of pectinase activity, it should be possible to obtain pectins with high molecular weights and low contents of neutral sugars. This method of releasing pectins from citrus peels, apple marks and sugar-beet pulp has been investigated.

MATERIALS AND METHODS

Raw materials

Dried apple pulp (Obi Pektin, Switzerland), dried citrus pulp (Copenhagen Pektin Fabrik, Denmark) and pressed sugar-beet pulp, stored under ethanol (Générale Sucrière, Eppeville, France) were used as raw materials. Before use, the latter was washed with acetone and ether on a glass filter and allowed to dry at $< 35°C$. All the pulps were ground in an IKA-mill for 1·5 min and then passed over a 1 mm sieve.

Enzymes

Two enzyme preparations (A and B) were obtained from *Bacillus subtilis* 34 (Rombouts *et al.*, 1984). They contained *endo*-galactanase, *endo*-arabinanase and residual pectate lyase activities (Table 1).

TABLE 1
Maximum Activities (mU ml^{-1}) Contained in the Enzyme Preparations from *Bacillus subtilis* 34

Preparation	endo-*Arabinanase*	endo-*Galactanase*	endo-*Pectate lyase*
A	14·5	6·0	20·0
B	22·7	24·8	40·0

Galactanase and arabinanase activities were measured according to Rombouts *et al.* (1988). In standard conditions for pectate lyase, the reaction mixture contained enzyme, sodium polygalacturonate (0·1%), 0·05 M glycine–NaOH buffer (pH 9·4), 0·25 mM $CaCl_2$. The increase in absorbance at 235 nm was measured at 30°C and the units were calculated using a molar extinction coefficient of 4800 M^{-1} cm^{-1} (Rombouts *et al.*, 1978). Pectate lyase activity was also measured in the conditions used for the enzymic extraction of pectins, i.e. with different buffers and without calcium addition.

Chemical extraction of the pectins

Each of the following extractions was repeated three times. The ground pulps were stirred with distilled water (50 ml g^{-1} of pulp) for 30 min at room temperature. The slurry was centrifuged at 3000 *g* for 10 min and the supernatant containing the water-soluble pectins was collected. The water-insoluble residue was mixed for 30 min with buffer (0·1 M succinate–oxalate pH 6·0, or 0·03 M acetate pH 5·0, see below; 60 ml g^{-1} of residue) and centrifuged. The supernatant containing the buffer-soluble pectins was collected. The buffer-insoluble residue was heated with 0·05 N HCl (57 ml g^{-1} of residue) for 30 min at 85°C, then cooled and centrifuged, leading to a supernatant containing the acid-soluble pectins.

The supernatants were concentrated on a rotary evaporator at a temperature below 40°C and precipitated by the addition of four volumes of 96% ethanol. The precipitates were collected and dried by solvent exchange. When necessary, residues were also collected and dried by the same procedure and submitted to enzymic extraction.

Enzymic extractions

For all the enzymic extractions, merthiolate, as antimicrobial agent, was added to a final concentration of 0·01%.

The release of galacturonic acids and neutral sugars as a function of time was studied as follows. In a centrifuge tube, exactly 100 mg of the

pulp to be examined was placed and 17 ml of buffer added. At time zero, 3 ml of the enzyme preparation A or 2 ml of the enzyme preparation B were added, or the same quantity of water for the control. The centrifuge tubes were incubated at 30°C, and the mixtures were magnetically stirred. At certain time intervals, the mixture was centrifuged at 8000 *g* for 10 min and an aliquot of the supernatants was analysed as described below.

The final enzymic extraction of pectins from citrus pulp was carried out on a residue, insoluble in 0·03 M acetate buffer, pH 5·0. Enzyme preparation B (45 ml) in the same buffer was added to 2·25 g of the residue; the pH was adjusted to 5·0. Incubation took place at 30°C for 12 h, 24 h and 48 h, while the slurry was continuously stirred. The mixtures were centrifuged at 8000 *g* for 10 min, the supernatants were concentrated, dialysed for three days at 4°C against distilled water and added to four volumes of ethanol. The precipitates were dried by solvent exchange.

Purification and analysis of the pectins

Pectins were purified by precipitation with copper acetate (Rombouts & Thibault, 1986). The anhydrogalacturonic acid content of pectins was determined with the automated *m*-hydroxydiphenyl method (Thibault, 1979) and their neutral sugar content (expressed as anhydroarabinose) was estimated with the automated orcinol method (Tollier & Robin, 1979) after corrections for galacturonic acid interference. These methods were also used in studying the kinetics of the release of pectins from pulps with enzymes.

For the determination of neutral sugar residues, pectins were hydrolysed with 2 M trifluoroacetic acid for 1 h at 120°C. The liberated sugars were reduced, acetylated and analysed by gas liquid chromatography (Albersheim *et al.*, 1967). The amounts of methyl and acetyl groups were measured by HPLC on an Aminex HPX 87H column after de-esterification of the pectins with 0·4 M NaOH in 50% aqueous isopropanol (Voragen *et al.*, 1986). Unsaturated uronides were detected by the thiobarbituric acid method (Weissbach & Hurwitz, 1959) and by UV absorption. Viscosity-average molecular weights were calculated (Owens *et al.*, 1946) from the intrinsic viscosity as measured in 0·155 M NaCl at 25°C with an automatic viscometer (Amtec, Villeneuve-Loubet, France). The amount of proteins was measured by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Crude pectins were chromatographed on a column (11·0 cm × 1·6 cm) of DEAE Sephacel (Pharmacia, Uppsala, Sweden). After equilibrating

with sodium acetate buffer (pH 4·8, ionic strength 0·03 M), the column was loaded with sample (5 ml of a 1 mg ml^{-1} pectin solution) and washed for 1 h with the same buffer. Elution of the bound materials was done with a linear gradient of 90 ml of sodium acetate buffer (pH 4·8, ionic strength 0·03–0·06 M). The flow rate was 45 ml h^{-1}. Fractions (5 ml) were assayed for galacturonosyl and neutral sugar residues, as described above.

Gel chromatography of purified pectins was carried out on Sepharose CL-2B (Pharmacia). Pectin solution (2·5 ml, 1·5 mg ml^{-1}) was loaded onto a column (98·5 cm × 1·6 cm) and eluted at a flow rate of 20 ml h^{-1} with a sodium acetate buffer (pH 4·0, ionic strength 0·1 M). Fractions (4 ml) were assayed for galacturonosyl and neutral sugar residues. The void volume (V_0) and the total volume (V_t) of the column were determined with amylopectin and glucose, respectively. The K_{av} values of the fractions were calculated as $(V_e - V_0)/(V_t - V_0)$, V_e being the elution volume of the fraction considered.

The elution profiles were drawn for 5 mg (DEAE Sephacel) or 10 mg (Sepharose CL-2B) of pectin injected.

RESULTS

Determination of the optimal conditions for enzymic extraction of pectins

Because of the presence of residual pectate lyase activity in the enzyme preparations, special attention was paid to the experimental conditions for the extraction of pectins. As pectate lyase needs calcium to be active, one way to inhibit the activity could be the use of chelating agents. But these agents also lead to the extraction of pectins. For example, 0·1 M oxalate–succinate buffer at pH 6·0 and 30°C released 9·5, 6·6 and 2·9% of pectins from water-extracted pulps of citrus, apple and sugar-beet, respectively. It can be noted that water also had a slight effect, solubilising 3·4, 3·9 and 0·8% of pectins, respectively. Moreover, the enzymes, when acting on these water- and buffer-extracted materials, liberated only a very small amount of additional material, up to 2% of anhydrogalacturonic acid for sugar-beet pulp. These results show that it is not possible to use a chelating agent in the buffer.

Therefore, the residual pectate lyase activity (optimum pH 9·4) must be inhibited only by changes in the pH value and a balance must be found between the inhibition of the enzyme and the extraction of pectins. For these reasons, various conditions of pH and ionic strength for acetate buffers were used for the extraction and the pectate lyase activity

was measured under these conditions (Tables 2 and 3). Table 2 shows that the pH of the buffer must be higher than 4 and that increasing of the pH results in the release of larger amounts of anhydrogalacturonic acids, as does decreasing of the ionic strength, up to an optimum of approximately 0·03 M. Hence the optimum conditions for enzymatic extraction seem to be (Table 2) pH 5·6 and ionic strength 0·03 M. The extraction of pectins from citrus pulp gave good results, while the extraction from apple and sugar-beet pulps was very poor. After five days under the

TABLE 2

Anhydrogalacturonic Acids Released during Incubation of Pulp Samples with Enzymes at 30°C Using Sodium Acetate Buffer Differing in pH and Ionic Strength (Figures Given are Percentages of Starting Material)

		Citrus		*Apple*		*Sugar-beet*	
		2 days	*5 days*	*2 days*	*5 days*	*2 days*	*5 days*
0·2 M pH 4·0	Control	0·2	0·3	0·9	1·0	0·4	0·6
	Preparation A	0·5	0·5	0·9	1·1	0·4	0·8
0·2 M pH 5·0	Control	0·4	0·4	1·0	1·2	1·0	1·5
	Preparation A	2·7	5·2	1·5	2·0	1·5	2·5
0·2 M pH 5·6	Control	0·5	0·5	1·0	1·5	1·0	1·0
	Preparation A	4·0	6·5	1·5	2·5	1·5	1·6
0·1 M pH 4·0	Control	0·5	0·6	0·8	1·0	0·4	0·8
	Preparation A	0·5	0·8	0·9	1·4	0·8	1·0
0·1 M pH 5·0	Control	0·6	1·0	1·0	1·2	1·0	1·5
	Preparation A	4·2	7·7	1·8	2·1	2·3	2·7
0·1 M pH 5·6	Control	0·8	1·0	2·0	1·5	1·0	2·0
	Preparation A	7·0	11·5	2·2	2·7	2·0	3·1
0·05 M pH 4·0	Control	0·6	0·7	0·8	1·2	0·3	0·6
	Preparation A	0·8	1·0	1·0	1·4	0·6	1·0
0·05 M pH 5·0	Control	0·7	0·8	1·0	1·2	0·7	1·6
	Preparation A	4·2	8·1	1·8	2·2	2·1	3·5
0·05 M pH 5·6	Control	0·9	1·1	1·0	1·2	0·9	2·0
	Preparation A	7·7	12·0	2·1	3·0	2·6	4·0
0·03 M pH 5·6	Control	1·0	1·0	0·8	1·0	0·8	1·0
	Preparation A	9·0	12·8	2·5	2·8	2·5	2·9
	Preparation B	10·2	14·4	2·6	4·0	2·6	3·2
0·01 M pH 5·6	Control	1·0	1·0	1·1	1·2	0·8	1·0
	Preparation A	8·0	10·5	2·5	2·5	2·5	3·5

optimum conditions, the amount of anhydrogalacturonic acid was 12·8, 2·8 and 2·9% of the initial weight of citrus, apple and beet pulps, respectively. Under these conditions, enzyme preparation B liberated more anhydrogalacturonic acid than enzyme preparation A, which is in agreement with its higher pectate lyase activity (Table 2).

However, this 0·03 M acetate buffer at pH 5·6 was not used for further studies. As the absence of pectate lyase activity is absolutely necessary to obtain pectins with a high molecular weight, the action of the enzyme preparation on pectate was studied using different buffers, with or without the addition of supplementary calcium ions. The activities found for several buffers are summarised in Table 3. Using the sodium acetate buffers, some activity was found at pH 5·6 and none at pH 5·0 without additional calcium. It was concluded from these results that enzymatic extractions could be best performed at pH 5·0.

Studies on enzymatically extracted pectins from citrus

Citrus pulp was first extracted with the sodium acetate buffer 0·03 M pH 5·0, and from the residue pectin was extracted either with acid or enzymatically. After concentration of the supernatant, the pectins were precipitated, washed with alcohol, acetone and ether, and dried. The yield of pectins obtained after the different extractions, the anhydrogalacturonic acid content and neutral sugar content of these pectins are indicated in Table 4. It must be noted that the 0·03 M acetate buffer pH 5·0 extracted less pectins than the 0·1 M oxalate–succinate buffer pH 6·0. Table 4 shows that the yield of pectin after incubation with enzymes for 12 h is rather low, especially as compared to the amount of anhydrogalacturonic acid found before the alcoholic precipitation. This indicates

TABLE 3
Activity of Pectate Lyase in Preparation A in Different Buffers

Buffer	*Activity (mU ml^{-1})*
Oxalate–succinate 0·1 M pH 6·0	0
Oxalate–succinate 0·1 M pH 6·0[a]	18·0
Sodium acetate 0·03 M pH 4·5[a]	0
Sodium acetate 0·03 M pH 5·0[a]	0·2
Sodium acetate 0·03 M pH 5·0	0
Sodium acetate 0·03 M pH 5·6[a]	2·5
Sodium acetate 0·03 M pH 5·6	0·7

[a] Ca^{2+} added.

TABLE 4
Yield of Pectins Obtained from Citrus Pulp, Anhydrogalacturonide (AGA) Content and Neutral Sugar Content of these Pectins

Extraction medium and conditions	*Yield (%)*[a]	*AGA content (%)*[b]	*Neutral sugar (%)*[b]
Sodium acetate buffer, 0·03 M pH 5·0	4·9	32·5 (0·5)	15·1 (0·8)
Acid	24·1	47·6 (0·6)	10·5 (0·9)
Enzymes, 12 h	1·3	35·9 (1·2)	8·2 (0·3)
Enzymes, 24 h	9·5	36·8 (1·8)	8·1 (0·4)
Enzymes, 48 h	10·8	50·0 (0·7)	4·9 (0·2)
Control	0·4	22·5 (1·1)	6·0 (0·5)

[a] Percentages are based on the initial pulp.
[b] Values in parentheses are standard deviations (five determinations).

the enzyme preparation released small fragments of pectins which are lost during the dialysis or which cannot be precipitated.

Enzymatically extracted pectins had an anhydrogalacturonic acid content which increased with the course of extraction, while the neutral sugar content decreased. The pectins extracted with buffer, acid and enzymes were chromatographed on DEAE Sephacel. The chromatography patterns are shown in Fig. 1. Recovery values for anhydrogalacturonic acids higher than 80% were found except for the buffer-extracted pectin, which gave a recovery of about 10%. This result suggests that these pectins have a low degree of methylation. The pectins extracted with acid and with the enzymes after 12 h were characterised by two peaks eluted during the gradient. In contrast, the pectins extracted with enzymes for a longer time tended to be eluted in a single peak for both the anhydrogalacturonic acid and the neutral sugars. All the pectins tested contained some neutral polysaccharides which were not bound to the gel and which amounted to 5–10% of the initial weight, indicating that a purification step was necessary.

Four pectins were purified by copper precipitation: buffer-soluble pectin, acid-soluble pectin and pectin solubilised with enzymes after 24 h and 48 h of time of reaction. Of the other two pectins ('Enzymes, 12 h' and 'Control', Table 4), sufficient material was not available. The

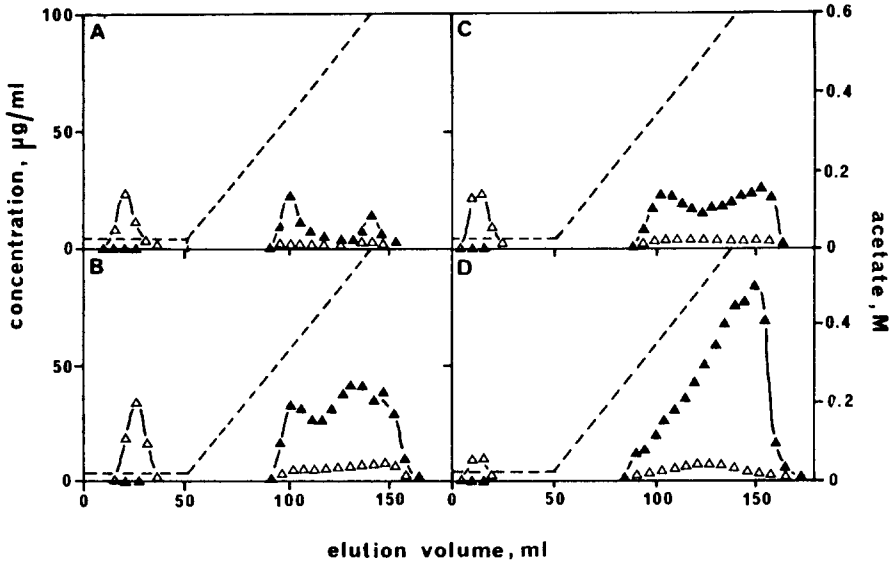

Fig. 1. Ion-exchange chromatography on DEAE Sephacel of citrus pectins extracted with buffer (A), with acid (B), with enzymes after 12 h (C), and after 48 h (D). (▲) anhydrogalacturonic acid, (△) neutral sugars.

TABLE 5
Characteristics of the Purified Citrus Pectins

	Extraction medium and conditions			
	Buffer	*Acid*	*Enzymes (24 h)*	*Enzymes (48 h)*
Galacturonic acid	58·5 (1·0)[a]	66·3 (3·5)[a]	56·7 (2·0)[a]	59·3 (1·7)[a]
Rhamnose	0·8	0·6	0·6	0·3
Arabinose	5·4	1·9	1·6	1·4
Xylose	0·3	0·2	0·2	0·2
Mannose	0·2	0·1	0·1	0·1
Galactose	2·9	2·7	0·9	0·7
Glucose	0·3	0·2	0·5	0·2
Proteins	9·4	11·5	13·7	8·4
Degree of methyl-esterification	28·3	79·1	82·3	nd
Degree of acetyl-esterification	2·9	2·0	< 1	1·5
Intrinsic viscosity (ml g^{-1})	352	533	314	252
Viscosity-average molecular weight	59 800	81 500	54 800	46 500

nd = Not determined.
[a] Values in parentheses are standard deviations (five determinations).

recoveries for pectins after purification were 42%, 42%, 27% and 31%, respectively, which indicated an important loss of material during the purification procedure. In our previous experience (Rombouts & Thibault, 1986) of purification of pectins with copper acetate, such losses were not encountered.

The chemical characteristics of these pectins were determined (Table 5). The anhydrogalacturonic acid content was increased by purification, in contrast with the neutral sugar content. The substantial increase in anhydrogalacturonic acid indicates that the greatest part of the losses during purification was not pectinaecous in nature. It is noted that for the four pectins a low neutral sugar content was found, and that the contents in arabinose and in galactose decreased with the course of enzymatic extraction, in contrast with mannose, xylose and glucose content, which remained constant. In all these pectins arabinose and galactose are the major neutral sugars. The amounts of arabinose and galactose decreased after enzymatic extraction, showing that the enzymes were active on these pectins.

As expected from the results obtained by DEAE chromatography, the degree of methylesterification of the buffer-extracted pectin is rather low. In contrast, the other pectins extracted either with acid or with enzymes are highly methylated. In all these pectins, acetic acid was found in low but significant amounts, and the corresponding degrees of acetylation (as moles of acetic acid per mole of anhydrogalacturonic acid) were in the range 1–3%.

The viscosity-average molecular weight of enzymatically extracted pectins is lower than for pectin extracted with acid and it seems that this molecular weight decreases further in the course of enzymatic extraction.

Gel filtration was performed with the four purified pectins and the chromatograms are shown in Fig. 2. Also from this figure it is clear that the peak for enzymatically extracted pectin shifts towards a higher K_{av} value, indicating a lower degree of polymerisation. From these results, it seems that although the conditions during enzymatic extraction were chosen to be unfavourable for pectate lyase activity, either this was still not sufficient to suppress this activity completely, or chemical β-elimination occurred under these conditions (Aspinall & Cottrell, 1970).

Two tests were performed to measure the amount of unsaturated uronides: neither of these methods can distinguish between chemical and enzymatic β-elimination. With the UV test no peaks were recorded between 220 and 260 nm. In contrast, small peaks at 550 nm were found in the thiobarbituric acid test for the enzymatically extracted pectins, and it can be calculated that these pectins contain less than 1·6% of unsaturated galacturonic residues, which could account for the drop in

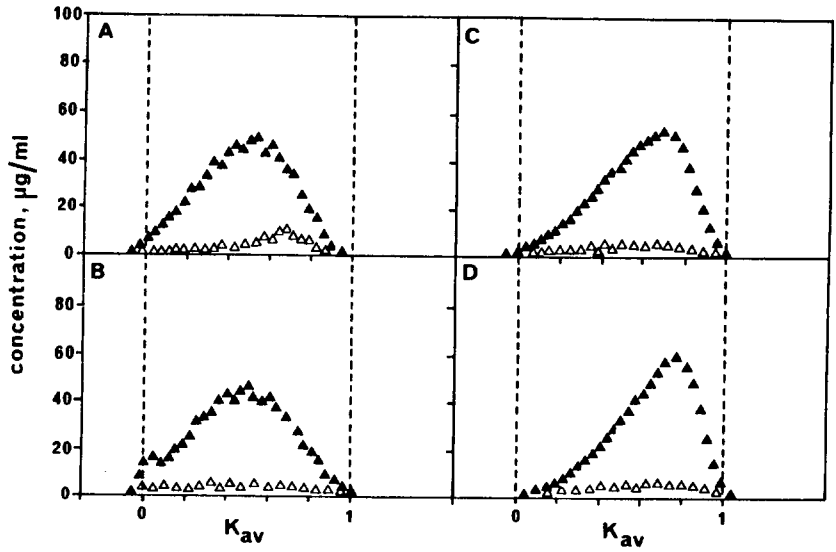

Fig. 2. Sepharose CL-2B chromatography of citrus pectins extracted with buffer (A), with acid (B), and with enzymes after 24 h (C), and after 48 h (D). (▲) anhydrogalacturonic acid, (△) neutral sugars.

molecular weight of these pectins. A similar level of β-elimination must have occurred during the enzymatic extraction of apple and sugar-beet pulps, but very little pectin was extracted at all from these two pulps (Table 2).

With citrus pulp the amount of pectin enzymatically extracted is not much higher than that extracted with chelating agents (15·7% including the effect of the buffer and 12·9% including the effect of water, respectively) and with apple pulp chelating agents extract even more pectin than enzymes (10·5% and 4·0%, respectively). The fact that pectins from citrus pulp were readily extracted with enzymes and buffers in contrast to those of apple or sugar-beet pulps can possibly be ascribed to differences in the fine structure of their neutral sugar side-chains, although the neutral sugar compositions are very similar (Rombouts & Thibault, 1986; De Vries *et al.*, 1984). Another reason for differences in ease of pectin extraction could be possible differences in the drying processes used for the industrial preparation of the citrus or apple pulps. Finally, oxidation and condensation of phenolic substances, which is particularly evident in beet and apple pulps (browning), may have an effect on pectin extraction.

From these results, the conclusion seems justified that pectins cannot be efficiently extracted with *endo*-arabinanase and *endo*-galactanase.

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