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Extraction of Green Labeled Pectins and Pectic Oligosaccharides from Plant Byproducts

AGATA ZYKWINSKA,[†] MARIE-HÉLÈNE BOIFFARD,[†] HANNA KONTKANEN,[‡] JOHANNA BUCHERT,[‡] JEAN-FRANÇOIS THIBAULT,[†] AND ESTELLE BONNIN*,[†]

INRA, UR1268 Biopolymères Interactions Assemblages, F-44300 Nantes, France, and VTT Technical Research Centre of Finland, P.O. Box 1000, FIN-02044 VTT, Finland

Green labeled pectins were extracted by an environmentally friendly way using proteases and cellulases being able to act on proteins and cellulose present in cell walls. Pectins were isolated from different plant byproducts, i.e., chicory roots, citrus peel, cauliflower florets and leaves, endive, and sugar beet pulps. Enzymatic extraction was performed at 50 °C for 4 h, in order to fulfill the conditions required for microbiological safety of extracted products. High methoxy (HM) pectins of high molar mass were extracted with three different enzyme mixtures. These pectins were subsequently demethylated with two pectin methyl esterases (PMEs), either the fungal PME from *Aspergillus aculeatus* or the orange PME. It was further demonstrated that high molar mass low methoxy (LM) pectins could also be extracted directly from cell walls by adding the fungal PME to the mixture of protease and cellulase. Moreover, health benefit pectic oligosaccharides, the so-called modified hairy regions, were obtained after enzymatic treatment of the residue recovered after pectin extraction. The enzymatic method demonstrates that it is possible to convert vegetable byproducts into high-added value compounds, such as pectins and pectic oligosaccharides, and thus considerably reduce the amount of these residues generated by food industries.

KEYWORDS: Enzymatic extraction; byproduct upgrading; cellulase; protease; cell wall polysaccharides

INTRODUCTION

Primary cell walls of fruits and vegetables are a rich source of pectins, hemicelluloses, and cellulose. Among all primary cell wall components, pectins seem the most complex and heterogeneous because of the presence of distinctive covalently linked domains. According to the most cited pectin model (*1*), the pectin backbone is mainly composed of two structural domains: homogalacturonan (HG) and type I rhamnogalacturonan (RG I). HG constitutes a linear chain composed of (1→4)-linked α-D-GalA*p* units that are often methyl-esterified at *O*-6 and sometimes acetyl-esterified at *O*-2 or *O*-3 (*2*). RG I contains a backbone of the repeating disaccharide unit: (1→2)-α-L-Rha*p*-(1→4)-α-D-GalA*p* (*3*) that is predominantly substituted at *O*-4 of Rha*p* residues by neutral sugar side chains, among which arabinan and galactan are the most abundant (*4*). Arabinan side chains are composed of (1→5)-α-L-Ara*f* residues, which can be branched by α-L-Ara*f* units attached at *O*-2 and/or *O*-3, whereas galactan side chains constitute (1→4)-linked β-D-Gal*p* units. A type II rhamnogalacturonan (RG II), a complex polysaccharide composed of GalA, Rha, Gal, and some unusual sugars, may also constitute a part of the pectin molecule (*5*). Besides pectin, the other major polysaccharide of the primary cell wall is cellulose. The cellulosic linear chains are composed of (1→4)-linked β-D-Glc*p* residues, which are tightly linked by hydrogen bonds to form microfibrils. Xyloglucan, the most abundant hemicellulosic polysaccharide in some primary cell walls of dicotyledons, is composed of a cellulose-like backbone, branched at *O*-6 by α-D-Xyl*p* residues, which can be further substituted at *O*-2 by β-D-Gal*p* residues (*6*). Some of the Gal residues may further be substituted at *O*-2 by α-D-Fuc*p* residues. Although cell walls are mostly composed of polysaccharides, structural proteins may also be present and form networks. Four major classes of structural proteins can be distinguished: the hydroxy-proline-rich glycoproteins (HRGPs), the proline-rich proteins (PRPs), the glycine-rich proteins (GRPs), and arabinogalactan-proteins (AGPs) (*7*).

Industrial pectins are extracted from the byproducts of the fruit juice industry (apple pomace and citrus peels). They are extracted in acidic conditions and are chemically modified to give high methoxy (HM) and low methoxy (LM) pectins. In the food industry, HM pectins are widely used as gelling agents in the production of jams, marmalades, jellies, and confectionery, while LM pectins are often used as stabilizers in acidified milk drinks including sour milk products and mixtures of fruit juice and milk (*8, 9*). The industrial method used for pectin manufacture, however, has some limitations. It generates large amounts of effluents that need further

* Corresponding author. Tel: 33-2-40-67-50-58. Fax: 33-2-40-67-50-84. E-mail bonnin@nantes.inra.fr.
[†] INRA.
[‡] VTT Technical Research Centre of Finland.

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treatment. It may also provoke undesired degradation of pectins during extraction. Moreover, nowadays there is a growing demand for so-called green chemistry, encouraging the design of products and processes that reduce or eliminate the use and generation of hazardous substances. Following this idea, a new environmentally friendly method for pectin extraction was proposed (*10*). This method was based on the assumption that enzymatic deconstruction of cellulose/xyloglucan and protein networks facilitates pectins liberation from the cell walls. This deconstruction was carried out using appropriate nonpectinolytic enzymes: cellulases, which are able to degrade cellulose and the cellulose-like backbone of xyloglucan, as well as proteases that are able to degrade proteins. The feasibility of the enzymatic method was demonstrated on two plant byproducts, chicory root and a mixture of cauliflower florets, leaves, or stamps (*10*), and the enzymatic method was validated by higher extraction yields obtained with enzymes compared to those obtained by acid treatment, usually used for pectin extraction.

In the present study, the enzymatic method was applied to extract green labeled pectins and pectic oligosaccharides from different plant byproducts, such as chicory root, citrus peel, cauliflower florets and leaves, endive pulp, and sugar beet pulp. Extractions were performed in the conditions required for the microbiological safety of extracted products. Various protease and cellulase mixtures were tested for the extraction of functional pectins, e.g., high molar mass pectins that can be further used as food ingredients. In particular, the addition of pectin methyl esterases (PMEs), having two different modes of action, to the enzymatic mixture was tested in order to obtain LM pectins with various degrees of methylation (DM) and distribution of methyl groups. In addition, pectic oligosaccharides with potential health benefit effects, the so-called modified hairy regions (MHR) (*11, 12*), were obtained as process coproducts after a secondary enzymatic treatment of the residue with a multienzyme mixture.

MATERIALS AND METHODS

Raw Materials. Chicory roots were obtained from Lemaître SA, France. Dried citrus peels were obtained from Cargill Texturizing Solutions (France). Cauliflower sample as florets and leaves was provided by Bretagne Biotechnologie Végétale (France). Dried endive pulp was obtained from Orafti (Belgium). Fresh sugar beet pulp was provided by Cagny sugar factory (France).

Preparation of Cell Wall Material (CWM). Chicory root, citrus peel, cauliflower (florets and leaves), endive, and sugar beet CWMs were prepared by boiling in five volumes of 96% (v/v) ethanol for 30 min. The slurry was filtered on a G3 sintered glass, and the insoluble material was washed with 70% (v/v) ethanol until the filtrate gave a negative reaction to the phenol−sulfuric acid test (*13*). The residue was then dried by solvent exchange (ethanol, acetone) and left overnight at 40 °C.

Enzymes. *Proteases.* Neutrase (N) preparation produced by *Bacillus subtilis* was supplied by Novozymes (Copenhagen, Denmark). Promod 298L, 648L, and 24L (two batches: 24L* and 24L) preparations produced by *Bacillus* sp. were provided by Biocatalyst (Cardiff, Wales, UK).

Cellulases. Cellulyve TR400 (CL), a solid preparation produced by solid-state fermentation by *Trichoderma reesei*, was provided by Lyven (Colombelle, France). A mixture of purified *Trichoderma reesei* enzymes (PC) containing cellobiohydrolases (CBH I, CBH II) and endoglucanases (EG I, EG II) was prepared. The relative proportions of CBH I, CBH II, EG I, and EG II were 42, 21, 21, and 16%, respectively. The enzymes were purified as described previously (*14*).

Endopolygalacturonase II (endo-PG II). The enzyme was purified as described (*15*) from a liquid preparation from *Aspergillus niger* (Novozymes).

Rhamnogalacturonan Hydrolase (RGase). The enzyme from *Aspergillus aculeatus* was expressed in *Aspergillus oryzae* and provided by Novozymes as a solid preparation.

Pectin Methyl Esterases (PMEs). The orange peel PME (PME-*O*) was purchased from Sigma (France) as a solid preparation. A fungal PME from *Aspergillus aculeatus* (PME-*A*) was expressed in *Aspergillus oryzae* and was provided by Novozymes as a solid preparation.

Rapidase Liq+ Rapidase Liq+, a large spectrum of cell wall degrading enzymes from a mixture of *Trichoderma longibrachiatum* and *Aspergillus niger*, was obtained from DSM (Delft, The Netherlands).

Solid preparations were dissolved at 10 mg/mL in sodium acetate buffer (50 mM, pH 5.5), with the exception of PME-*O* dissolved at 0.5 mg/mL and RGase dissolved at 20 mg/mL. Enzyme solutions as well as liquid preparations were dialyzed against the same buffer before use.

Enzymatic Assays. All activities were measured at 50 °C in 50 mM sodium acetate buffer, pH 5.5, for the incubation time requested for initial velocity measurements. Protease activities were determined by colorimetry using azocasein (*16*) and expressed in μg/mL/s. All other activities are expressed in nkat, 1 nkat being the amount of enzyme that releases 1 nmol of reducing ends per second. The enzymes were assayed toward substrates from commercial origin (carboxymethylcellulose (CMC) and polygalacturonic acid from Sigma Chemicals, France) or from the laboratory collection (rhamnogalacturonan (*17*), arabinan (*18*), galactan (*19*), and xyloglucan), which were used, respectively, to measure cellulase (CMCase), polygalacturonase (PGase), rhamnogalacturonase (RGase), arabinanase, galactanase, and xyloglucanase (XGase) activities. Activities were calculated from the increase in reducing ends (*20, 21*) using appropriate sugars for standard curves.

Enzymatic Extraction of Pectins. *Small Scale Extraction.* Fifty milligrams of CWM was suspended in sodium acetate buffer (50 mM, pH 5.5). Two hundred fifty microliters of protease and 250 μL of cellulase were then added. In some experiments, endo-PG II (5 nkat) and RGase (1 nkat) were added to the enzymatic mixture containing protease and cellulase. The final volume was 5 mL. The resulting mixture was incubated for 4 h at 50 °C and then heated for 5 min at 100 °C to inactivate enzymes. Soluble compounds were separated from the residue by centrifugation (15 min at 3800*g*). An aliquot of supernatant was taken, and the remaining supernatant was dialyzed against water in order to eliminate sodium acetate as well as mono and/or oligosaccharides generated by the enzymatic treatment. The chemical composition was determined before and after the dialysis step. In parallel to the enzymatic treatment, CWM was incubated without any enzymes (blank).

Enzymatic Extraction with Protease + Cellulase. Five grams of CWM was suspended in 500 mL of sodium acetate buffer (50 mM, pH 5.5) for 30 min at ambient temperature. Two hundred fifty microliters of protease and 250 μL of cellulase were then added. The suspension was incubated for 4 h at 50 °C at a constant mixing rate (120 rpm). Enzymes were inactivated by boiling for 10 min. The residue was separated from the soluble compounds by centrifugation (15 min at 9000*g*). The residue was washed three times with water. Supernatants were pooled and extensively dialyzed against water before freeze-drying.

Enzymatic Extraction with Protease + Cellulase + PME. Five grams of CWM was suspended in 250 mL of sodium acetate buffer (50 mM, pH 5.5) for 30 min at ambient temperature. One hundred twenty-five microliters of protease and 125 μL of cellulase were then added. One hundred twenty-five microliters of the PME-*A* was added twice, at the beginning and after 2 h of incubation. After incubation for 4 h at 50 °C, enzymes were inactivated by boiling for 10 min. Soluble compounds were separated from the residue by centrifugation (15 min at 9000*g*). The residue was washed three times with water. Supernatants were pooled, extensively dialyzed against water, and freeze-dried prior to analysis.

Enzymatic Demethylation of Extracted Pectins with PMEs. Enzyme-extracted pectin (150 mg) was solubilized in 30 mL of sodium acetate buffer (50 mM, pH 5.5) for the treatment with PME-*A* or 30 mL of sodium phosphate buffer (100 mM, pH 7) for the treatment with PME-*O*. Two dosages of PMEs were used: 12 nkat or 240 nkat of PME-*A* and 9 nkat or 180 nkat of PME-*O*. Pectin solutions were

Table 1. Sugar Composition of Cell Wall Materials

	neutral sugars and galacturonic acid (mg/g dry matter)							
cell wall material	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
chicory root	14	3	72	33	17	39	232	279
citrus peel	9	6	84	37	30	64	258	329
cauliflower (florets, leaves)	10	4	75	24	12	43	160	150
endive pulp	12	2	84	25	16	50	206	260
Sugar beet pulp	16	2	225	19	14	54	250	225

Table 2. Enzyme Activities toward Protein and Cell Wall Polysaccharides

enzymes/abbreviation	protease[c]	PME	CMCase[d]	XGase[d]	PGase[d]	RGase[d]	arabinanase[d]	galactanase[d]
Proteases								
Promod 298L/298L	1276	nd[e]	<1	0	2	2	<1	14
Promod 648L/648 L	1675	nd	1	0	<1	3	1	7
Promod 24L*/24L*	1640	nd	<1	0	10	37	39	35
Promod 24L/24L	1375	nd	0	0	5	1	2	8
Neutrase/N	135	nd	1	<1	1	9	14	9
Cellulases								
purified cellulase/PC	0	nd	1500	605	0	1	<1	0
Cellulyve[a]/CL	<1	nd	670	380	11	9	<1	<1
PMEs								
PME*Aspergillus*[a]/PME-*A*	nd	2400	0	1	0	<1	<1	0
PMEorange[b]/PME-*O*	nd	890	0	0	0	5	1	0
Rapidase Liq+	4	nd	4170	2990	13210	16310	1360	2050

[a] The solid preparation was solubilized at 10 mg/mL. [b] The solid preparation was solubilized at 0.5 mg/mL [c] Expressed in μg/mL/s. [d] Expressed in nkat/mL. [e] nd = not determined.

incubated at 40 °C with PME-*A* and at 30 °C with PME-*O* under a constant mixing rate. A new enzyme portion was added after 2 h. After 4 h of incubation, solutions were dialyzed against water and freeze-dried.

Enzymatic Extraction of MHR. The total residue recovered after enzymatic extraction of pectins was suspended in 250 mL of sodium acetate buffer (50 mM, pH 4.5) and incubated with 1 mL of Rapidase Liq+ for 4 h at 50 °C. A new enzyme portion was added after 2 h of incubation. Enzymes were inactivated by boiling for 10 min. The soluble compounds were separated from the residue by centrifugation. The residue was washed three times in water. Supernatants were pooled, dialyzed against water, and freeze-dried.

Analytical. The individual neutral sugars were analyzed as their alditol acetate derivatives by gas−liquid chromatography (*22*). Soluble samples were hydrolyzed by 2 M H_2SO_4 at 100 °C for 2 h. Insoluble samples were first prehydrolyzed by 72% (w/v) H_2SO_4 at 25 °C for 1 h and 30 min. Inositol was added as the internal standard. Uronic acid content (as GalA) was determined colorimetrically by the automated *m*-hydroxybiphenyl method (*23*). Proteins in soluble samples were measured colorimetrically (*24*) with bovine serum albumin as standard.

Methanol and acetic acid were released by alkaline deesterification in the presence of $CuSO_4$ and quantified by HPLC on a C18 column as previously described (*25*). Isopropanol was used as the internal standard. Degree of methylation (DM) and degree of acetylation (DA) were calculated as the molar ratio of methanol and acetic acid to GalA, respectively.

Molar mass (M_W, weight-average molar mass) and intrinsic viscosity [η] were determined, respectively, by light scattering detection and viscometer detection after high performance size exclusion chromatography (HPSEC). The system used was composed of one Shodex SB-G precolumn followed by two Shodex OH-pak SB HQ 805 and 804 columns in series with a multiangle laser light scattering detector (MALLS; mini Dawn, Wyatt, Santa Barbara, CA, USA), a differential refractometer (ERC 7517 A), a differential viscometer (T-50A, Viscotek), and a UV detector (LDC Milton Roy), operating at $\lambda = 280$ nm. Elution was performed with 50 mM $NaNO_3$ containing 0.02% NaN_3 at a flow rate of 0.7 mL/min at room temperature. The system was calibrated using pullulan standards. The weight-average molar mass was calculated with ASTRA 1.4 software (Wyatt Technology) using a refractive index increment $dn/dc = 0.146$ g/mL. The intrinsic viscosity was determined with TRISEC software (Viscotek Corp.).

RESULTS

Composition of Cell Wall Materials (CWMs). Chemical compositions of chicory root, citrus peel, cauliflower florets and leaves, endive, and sugar beet CWMs reveal that they are particularly rich in pectins (**Table 1**). The amount of GalA, the major pectic sugar, varies depending on the cell wall, from 150 mg/g in cauliflower to 329 mg/g in citrus peel CWMs. It appears that pectins contain various amounts of neutral sugar side chains, as some Ara and Gal were also present. The CWMs are also rich in Glc, mainly representative of the cellulose present in cell walls, as 160 mg/g, 206 mg/g, 232 mg/g, 250 mg/g, and 258 mg/g of this sugar was quantified, respectively, in cauliflower, endive pulp, chicory root, sugar beet pulp, and citrus peel CWMs.

Enzyme Activities. Main and side activities of selected enzymes tested on appropriate substrates are presented in **Table 2**. Promod 298L and 648L have high protease activities and very low contaminant activities against the pectic backbone (PGase and RGase) and arabinan side chains (arabinanase). They also contain activity against galactan side chains (galactanase). Promod 24L* contains not only high protease activities but also pronounced PGase, RGase, arabinanase, and galactanase side activities. On the contrary, Promod 24L and Neutrase are less contaminated by these activities. The purified *T. reesei* enzyme mixture (PC) has high activities toward cellulose (CMCase) and the cellulose-like backbone of xyloglucan (XGase). However, the Cellulyve preparation contains some PGase and RGase activities. PME-*A* and PME-*O* have high PME activity and low RGase and arabinanase side activities. Rapidase Liq+ is a multienzyme mixture containing high activities toward cellulose, xyloglucan, the pectic backbone, and pectic side chains.

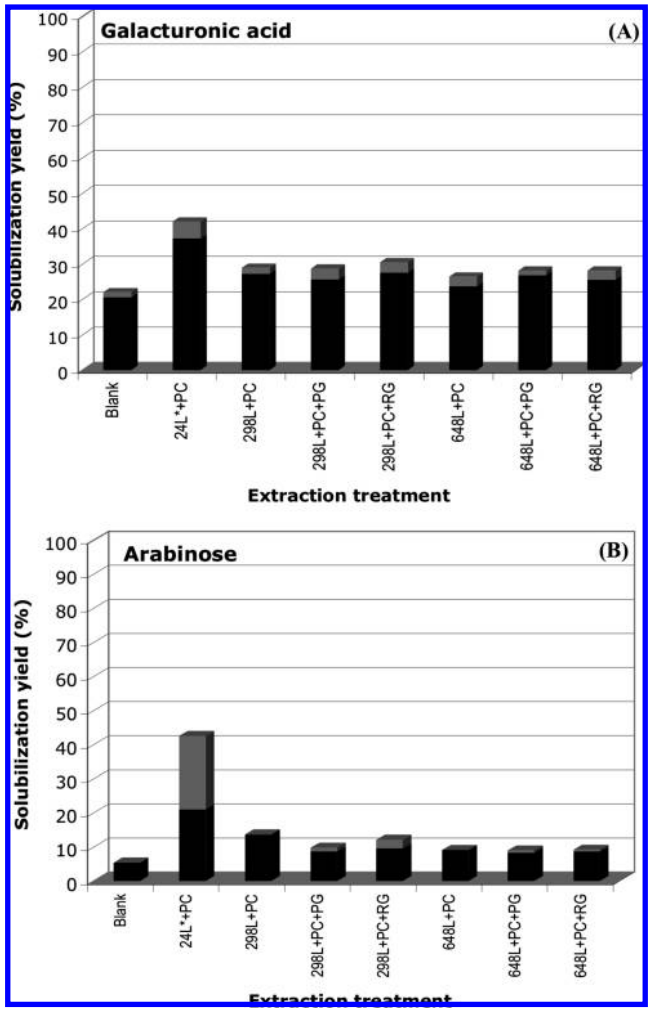

Figure 1. Solubilization yields (expressed in % of dry chicory root CWM) of (**A**) galacturonic acid and (**B**) arabinose obtained after different enzymatic treatments. Black bars represent the polymer part, and gray bars represent the oligomer part.

Enzymatic Extraction of Pectins. *Optimization Tests.* Optimization tests were first performed on chicory root CWM. Three proteases (Promod 24L*, Promod 298L, and Promod 648L) and purified *T. reesei* cellulase mixture were used in the following combinations: 24L* + PC, 298L + PC, and 648L + PC. Solubilizations of GalA and Ara, obtained for each enzyme mixture, are presented in **Figure 1 A** and **B**. Analyses were performed before and after the dialysis step, which allowed us to differentiate polymeric pectins from oligo- and monomers. Around 26% and 28% of the initial GalA was solubilized, respectively, with 648L + PC and 298L + PC, in comparison to 20% of the initial GalA liberated by incubation with the buffer alone. The highest solubilization of GalA was observed for the association 24L* + PC, where 42% of the initial GalA was extracted from the cell walls. Even if the majority of GalA was quantified as polymers, some minor part of GalA (1−5%) was also present as mono and/or oligomers, and was then eliminated during dialysis (**Figure 1 A**). Their presence is expected to be due to the slight degradation of the pectic backbone induced by pectinolytic activities present in the proteases used (**Table 2**). In order to exclude the fact that pectin extraction was enhanced by pectinolytic activities, endo-PG II and RGase were added to the enzyme mixtures characterized by low amounts of contaminant activities: 298L + PC and 648L + PC. No significant increase of GalA solubilization was observed (**Figure 1A**). This result indicates that pectinolytic activities against the pectic backbone are not responsible for pectin extraction.

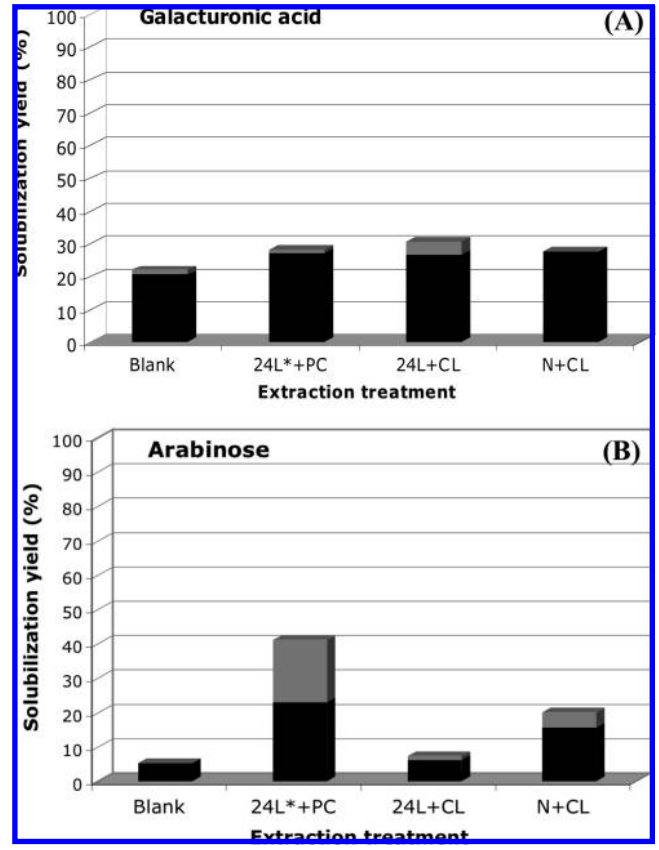

Figure 2. Solubilization yields (expressed in % of dry chicory root CWM) of (**A**) galacturonic acid and (**B**) arabinose obtained after different enzymatic treatments (enzyme 10-fold diluted). Black bars represent the polymer part, and gray bars represent the oligomer part.

The use of different enzyme mixtures allowed the solubilization of pectins more or less rich in Ara side chains. Indeed, the enzyme combinations with the lowest content of contaminant activities, i.e., 298L + PC and 648L + PC, solubilized around 13% and 9% of the initial Ara, respectively (**Figure 1B**). Neither endo-PG II nor RGase influenced the solubilization of arabinan side chains. In comparison, 24L* + PC with the highest activities against pectic side chains allowed the highest arabinan solubilization (43% of the initial Ara). It led also to the most important arabinan degradation, as more than 21% of the initial Ara was lost during dialysis (**Figure 1B**). This degradation was most likely induced by pectic side chain degrading enzymes present in the protease 24L* (**Table 2**).

According to these results, the association 24L* + PC was selected for further studies together with two new enzyme mixtures: Promod 24L and Cellulyve (24L + CL), Neutrase and Cellulyve (N + CL) (**Table 2**). These new enzyme mixtures were selected in order to enlarge the spectrum of enzymes that can efficiently be used for pectin extraction. In a view of reducing the extraction cost, the amount of enzymes used for pectin extraction was 10-fold reduced compared to optimization tests described above.

It appears that around 30% of the initial GalA was solubilized with 24L + CL and N + CL, in comparison to 20% solubilized by incubation with the buffer alone (**Figure 2A**). Only slight differences in solubilization of GalA were observed after treatment with different enzyme mixtures, and the majority of GalA was quantified as polymers. It comes out that the use of 10-fold lower quantities of selected enzymes only slightly

Table 3. Extraction Yields, Chemical Composition, and Macromolecular Features of Enzyme-Extracted pectins

		composition (mg/g)									degrees of esterification		macromolecular characteristics	
pectins (enzymes)	yield (%)[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	protein	DM	DA	M_W[b]	$[\eta]$[c]
chicory pectin (24L* + PC)	12	6	2	117	3	20	33	30	459	23	59	11	250	260
chicory pectin (24L + CL)	13.1	6	2	32	4	23	39	32	551	23	52	11	250	260
chicory pectin (N + CL)	11.4	6	2	61	4	21	36	30	528	19	45	9	230	260
citrus pectin (24L + CL)	12.6	10	2	71	7	10	34	12	756	4	68	2	180	900
cauliflower pectin (24L + CL)	6.3	4	2	53	8	11	63	15	156	33	56	50	140	200
endive pectin (24L + CL)	7.8	8	0	80	5	25	41	45	233	9	54	19	nd[d]	nd
sugar beet pectin (24L + CL)	4	11	2	103	4	16	40	52	403	17	68	44	310	400

[a] Extraction yield, expressed in % (w/w) of dry CWM. [b] M_W: weight average molar mass (kDa). [c] $[\eta]$: intrinsic viscosity (mL/g). [d] nd: not determined.

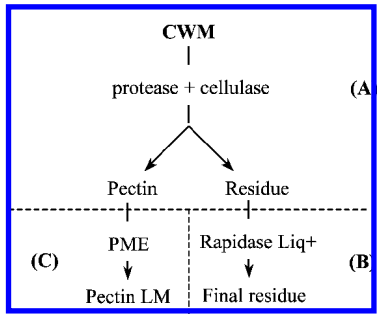

Figure 3. Overall scheme of enzymatic extractions: (**A**) enzymatic extraction of cell walls with protease and cellulase, (**B**) Rapidase Liq+ treatment of the residue recovered after extraction with protease and cellulase, and (**C**) demethylation of extracted pectin with PME.

decreased the GalA yield, showing that the enzymes were used in large excess in the previous experiments. In comparison, the solubilization of Ara varied, and pectins were found more or less enriched in side chains (**Figure 2B**). The highest solubilization of Ara was obtained after extraction with the enzyme mixture 24L* + PC, where 40% of the initial Ara was solubilized. Enzyme mixtures 24L + CL and N + CL allowed solubilizing less Ara; 7% and 20% of the initial Ara was liberated by each treatment. It appears that pectic side chains were degraded during the treatment, as 18% (with 24L* + PC) and 4% (with N + CL) of the initial Ara were lost during dialysis and were thus quantified as mono and/or oligomers. This degradation was most likely induced by the pectic side chain degrading enzymes present in commercial preparations (**Table 2**).

The results of optimization tests performed on chicory root CWM demonstrate that pectin can efficiently be extracted with the three enzyme mixtures: 24L* + PC, 24L + CL, and N + CL. Thus, these enzyme combinations were used to extract pectin from chicory root CWM in large amounts.

Enzymatic Extraction with Protease + Cellulase from Chicory Root CWM. The extraction scheme followed for pectin extraction is shown in **Figure 3A**. Yields, chemical compositions, and macromolecular features of chicory pectins extracted by 24L* + PC, 24L + CL, and N + CL are presented in **Table 3**. Similar extraction yields (11.4, 12.0, and 13.1% of dry CWM) were obtained whatever the enzyme mixture used. Chemical compositions revealed that pectins (24L* + PC), (N + CL), and (24L + CL) were mainly composed of GalA, which represents about 20%, 22%, and 26% of the initial GalA present in chicory CWM. These results correspond well to the results of optimization tests (**Figure 2A**). The three pectins contained small amounts of Rha, corresponding to 6% of the initial Rha. The pectin extracted with 24L* + PC was rich in arabinan side chains (117 mg/g of Ara, representing about 19.5% of the initial Ara), whereas the pectin extracted with 24L + CL and N + CL contained less Ara, corresponding to about 5.8% and 9.6% of the initial Ara present in CWM. These results are in agreement with the results of optimization tests (**Figure 2B**). The amount of galactan side chains remained constant, between 33 mg/g for pectin (24L* + PC) and 39 mg/g for pectin (24L + CL). The protein content varied only slightly between 19 mg/g and 23 mg/g. Pectins extracted with 24L* + PC and 24L + CL had DM of 59 and 52, respectively, while the pectin (N + CL) had DM of 45, suggesting that Neutrase could contain some PME activity. Contrary to DM, the DA of all pectins remains constant (around 10). The weight-average molar masses and the intrinsic viscosities of pectins were around 250 kDa and 260 mL/g, respectively, for all pectins. HPSEC profiles of pectins indicated the presence of different fractions eluted between 12 and 17 mL (**Figure 4**). The peak eluting between 18 and 21 mL was associated with a UV signal of high intensity (data not shown), suggesting the presence of proteins and/or some colorants.

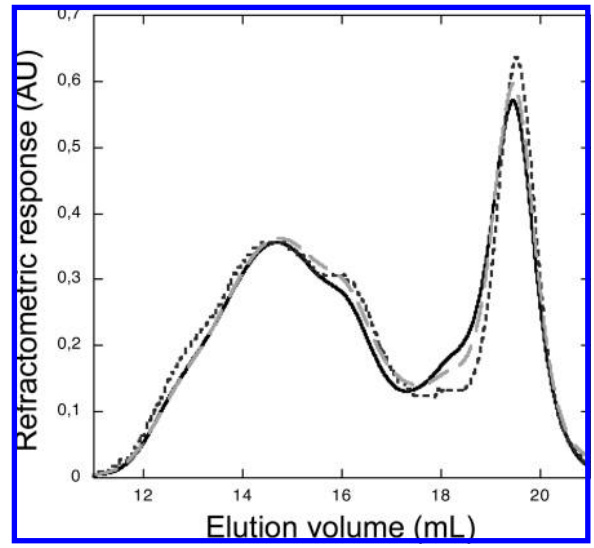

Figure 4. HPSEC profiles of chicory pectins (24L* + PC) (—), (24L + CL) (---), and (N + CL)(— — —).

Yields and chemical compositions of the residues are presented in **Table 4**. The yields were similar for the three residues recovered. The analyses showed that important amounts of pectins were still present in these residues. Indeed, the residue (24L* + PC) contained 311 mg/g of GalA, which represents 80% of the initial GalA, whereas 256 mg/g of GalA were quantified in the residues (24L + CL) and (N + CL), corresponding, respectively, to 66.4% and 67.5% of the initial GalA. High amounts of other pectic (Rha, Ara, and Gal) and cellulosic (Glc) sugars were also detected. Indeed, from 73.5%

Table 4. Recovery Yield and Sugar Composition of Residues Recovered after Pectin Extraction

residues (enzymes)	yield (%)[a]	sugar composition (mg/g)							
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
chicory residue (24L* + PC)	71.7	11	3	63	35	17	40	238	311
chicory residue (24L + CL)	72.4	13	2	84	36	17	45	272	256
chicory residue (N + CL)	73.6	12	3	76	36	17	44	270	256
citrus residue (24L + CL)	76.6	15	6	97	36	30	72	280	284
cauliflower residue (24L + CL)	77.5	12	5	93	27	15	53	205	169
endive residue (24L + CL)	80.8	18	4	86	27	14	55	260	274
sugar beet residue (24L + CL)	86.4	20	0	215	15	12	57	228	181

[a] Recovery yield, expressed in % (w/w) of dry CWM.

Table 5. Yields and Sugar Composition of MHR Extracted with Rapidase Liq+ from Chicory and Citrus Residues Recovered after Extraction with 24L + CL

products of Rapidase Liq+ treatment	yield (%)	sugar composition (mg/g)							
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA
chicory MHR	15.2[a]	86	7	57	9	9	119	16	433
citrus MHR	6.2[a]	45	6	96	13	15	69	24	420
chicory residue	31.1[b]	0	0	13	52	27	14	408	156
citrus residue	30.0[b]	9	6	15	64	45	29	515	60

[a] Extraction yield, expressed in % (w/w) of dry CWM. [b] Recovery yield, expressed in % (w/w) of dry CWM.

(in the residue (24L* + PC)) to 85.6% (in the residue (R-N + CL)) of the initial Glc were present, suggesting that cellulase treatment resulted in only partial cellulose degradation.

Enzymatic Extraction with Protease + Cellulase from Other CWMs. According to the results obtained on chicory root CWM, the enzyme mixture 24L + CL was selected to extract pectin from citrus peel, cauliflower florets and leaves, endive, and sugar beet pulps. Yields, chemical compositions, and macromolecular characteristics of extracted pectins are presented in **Table 3**. Extraction yields varied from 4% for sugar beet to 12.6% for citrus peel. Intermediate extraction yields were obtained for cauliflower (6.8%) and endive (7.8%). The extracted pectins had different compositions. The highest GalA content was measured for citrus pectin (756 mg/g), which corresponds to 29% of the initial GalA. Cauliflower, endive, and sugar beet extracts contained less GalA, as only 156 mg/g, 233 mg/g, and 403 mg/g of GalA were quantified. Citrus, endive, and sugar beet extracts contained similar amounts of Rha. All extracts were rather rich in arabinan side chains, especially the sugar beet extract (103 mg/g of the Ara), and possessed some galactan side chains as well. Citrus and endive pectins were poor in proteins (<10 mg/g), contrary to cauliflower pectin, which contained about 33 mg/g of protein. Citrus and sugar beet pectins had DM of 68, while cauliflower and endive pectins had lower DM of 56 and 54, respectively. Citrus pectin had very low DA (2), whereas the highest DA were measured for cauliflower (DA 50) and sugar beet (DA 44) pectins. The weight-average molar masses and the intrinsic viscosities of pectins were also determined (**Table 3**). The lowest molar mass of 140 kDa was estimated for cauliflower pectin, intermediate molar mass of 180 kDa was obtained for citrus pectin, and the highest one of 310 kDa was obtained for sugar beet pectin. The weight-average molar mass of endive pectin was difficult to assess because of its high heterogeneity. Intrinsic viscosities varied from 200 mL/g for cauliflower pectin to 900 mL/g for citrus pectin.

Yields and chemical compositions of the residues are presented in **Table 4**. Citrus and cauliflower residues were recovered with lower yields (76.6 and 77.5% of dry CWM) compared to endive and sugar beet residues (80.8 and 86.4% of dry CWM). It can be calculated that high amounts of pectins were still present after extraction, e.g., the lowest GalA content (66.1% of the initial GalA) was detected in citrus residue, which is in agreement with the highest extraction yield of citrus pectin. In contrast, the highest GalA content (87.3% of the initial GalA) was quantified in the cauliflower residue. Important amounts of cellulose were also present.

Enzymatic Extraction of Modified Hairy Regions (MHR). In order to obtain an additional fraction of interest and thus to decrease the amount of the residue recovered after pectin extraction, chicory and citrus residues were further treated with the enzymatic cocktail Rapidase Liq+, containing enzymes with high activities toward cellulose, xyloglucan, pectic backbone, and pectic side chains (**Table 2**) (**Figure 3B**). The enzymatic treatment led to the extraction of a soluble fraction, containing pectic oligosaccharides of specific structure, known as MHR (*11, 12*).

Citrus and chicory MHRs were obtained with the yields of, respectively, 6.2% and 15.2% of dry CWMs and were mainly composed of GalA, Rha, Ara, and Gal (**Table 5**). HPSEC profiles of both MHRs (**Figure 5**) confirmed the presence of several oligosaccharide fractions, which differed by their hydrodynamic volumes. At least 3 main fractions can be distinguished with molar masses roughly estimated at 50 kDa (16−17.5 mL), 15 kDa (17.5−18.5 mL), and 5 kDa (18.5−20 mL).

The chicory and citrus final residues were recovered with very similar yields (around 30% of dry CWMs) (**Table 5**). Both residues contained lower amount of pectins, as 60 mg/g and 156 mg/g of GalA, corresponding to 5.5% and 17% of the initial GalA in citrus and chicory residues, respectively. The amount of cellulose was also reduced; 408 mg/g and 515 mg/g of Glc, which represents about 55% and 60% of the initial Glc, were detected.

Preparation of LM Pectins by Enzymatic Demethylation with PMEs of Extracted HM Pectins. In order to obtain LM pectins with various DM and distribution of methyl groups, chicory pectins extracted with the enzyme mixtures 24L* + PC, 24L + CL, and N + CL were demethylated using the PMEs from two different origins: the plant PME (PME-*O*) and the fungal PME (PME-*A*) (**Figure 3C**). The theoretical amount of PMEs necessary to demethylate the pectin (24L* + PC) was calculated, taking into account enzyme activities, total amount of GalA, and DM of pectin. On the contrary, the PMEs used

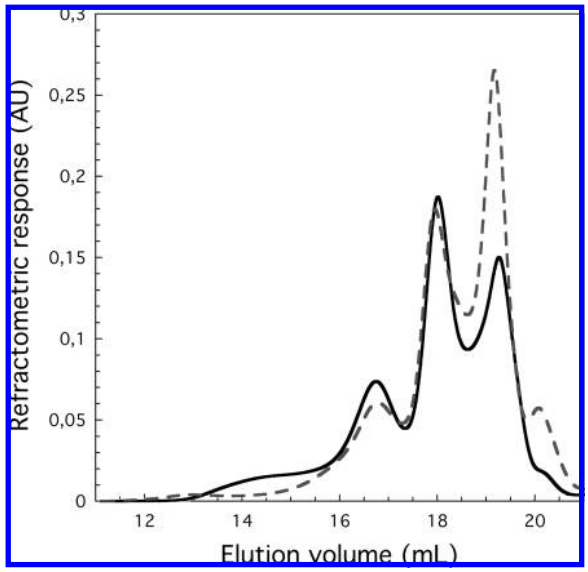

Figure 5. HPSEC profile of MHR extracted after Rapidase Liq+ treatment. Full line, chicory MHR; dotted line, citrus MHR.

for the demethylation of pectins (24L + CL) and (N + CL) were added in excess in order to reach the lowest possible DM.

Demethylation treatment with PMEs allowed to considerably decrease the DM of chicory pectins (**Table 6**). Indeed, pectins extracted with 24L + CL and N + CL, and demethylated with PME-*A* had their DM close to 10, whereas the same pectins demethylated with PME-*O* had their DM close to 20. DM of pectin extracted with 24L* + PC, demethylated by adding lower amount of PMEs, was determined at 23 after PME-*A* and 46 after PME-*O* treatments. DA of all demethylated pectins was constant and close to 10, as it is for the native pectins. The treatment led to some loss of pectic sugars. Indeed, loss of GalA residues was particularly observed for pectins (24L + CL-*O*) and (24L + CL-*A*) (about 4%), pectin (N + CL-*A*) (about 2%), and pectin (N + CL-*O*) (about 6%). On the contrary, only minor loss of Rha residues was observed after demethylation treatment (about 0.5%). This loss of pectic sugars can be due to some pectinolytic activities present in the PMEs used (**Table 2**). Low RGase activities detected in the PMEs may also cleave the pectin backbone, which results in pectins of lower molar masses. Indeed, slight degradation of pectins demethylated with PMEs used in excess, in comparison to their mother pectins, can be seen on HPSEC profiles (**Figure 6A** and **B**). Demethylated pectins are eluted in slightly higher volume, which suggests their lower masses. They appear also less heterogeneous, compared to their corresponding mother pectins. HPSEC profiles of demethylated pectin (24L* + PC) were exactly the same (data not shown) as that observed for its mother pectin (**Figure 4**).

Enzymatic Extraction of LM Pectins with Protease + Cellulase + PME. In order to extract LM pectins in only one step, the fungal PME-*A* being the most efficient was added directly to the enzymatic mixture containing protease and cellulase. The enzymatic treatment was first performed on chicory root CWM, and two pectins were obtained: pectin (24L + CL+PME-*A*) and pectin (N + CL+PME-*A*). Yields, chemical compositions and macromolecular characteristics of extracted pectins, are presented in **Table 7**. The enzymatic procedure led to the extraction of LM pectins with a DM of 23. The obtained yield of extraction was close to 10% of dry CWM for both enzymatic treatments and were thus comparable with that obtained for pectins demethylated after extraction (**Table 6**). However, both pectins contained less GalA, as 500 mg/g (instead of 547 mg/g, **Table 6**) in pectin (24L + CL+PME-*A*) and 506 mg/g (instead of 559 mg/g, **Table 6**) in pectin (N + CL+PME-*A*) were measured. The amount of other sugars was constant. HPSEC profile of pectin (24L + CL+PME-*A*) was very similar to that of pectin (24L + CL) (**Figure 7**); the weight-average molar mass of pectin (24L + CL+PME-*A*) was determined at 330 kDa. In the opposite, pectin (N + CL+PME-*A*) was degraded during the treatment, as a major pectic peak was eluted between 17 and 20 mL (**Figure 7**). Its weight-average molar mass was difficult to assess because of the very high heterogeneity of the pectin.

According to the demethylation results obtained on chicory root CWM, enzyme mixture 24L + CL+PME-*A* was applied to extract LM pectin from citrus peel. The addition of the PME allowed the direct extraction of LM pectin with DM of 30 (**Table 7**). LM citrus pectin was extracted with higher yield (13.8%) than chicory LM pectin (10.3%). Moreover, no sugar loss was observed when PME-*A* was added to the enzymatic mixture, and no change in molar mass was observed (180 kDa).

DISCUSSION

The principal aim of the present work was to transform vegetable byproducts into high-added value compounds, such as pectins and pectic oligosaccharides, and thus considerably reduce the amount of these residues generated by food industries. The use of enzymes appears as an environmentally friendly way to extract the products of interest. Physico-chemical characteristics of extracted pectins, i.e., chemical composition, molar mass, and viscosity, will determine the functional properties of pectins and their further use as gelling, emulsifying, and/or thickening agents.

Optimization tests performed on chicory root CWM revealed that higher pectin yields are obtained after extraction with enzymes presenting pectinolytic activities. The addition of PGase and RGase to the enzymes with very low contaminant activities discarded the hypothesis that these enzymes enhance pectin extraction. PGase and RGase led, however, to pectin degradation, especially if incubation times are longer. Indeed, important pectin degradation was observed after 16 h of incubation with Neutrase and Cellulyve by Panouillé et al. (*10*), compared to 4 h of incubation with the same enzymes performed in the present study. It was also demonstrated that the quantities of enzymes used for pectin extraction can be considerably reduced (10-fold) without an important decrease of extraction yields. This observation is of great importance as decreasing the enzyme amounts means decreasing the extraction costs. Therefore, an environmentally friendly process does not inevitably imply an excessive cost.

Moreover, we observed that pectins with different lengths of the neutral sugar side chains were obtained after extraction with different enzyme mixtures. Indeed, assuming that native chicory pectins always have the same degree of branching, it can be suggested that pectins with longer arabinan side chains were extracted with enzymes (especially proteases) presenting higher *endo*-arabinanase activity (pectins (24L* + PC) and (N + CL)) than enzymes containing only low *endo*-arabinanase activity (pectin (24L + CL)) (**Table 3**). This observation indicates an important heterogeneity of pectin molecules with populations more or less rich in side chains of different length. High pectin heterogeneity was also confirmed by HPSEC profiles (**Figure 4**). The presence of different pectin populations was also reported in other cell walls, e.g., sugar beet and potato (*26, 27*). Pectin population with long side chains was more integrated within the cell walls and thus more difficult to extract, which

Table 6. Extraction Yields, Chemical Composition and Macromolecular Features of Chicory Pectins Demethylated after Extraction with PME from Orange (PME-*O*) or PME from *Aspergillus aculeatus* (PME-*A*) (**Figure 3**B)

		composition (mg/g)									degrees of esterification		macromolecular characteristics	
enzymes	yield (%)[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	protein	DM	DA	M_W[b]	$[\eta]$[c]
24L* + PC-PME-*A*	10.8	7	2	117	3	20	23	30	495	27	23	9	250	240
24L* + PC-PME-*O*	11.4	7	2	107	3	19	32	28	486	25	46	9	250	220
24L + CL-PME-*A*	11.4	6	2	32	4	26	38	35	547	23	11	11	240	120
24L + CL-PME-*O*	12.3	6	2	34	4	26	41	36	490	23	22	9	240	128
N + CL-PME-*A*	10.1	6	2	62	4	24	37	32	559	20	10	11	260	120
N + CL-PME-*O*	10.8	6	2	51	3	18	31	27	430	21	23	10	205	120

[a] Extraction yield, expressed in % (w/w) of dry CWM. [b] M_W: weight average molar mass (kDa). [c] $[\eta]$: intrinsic viscosity (mL/g).

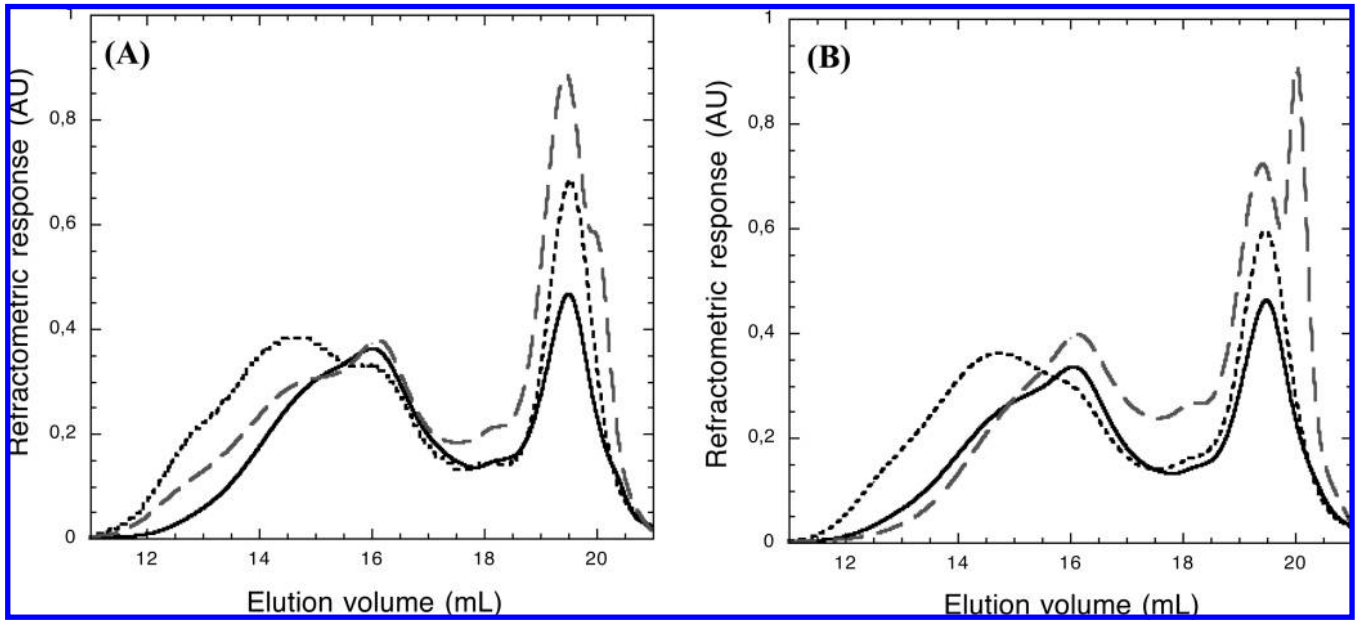

Figure 6. HPSEC profiles of chicory pectins (**A**) (24L + CL) (---), (24L + CL-*O*) (— — —), and (24L + CL-*A*) (—); (**B**) (N + CL) (---), (N + CL-*O*) (— — —), and (N + CL-*A*) (—).

Table 7. Extraction yields, Chemical Composition and Macromolecular Features of Chicory and Citrus Pectins Extracted with Protease, Cellulase and PME from *Aspergillus aculeatus* (PME-*A*)

		composition (mg/g)									degrees of esterification		macromolecular characteristics	
pectin sources (enzymes)	yield (%)[a]	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	protein	DM	DA	M_W[b]	$[\eta]$[c]
Chicory Pectin														
24L + CL+PME-*A*	10.3	7	2	27	3	15	34	20	500	20	24	9	330	380
N + CL+PME-*A*	10.1	7	2	62	3	15	40	20	506	19	23	8	nd[d]	nd
Citrus Pectin														
24L + CL+PME-*A*	13.8	6	2	75	10	10	32	14	709	5	30	2	180	800

[a] Extraction yield, expressed in % (w/w) of dry CWM. [b] M_W: weight average molar mass (kDa). [c] $[\eta]$: intrinsic viscosity (mL/g). [d] nd: not determined.

was explained by higher entanglement and/or association with other primary cell wall components, such as cellulose micro fibrils (*27, 28*). Therefore, *endo*-arabinanase may act in synergy with protease and cellulase for releasing pectin molecules, in particular those that are embedded and/or associated with cellulose. Protease and cellulase with no or low *endo*-arabinanase activity may liberate the pectin population with short side chains that play a role in the matrix by filling the interstices within cellulose/xyloglucan and protein networks.

The same enzymatic method applied to different cell wall materials gave pectins with various yields. This finding emphasizes the important heterogeneity of cell walls, their elementary components, and their organization. Therefore, it is very unlikely that one can create one enzymatic method suitable for any plant matrix. However, appropriate association of protease and cellulase may lead to the extraction of pectins with desired structure in high amounts. It is important to note that the enzymatic method applied to citrus peel allows the extraction of pectins with similar characteristics than commercially available citrus pectins extracted by chemical means (*9, 29, 30*).

Proteases and cellulases allow the extraction from the cell walls of native pectins, which are characterized by high DM. The DM of pectins can be modified. This can be done during the extraction of pectins or after their extraction as an additional treatment. It was demonstrated in the present study that LM pectins with various DM and distribution of methyl groups can be obtained with the PMEs from two different origins: (i) the plant PME from orange and (ii) the fungal PME from *Aspergillus aculeatus*. It is generally proposed that plant PMEs remove methyl esters in a processive way, resulting in contiguous domains of de-esterified GalA in HG regions (*30*), whereas the action of fungal PMEs is regarded rather as random (*32*). The

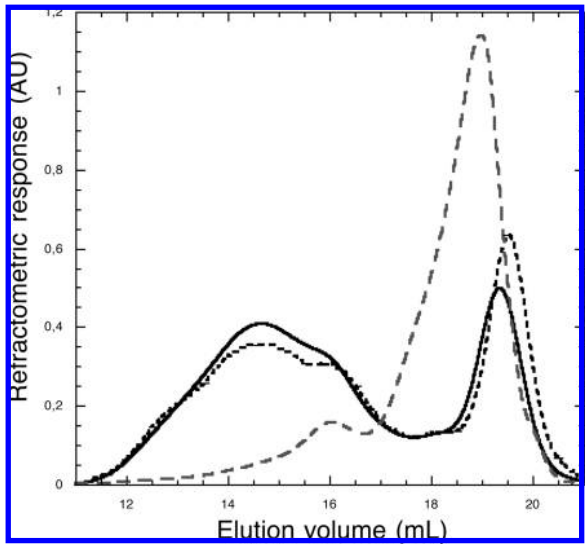

Figure 7. HPSEC profiles of chicory pectins (24L + CL) (---), (24L + CL+*A*) (− − −) and (N + CL+*A*) (—).

de-esterification pattern influences the gelling properties of pectins, and generally stronger gels are obtained with plant PME-demethylated pectins (*33, 34*). In the present work, we noticed that the theoretical amount of PMEs was not sufficient to considerably lower the pectin DM, especially if LM pectins are to be obtained. In order to improve the demethylation of pectins, the PMEs were added in excess. It appears that demethylation performed onto chicory pectins was more efficient with the fungal PME than with the plant PME. Therefore, the fungal PME was selected and added directly to the enzymatic mixture containing protease and cellulase in order to extract LM pectins in one step. It appears that only one enzyme mixture (24L + CL+PME-*A*) can successfully be applied to extract high molar mass LM pectins. The important pectin degradation observed with the combination N + CL+PME-*A* can be due to the synergy of pectinolytic side activities with PME, particularly PGase. Moreover, the DM of LM pectins extracted in one step was about 2 times higher than the DM of pectins demethylated after extraction. The difference in demethylation efficiency might be due to lower accessibility of the PME to pectins because of their important entanglement with other cell wall components during extraction.

The enzymatic extraction of pectins decreases the starting CWM by 25%. Therefore, in order to further decrease the amount of generated residue, a liquefying enzyme preparation commonly used in fruit juice manufacturing (Rapidase Liq+) was sequentially applied. The use of the combined action of pectinolytic and cellulolytic enzymes allowed not only to considerably decrease the amount of the final residue but also to extract pectic oligosaccharides, the so-called MHRs (*11, 12*). MHRs have peculiar structure; they are mainly composed of RG I regions branched with arabinan and galactan side chains. They are presumed to have some beneficial health effects, e.g., prebiotic effects. Indeed, a prebiotic effect of pectin oligosaccharides from orange albedo (arabinans and/or arabinogalactans attached to the RG I backbone) was recently reported (*35*). The chicory and citrus MHRs had similar compositions as already described for the apple MHR (*11*). In chicory MHR, the molar ratio Rha/GalA (0.24) was in the same range as the value given for the apple MHR (0.3) (*11*). The molar ratio was slightly lower in the citrus MHR (0.13), but one can think that it will not influence the biological activity of this fraction.

In the present study, we put forward that the way of extraction influences the chemical composition and macromolecular features of pectins. In the case of enzymatic extraction, these characteristics depend not only on the main activities of enzymes used but also on their contaminant activities present. Only an appropriate enzyme mixture allows the extraction of high molar mass HM or LM pectins, which can be further used as food ingredients. Sequentially, oligosaccharides with potential prebiotic activity were extracted. Thus, 70% of the byproducts were consumed to extract two different products of interest.

Functional properties, e.g., gelling and/or emulsifying properties of enzyme-extracted pectins obtained in this study will be described in a subsequent article.

ABBREVIATIONS USED

Ara, arabinose; CWM, cell wall material; DA, degree of acetylation; DM, degree of methylation; endo-PG, endopolygalacturonase; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; Glc, glucose; HG, homogalacturonan; HM, high methoxy; HPSEC, high performance size exclusion chromatography; LM, low methoxy; Ma, mannose; MHR, modified hairy region; PC, purified cellulase; PME, pectin methylesterase; PME-*A*, pectin methylesterase from *Aspergillus aculeatus*; PME-*O*, pectin methylesterase from orange; RG I, rhamnogalacturonan I; RG II, rhamnogalacturonan II; RGase, rhamnogalacturonase; Rha, rhamnose; XGase, xyloglucanase; Xyl, xylose.

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