Cellulase and Protease Preparations Can Extract Pectins from Various Plant Byproducts

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The use of protease and cellulase preparations to extract pectins from plant byproducts (chicory, cauliflower) was investigated. Different enzymatic preparations were characterized by their activities toward proteins, cellulose, and pectins. These preparations were then tested regarding pectin extraction, and extraction conditions (nature and concentration of enzyme, incubation time) were optimized. Enzymatic and acidic extractions were compared and also combined in sequential extractions. This study shows that it is possible to extract pectins by using cellulases and proteases. Enzymes can extract pectins with a higher yield (∼35%) than acid (∼28%) but enzyme-extracted pectins have a smaller molar mass (300 000 g/mol) than acid-extracted pectins (500 000 g/mol). Different hypotheses are tested and discussed to explain this mass difference.

KEYWORDS: Pectins; extraction; enzymes; cellulase; protease; byproducts; cell wall; *Cichorium intybus*; chicory; *Brassica oleracea*; cauliflower

INTRODUCTION

Pectins are a family of complex polysaccharides found in the cell wall of higher plants. Pectin molecules are composed of different structural regions, which have been extensively described in previous reviews (*1–3*). The homogalacturonan regions (HG), generally called "smooth regions", consist in a repetition of $\alpha(1 \rightarrow 4)$-linked D-galacturonic acid. The degree of polymerization is commonly between 75 and 100. Galacturonic acid residues can be partially methyl and/or acetyl-esterified. The degree of methylation (acetylation) is defined as the number of methyl (acetyl) groups for each 100 galacturonic acids. The rhamnogalacturonan regions *I* (RG*I*), also called "hairy regions", are constituted by an alternating sequence of $\alpha(1 \rightarrow 4)$-linked D-galacturonic acid and $\alpha(1 \rightarrow 2)$-linked rhamnopyranosyl residues. Various side chains (mainly arabinan, galactan, and arabinogalactan) can be linked to rhamnose residues. The nature and lengths of the side chains vary among plant species. Another type of rhamnogalacturonan (RGII) is a minor component of pectins; its structure is very complex and it is composed of at least 12 different monomers. The structure of the HG and RG regions is quite well-known, but their way of combination remains unclear (*4*).

In the food industry, pectins are used for their gelling and thickening properties. Apple pomace and citrus peels, which are byproducts from the juice industry, are generally used as raw material for pectin extraction. Pectins can be extracted from the cell walls by physical, chemical, as well as enzymatic ways. Physical methods, such as extrusion-cooking (*5*) or microwave-

assisted extraction (*6*), can be used. Industrial pectins are extracted in acidic conditions. The pH (between 1 and 3), the temperature (50–100 °C), and the extraction time (1–12 h) are chosen depending on the functional properties required (*3, 7, 8*). Too strong conditions can lead to depolymerization and de-esterification of pectins.

Acidic extraction generates large amounts of effluents that require treatment. Moreover, the consumer demand for "green" products stimulates the search for alternative means of extraction. Therefore, enzymes could represent, despite their potential cost, an alternative and environmentally friendly way to extract "green labeled" pectins. Two different approaches can be considered. The first one involves enzymes to degrade the pectins and isolate pectin fragments. Enzymes degrading the pectin backbone (endo-polygalacturonase together with pectin methyl esterase, or endo-pectin lyase) are able to solubilize all the galacturonic acid (*9*). These enzymes extract high molecular mass fragments of "hairy regions", carrying many side chains, but also galacturonic acid oligomers from "smooth regions" (*10*). The yield of extraction obtained with pectinolytic enzymes is higher than that with chemical means (*10, 11*). Some studies have also been done with various "protopectinases" (*12, 13*). According to their authors, these enzymes are a heterogeneous group that solubilizes pectins from the insoluble pectin in plant tissues (the so-called "protopectin") by restricted depolymerization. They can be active against the pectin backbone or against the pectin side chains. Their action is strongly dependent on the type of enzyme and the nature of substrate (*12*). Some "protopectinases" can be more efficient to extract pectin than acid, depending on the substrate. This is the case for protopectinases having a pectin lyase or an endo-polygalacturonase activity, but also an arabinanase activity (*13*). However, pure

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arabinanase and galactanase were found to be inefficient to extract galacturonic acid-containing polymers and oligomers (*14*, *15*).

The second approach consists of using enzymes able to deconstruct the plant cell wall and isolate pectins. The primary plant cell wall of dicotyledons is composed of various polysaccharides (cellulose, xyloglucan, pectin) and proteins, which form entangled networks (*16*). Xyloglucan is known to associate to cellulose microfibrils, probably via hydrogen bonding (*17*). The cellulose/xyloglucan network is embedded in a pectic matrix, together with a protein network. However, recent studies have shown the possibility to form interactions between cellulose and pectin side chains (*18*, *19*). Regarding pectin extraction, the combined use of cellulases and proteases could allow the isolation of pectic polysaccharides by degrading the cellulose/xyloglucan and protein networks.

The aim of this study is to use commercial cellulases and proteases to extract pectins from different byproducts. The enzymatic extraction will be compared with acidic extraction. First, experiments were done on chicory roots, and then a similar procedure was further applied on cauliflower byproducts to check the accuracy of the method. Chicory roots have been chosen as the study material because they are very rich in pectins, but also because chicory production generates very large amounts of these byproducts. Seven hundred thousand tons of chicory are produced each year in the EC, resulting in 700 000 tons of roots that are, such as cauliflower byproducts, generally not valorized. They are sometimes used for animal feeding or landfill, but European legislation concerning landfill becomes more and more restrictive and other solutions for byproduct treatment have to be found. Extraction of compounds with (high) added value from these byproducts could be an interesting alternative.

MATERIALS AND METHODS

Raw Materials. Chicory (*Cichorium intybus*) roots were obtained from a chicory producer by hydroponic forcing (Lemaître SA, Crecy sur Serre, France). Two different samples of cauliflower (*Brassica oleracea*) byproducts (sample 1: small pieces of florets and leaves; sample 2: small pieces of florets and stumps) were obtained from Bretagne Biotechnologie Végétale (BBV, Saint Pol de Léon, France).

Preparation of Alcohol Insoluble Residue (AIR). The raw materials were sliced and treated in five volumes of boiling ethanol at 96 °C for 30 min, then washed in cold ethanol at 70 °C as many times as necessary to release all soluble sugars. The soluble sugars were quantified with the phenol/sulfuric acid method (*20*). The residue was washed twice in ethanol at 96 °C and dried by solvent exchange with acetone. AIR yields were 5.4% (w/w fresh matter) for chicory roots, 4.4% for cauliflower sample 1, and 4.7% for cauliflower sample 2. AIR was used as the starting material for all extraction experiments.

Enzymes. Neutrase, Celluclast 1.5L, and Pectinex AR were obtained from Novozymes (Copenhagen, Denmark). Neutrase is a liquid protease preparation produced by *Bacillus subtilis*. Celluclast 1.5L is a liquid cellulase preparation made by submerged fermentation of the fungus *Trichoderma reesei*. Pectinex AR is a liquid pectinolytic enzyme preparation produced by *Aspergillus niger*. Cellulyve TR400 was obtained from Lyven (Colombelle, France). This solid cellulase preparation is produced by solid-state fermentation by *T. reesei*. Papain and Maxazyme CL2000 were obtained from DSM (Seclin, France). Maxazyme is a cellulase preparation produced by *Trichoderma viride*. Papain is a solid preparation from *Carica papaya*.

Solid preparations were dissolved at 10 mg/mL in sodium acetate buffer (50 mM, pH 5.5). All solutions, including liquid preparations, were dialyzed against the same acetate buffer before use. Depending on experiments, enzymes were used undiluted, 1/10 diluted, or 1/100 diluted.
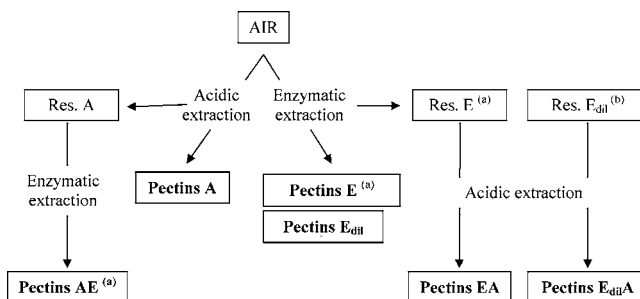
Figure 1. Scheme of sequential extractions. [a]Pectins E and AE were extracted with 10-fold diluted enzymes solutions. [b]Pectins E$_{dil}$ were extracted with 100-fold diluted enzymes solutions.

Enzymatic Assays. All activities were measured at 40 °C in 50 mM sodium acetate buffer, pH 5.5. Protease activities were determined by colorimetry using azocasein (*21*) and expressed in μg/mL/s. All other activities are expressed in nkatals, 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 orange polygalacturonic acid, Sigma Chemicals, L'Isle d'Abeau, France) or from the laboratory collection (rhamnogalacturonan (*22*), arabinan (*23*), galactan (*24*), and xyloglucan), which were used, respectively, to measure cellulase, polygalacturonase (PG), rhamnogalacturonase (RG), arabinanase, galactanase, and xyloglucanase (XG) activities. Activities were calculated from the increase in reducing ends (*25*, *26*) using appropriate sugars for standard curves.

Extractions. *Acidic Extraction.* Pectins were extracted from AIR with HCl. Extraction conditions were chosen to be in the range of pH (1.3), temperature (85 °C), and extraction time (3 h) generally used in industry (*3*). Five grams of AIR was dispersed in 150 mL of 50 mM HCl and treated at 85 °C for 1 h. After cooling, the extract was separated from the residue by filtration on sintered glass. The same procedure was repeated 3 times, and the final residue was washed with deionized water. The three extracts and washing waters were pooled together, and the pH was adjusted to 5.5 with 1 M NaOH. The extract was then extensively dialyzed against water at 4 °C and freeze-dried, giving pectins A.

Optimization of Enzymatic Extraction. Depending on the experiments, a combination of one, two, or three enzymes was used. Fifty milligrams of AIR was suspended in sodium acetate buffer (50 mM, pH 5.5), and 0.25 mL of each enzyme solution was added. The final volume was 5 mL. These resulting mixtures were incubated at 40 °C for a given time and heated at 100 °C for 5 min to inactivate the enzymes. Soluble compounds were separated from the residue by centrifugation. An aliquot of supernatant was taken, and the rest of the supernatant was dialyzed against water to eliminate sodium acetate and small oligosaccharides liberated by enzymatic reaction. Composition analyses were performed before and after the dialysis step.

Large-Scale Enzymatic Extraction. Five grams of AIR was incubated in a total volume of 500 mL of acetate buffer (50 mM, pH 5.5) with 2.5 mL of each diluted (1/10) enzyme solution. After inactivation of enzymes and separation of soluble compounds by centrifugation, the supernatant was extensively dialyzed against water and freeze-dried prior to other analysis, giving pectins E.

Sequential Extraction. Acidic and enzymatic extractions were combined as described in **Figure 1**. First, extractions were carried out as described above, leading to pectins A and E. The resulting residues were used for second extractions. Enzymatic extraction was performed on the residue resulting from acidic extraction, giving pectins AE, and acidic extraction on the residue resulting from enzymatic extraction, giving pectins EA. Another sequential extraction was done with more diluted enzymes (1/100), resulting in pectins E$_{dil}$ and E$_{dil}$A. All the extracts were dialyzed and freeze-dried before further analysis.

Analytical. Individual neutral sugars were quantified after hydrolysis (2 h in 1 M H$_2$SO$_4$, 100 °C), derivatized in their alditol acetates (*27*), and analyzed by gas liquid chromatography on a BP-225 fused-silica capillary column (SGE, Courtaboeuf, France; 25 m × 0.32 mm i.d.)

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Table 1. Enzymatic Activities of Commercial Enzyme Preparations

		protease[b]	CMCase[c]	XG[c]	PG[c]	RG[c]	arabinanase[c]	galactanase[c]
proteases	Papain[a]	**5**	3	0	3	4	0	1
	Neutrase	**140**	0	0	2	8	5	9
cellulases	Celluclast	<1	**990**	1120	4	10	1	0
	Cellulyve[a]	<1	**210**	80	83	30	1	0
	Maxazyme[a]	<1	**50**	55	0	1	1	<1
pectinase	Pectinex AR	<1	10	80	**18700**	12000	410	1200

[a] Solid preparations were suspended at 10 mg/mL. [b] Expressed in μg/mL/s. [c] Expressed in nkat/mL. XG = xyloglucanase. PG = polygalacturonase. RG = rhamnogalacturonase.

Table 2. Extraction Yields and Chemical Composition of Air and Extracted Pectins from Chicory Roots and Cauliflower Samples

			yield (%)[a]	composition (%)										DE (%)[b]	
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	protein	total	DM	DA
chicory	roots	AIR	5.4	1.4	0.3	7.2	3.3	1.7	3.9	23.2	27.9	7.4	83.1	68	36
		pectins A	27.8	1.7	0.2	12.8	0.3	0.7	5.9	0.9	53.1	7.5	83.1	49	12
		pectins AE	11.3	4.3	0.4	0.9	2.3	1.5	7.4	3.7	64.8	1.5	86.8	37	16
		total	39.1												
		pectins E	34.6	2.1	0.3	9.1	0.8	1.1	4.0	2.9	58.6	2.2	81.1	53	17
		pectins EA	3.8	3.3	0.2	4.2	1.1	0.8	8.0	1.1	54.3	13.1	86.1	31	20
		total	38.4												
		pectins E$_{dil}$	19.8	1.1	0.2	10.3	0.4	1.4	3.1	2.6	49.9	2.3	71.3	66	16
		pectins E$_{dil}$A	17.6	3.0	0.2	8.7	0.6	0.4	7.2	0.6	49.5	9.1	79.3	43	18
		total	37.4												
cauliflower	sample 1 (florets, leaves)	AIR	4.4	1.0	0.4	7.5	2.4	1.2	4.3	16.0	15.1	32.6	80.5	54	39
		pectin A	16.8	1.2	0.2	19.0	0.4	0.6	9.8	5.4	20.6	22.7	79.9	72	15
	sample 2 (florets, stumps)	AIR	4.7	1.1	0.4	10.0	3.4	1.7	6.1	19.9	18.8	17.5	78.9	64	42
		pectin A	22.2	1.3	0.1	18	0.5	0.6	10.2	7	26.6	55.6	119.9	59	17

[a] For AIR, yield is expressed as a percentage of fresh raw material. For pectins, yield is expressed as a percentage of dry AIR. [b] The confidence interval is less than ±0.7%.

mounted in a Perkin-Elmer Autosystem gas chromatograph (Courtaboeuf, France). For AIR, a prehydrolysis step (30 min in 13 M H_2SO_4, 25 °C) was added. Uronic acid content was colorimetrically determined by the automated *m*-hydroxybiphenyl method (*28*, *29*). Degrees of methylation (DM) of pectins A and all degrees of acetylation (DA) were calculated after HPLC determination of methanol and acetic acid released by alkaline de-esterification of pectins (*30*), respectively. In the case of pectins extracted with enzymes, the peak of methanol was hindered by other peaks and therefore the DM was determined by colorimetry (*31*). Soluble proteins were measured colorimetrically (*32*), with ovalbumin as standard. For AIR, protein content was calculated as ($N \times 6.25$), the N content being determined by Kjeldhal procedure.

Size Exclusion Chromatography and Molar Mass Determination. High-performance size exclusion chromatography (HPSEC) was performed at room temperature on two columns in series (Shodex OH-Pack SB-804 HQ and OH-Pack SB-805 HQ) eluted at 0.7 mL/min with 50 mM sodium nitrate containing 0.02% sodium azide. The column effluent was monitored using a differential refractometer (Erma 7512, Japan), an online multi-angle laser light scattering (MALLS) detector (Mini Dawn, Wyatt Technology Corp., Santa Barbara, CA). The weight average molar mass ($\overline{M_w}$) was calculated with Astra 1.4 software using a refractive index increment $dn/dc = 0.146$ g/mL. The molar mass of molecules eluting after 16 mL was not taken into account.
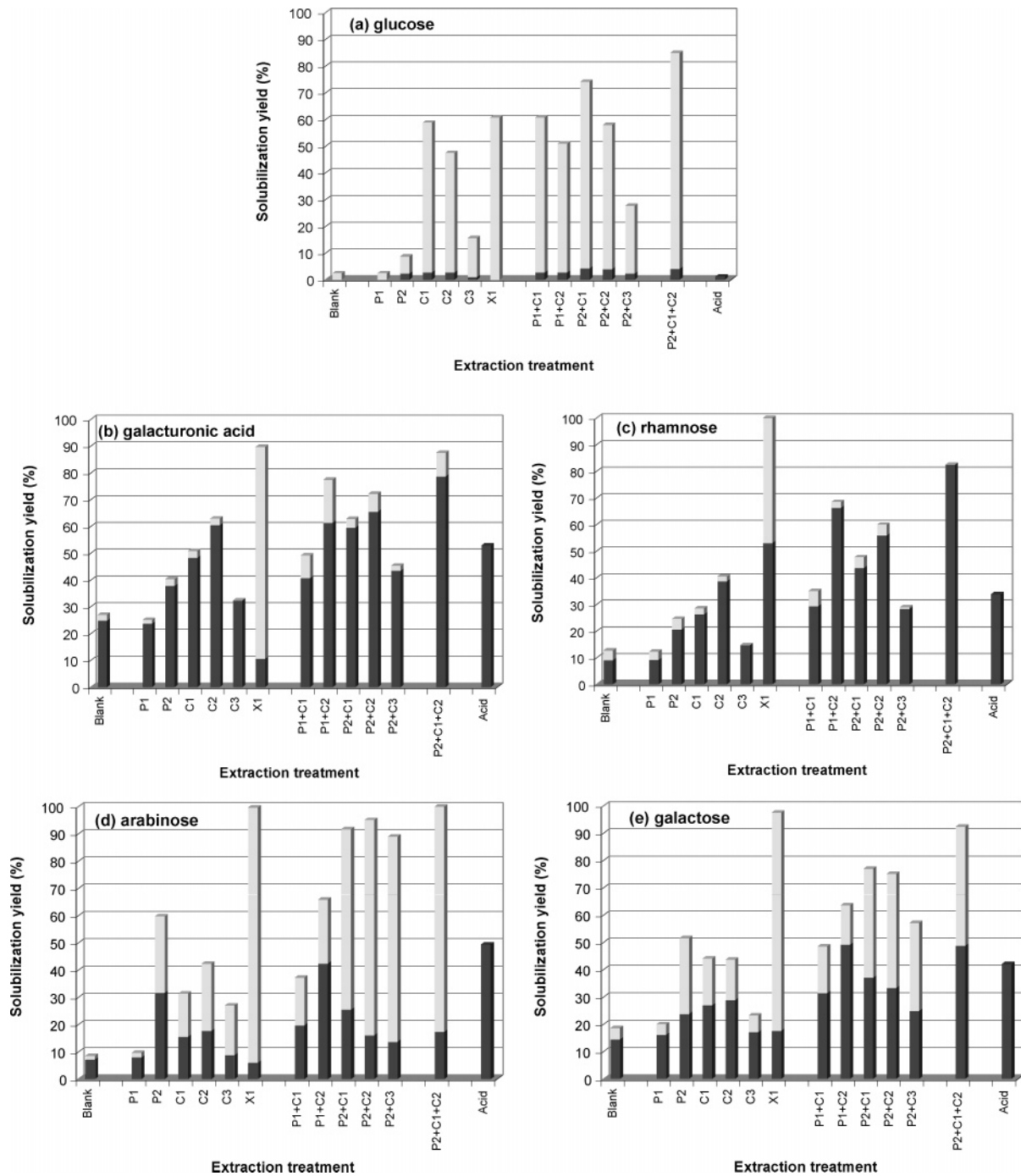
RESULTS

Enzyme Activities. Commercial enzymatic preparations were chosen instead of purified enzymes in order to limit the cost of extraction. The activities of enzyme preparations were measured using various substrates (**Table 1**). Pectinex AR was used as a reference for pectinolytic activities. It has activities degrading pectin backbone as well as pectin side chains, but also small

CMCase and xyloglucanase activities. Papain and Neutrase have a protease activity of 5 and 140 μg/mL/s, respectively. They have no or very low CMCase, xyloglucanase, and pectinolytic activities. Cellulase preparations have an activity toward CMC (ranging from 50 nkat/mL for Maxazyme to 990 nkat/mL for Celluclast), but also toward xyloglucan (from 55 nkat/mL for Maxazyme to 1120 nkat/mL for Celluclast). They have no protease activity. Celluclast and Maxazyme also have no or very low pectinase activities, contrary to Cellulyve, which has significant activities toward pectin backbone (83 and 30 nkat/mL for polygalacturonase and rhamnogalacturonase activities, respectively). However, these side activities are very low compared with activities found in the pectinolytic preparation Pectinex AR and lower than the main cellulase activities measured on CMC.

Composition of AIR and Pectins A from Chicory Roots. Compositions of AIR and pectins from chicory roots are given in **Table 2**. Chicory root AIR is very rich in galacturonic acid (27.9%) and in rhamnose (1.4%), arabinose (7.2%), and galactose (3.9%), suggesting that pectins are the major polysaccharides in chicory roots. AIR also contains a large quantity of glucose (23.2%), resulting mainly from cellulose but also probably from xyloglucan (xylose 3.3%). These results are in accordance with the data from Femenia et al. (*33*).

Acidic extraction has a yield of 27.8% (dry w/dry w AIR). Pectins A are rich in galacturonic acid (53.1%), which represents more than one half of galacturonic acid present in AIR. They contain 1.7% rhamnose and are also rich in neutral sugars, especially in arabinose (12.8%). The DM is around 50%, and the DA is around 13%.

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Figure 2. Solubilization yields for (**a**) glucose, (**b**) galacturonic acid, (**c**) rhamnose, (**d**) arabinose, and (**e**) galactose. They are expressed as a percentage of the initial concentration in AIR. Dark and clear bars describe the proportion of sugar released as polymers and oligomers, respectively. Enzymes codification: P1, Papain; P2, Neutrase; C1, Celluclast; C2, Cellulyve; C3, Maxazyme; X1, Pectinex AR.

Solubilization with Different Enzyme Combinations. Enzymatic extraction of pectins was studied first with undiluted enzymatic preparations, alone or in combination (one protease and/or one cellulase), after incubation for 16 h at 40 °C. Blanks were prepared without any enzyme. Incubation with the pectinolytic preparation Pectinex AR was also done for comparison. Solubilization yields are presented in **Figure 2** for glucose and pectin constituting sugars. Analyses were performed before and after the dialysis step, which allowed a differentiation between pectins extracted in an oligomeric or a polymeric state.

Cellulose Solubilization. The different cellulase preparations, used alone, solubilize various amounts of glucose (from 15% for Maxazyme to 60% for Celluclast; **Figure 2a**). The glucose

is only released in an oligomeric form, which demonstrates, together with the high proportion of glucose released, the efficiency of cellulose hydrolysis. Solubilization yields obtained with Celluclast and Cellulyve are 58 and 46%, respectively, although Celluclast is almost 5-fold more active than Cellulyve on CMC and has a higher xyloglucanase activity. Neutrase, despite having no activity detected toward CMC, solubilizes 10% of glucose, and glucose solubilization is increased when Neutrase is used together with Celluclast or Cellulyve. The combination of Neutrase, Celluclast, and Cellulyve gives the highest solubilization yield (more than 80% of glucose), suggesting that Celluclast and Cellulyve have different cellulase activities. Pectinex AR solubilizes as much glucose as Celluclast
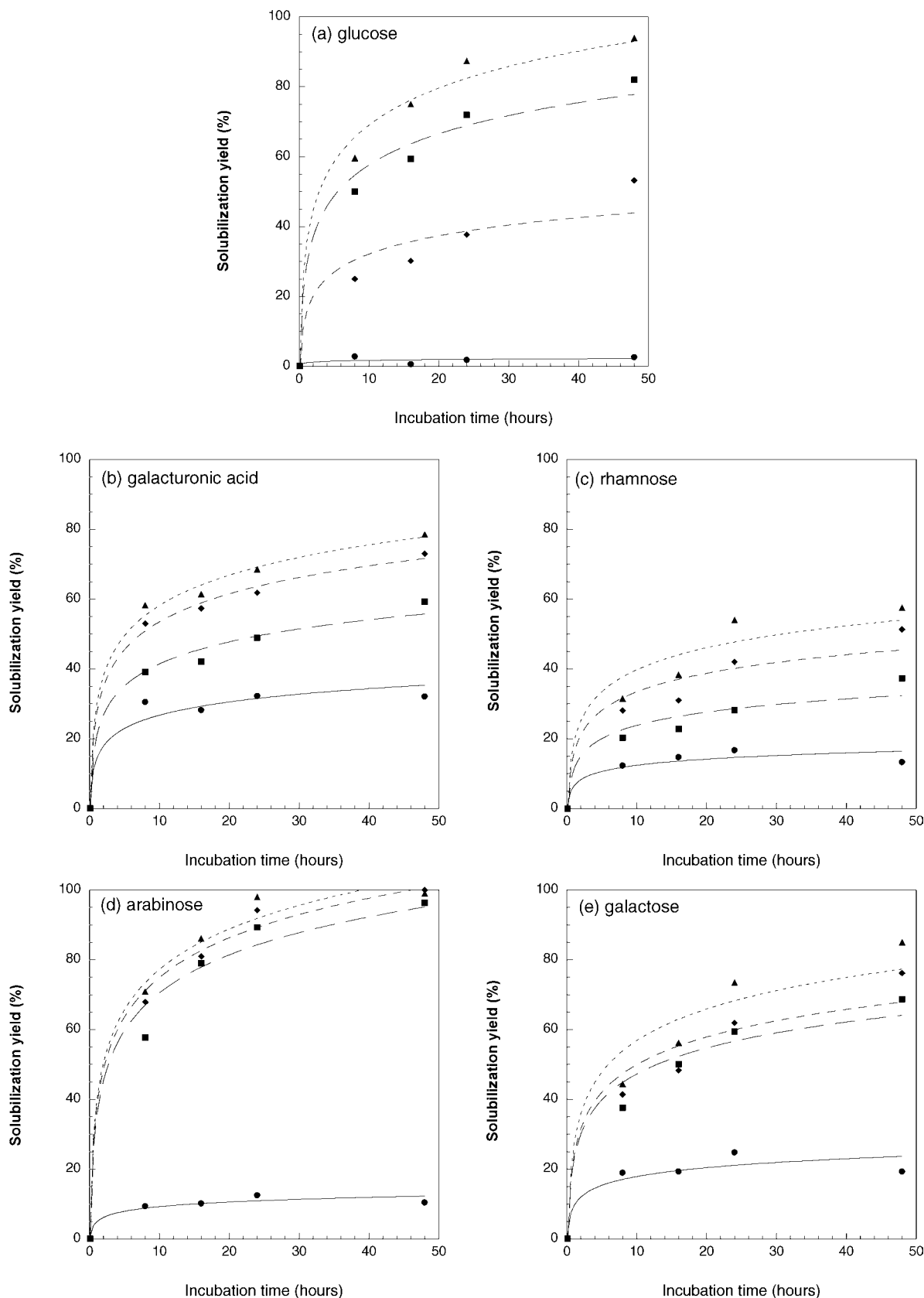
Figure 3. Total quantity of (**a**) glucose, (**b**) galacturonic acid, (**c**) rhamnose, (**d**) arabinose, and (**e**) galactose released as a function of time with different enzyme combinations. Solubilization yields are expressed as a percentage of the initial concentration in AIR. (●) No enzyme (blank). (■) Neutrase + Celluclast. (◆) Neutrase + Cellulyve. (▲) Neutrase + Celluclast + Cellulyve.

although it has a lower CMCase activity, probably because of a synergistic action between cellulases and pectinases (*9, 11, 34*). Xylose solubilization profiles are similar to glucose ones, with the best solubilization yield (45%) obtained for the

combination of Neutrase and Celluclast (results not shown). Xyloglucan is also degraded by cellulase preparations.

Pectin Solubilization. Solubilization patterns of galacturonic acid and rhamnose (**Figure 2b,c**) on the one side and arabinose

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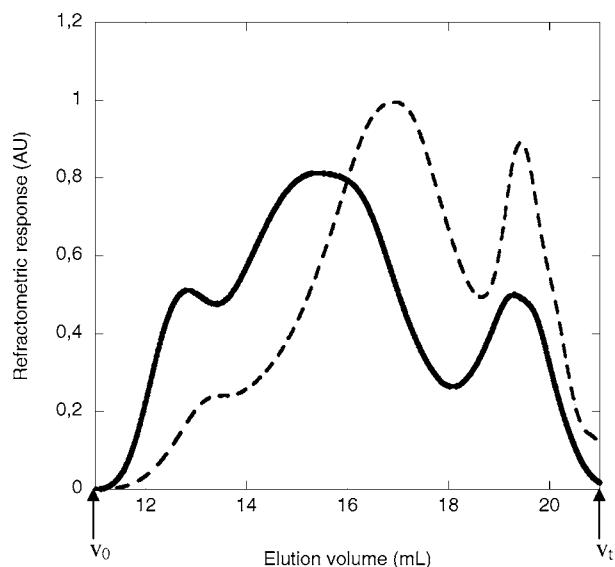
Figure 4. HPSEC profiles for pectins A (full line) and pectins E (dotted line).

and galactose on the other side (**Figure 2d**,**e**) are quite similar. Without enzyme, a significant quantity of galacturonic acid, rhamnose, arabinose, and galactose is solubilized (25, 10, 9, and 18% of the initial content of AIR in each sugar, respectively). Pectinex AR hydrolyzes almost all the pectin sugars as oligomers. Papain has no effect on pectin solubilization, but all the other preparations increase pectin solubilization. Pectin backbone, containing galacturonic acid and rhamnose, is released as polymers, but side chains are partially hydrolyzed. The combination of one protease and one cellulase improves pectin extraction, especially for rhamnose. As shown for glucose, the best extraction results are obtained with the combination of Neutrase, Celluclast, and Cellulyve (more than 80% for all pectic sugars).

Considering these results, the most efficient enzyme preparations (Neutrase, Celluclast, and Cellulyve) were selected for further experiments.

Influence of Enzyme Concentration and Kinetics of Solubilization. To optimize extraction conditions, enzyme concentration was decreased 10-fold and the kinetics of solubilization was studied from 8 to 48 h (**Figure 3**). The quantity of sugars released without enzyme is constant. On the other hand, the solubilization by enzymes increases up to 48 h. After 48 h, the solubilization yields obtained for all sugars are similar to those obtained with undiluted enzymes. **Figure 3** describes the total solubilization yield of each sugar; however, as previously shown in **Figure 2**, glucose is released only as oligomers. Pectins are solubilized as polymers (less than 5% of galacturonic acid and rhamnose are eliminated by dialysis), and hydrolysis of pectin side chains is less important than with undiluted enzymes: whatever the enzyme combination, less than 50% of arabinose and galactose are hydrolyzed in oligomers, with an exception for the combination of Neutrase + Celluclast + Cellulyve, which released almost 80% of arabinose as oligomers.

HPSEC profiles of hydrolyzates were studied as a function of reaction time between 8 and 48 h (data not shown). No significant change was observed, showing that pectins are not degraded during incubation, at least between 8 and 48 h. A typical profile, obtained for an incubation time of 48 h, is presented in **Figure 4** (pectins E).

Regarding the solubilization yields, the combinations Neutrase + Celluclast + Cellulyve and Neutrase + Cellulyve are the most efficient (up to 70% of galacturonic acid and 50% of rhamnose released). Decreasing the quantity of enzymes 10 times does not affect the final solubilization yield of pectin.

Comparison of Acidic and Enzymatic Extracts. To compare acidic and enzymatic extractions, a larger scale enzymatic extraction was performed with the condition selected to have the best extraction yield: chicory AIR was incubated with the combination of 1/10 diluted Neutrase and Cellulyve for 48 h at 40 °C, resulting in pectins E.

The composition and solubilization yields obtained for acidic as well as enzymatic extractions are presented in **Table 2**. Total extraction yield is higher for enzymatic extraction (34.6%) than for acidic extraction (27.8%). The compositions of the two extracted pectins are quite similar, even if pectins E are richer in galacturonic acid and poorer in protein (58.6 and 2.2%, respectively) than pectins A (53.1 and 7.5%). The solubilization yields of galacturonic acid and rhamnose are higher after enzymatic extraction. DM and DA are in the same range for both extractions but slightly higher in the case of pectins E.

HPSEC patterns for pectins A and E are shown in **Figure 4**. Molecules eluting after 16 mL are too small to give a correct signal by light scattering. The average molar mass ($\overline{M_w}$) of pectins was then calculated from 11 to 16 mL, corresponding to the high molar mass pectins. The following $\overline{M_w}$ values are therefore overestimated. Pectins A have a much higher molar mass than pectins E. The calculated $\overline{M_w}$ value is around 500 000 g/mol for pectins A and 300 000 g/mol for pectins E.

Sequential Extractions. Different sequential extractions were performed as shown in **Figure 1**, resulting in six different pectins. Pectins E_{dil} were extracted with 10 times fewer enzymes than pectins E (dilution 1/100). Compositions and solubilization yields are presented in **Table 2**.

Pectins E_{dil} are obtained with a lower yield (19.8%) than pectins E (34.6%), and galacturonic acid solubilization is much lower (35.4% instead of 72.7%). Pectins AE, EA, and E_{dil}A are extracted with a yield of 11.3, 3.8, and 17.6%, respectively. However, the total extraction yields are similar for the three sequential extractions (37−39%), showing that the same amount of pectins is extracted in all cases. All pectins are rich in galacturonic acid (50−65%) but differ in their individual sugar content, especially for rhamnose, arabinose, and galactose. Pectins extracted with HCl (pectins A, EA, and E_{dil}A) are quite rich in proteins. Pectins resulting from the second steps of extractions (pectins AE, EA, and E_{dil}A) are always richer in rhamnose and galactose. They also have a lower DM but a slightly higher DA. Solubilization yields of backbone sugars are very close for A + AE and E + EA extractions (80% for galacturonic acid, 60−70% for rhamnose) and a little lower for E_{dil} + E_{dil}A extraction (67% for galacturonic acid and 53% for rhamnose).

The HPSEC profiles of the six types of pectins are presented in **Figure 5a**. Pectins extracted with acid (pectins A, EA, and E_{dil}A) have a much higher molar mass (around 500 000, 1 000 000, and 800 000 g/mol, respectively) than pectins extracted with enzymes (pectins E, E_{dil}, and AE: around 300 000 g/mol). It should be noted that the peak of high molar mass pectins is associated with a UV peak, especially for acidic extracts (data not shown). As any phenolics were found in the AIR (<0.001%), this suggests an interaction between high molar mass pectins and proteins and is reliable to the highest amount of proteins in pectins A.
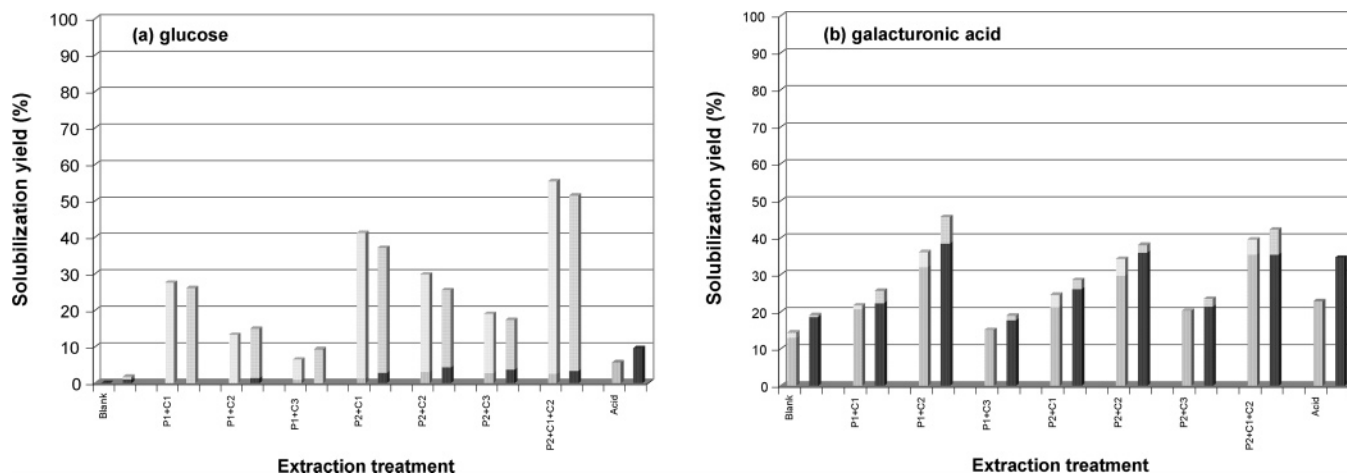
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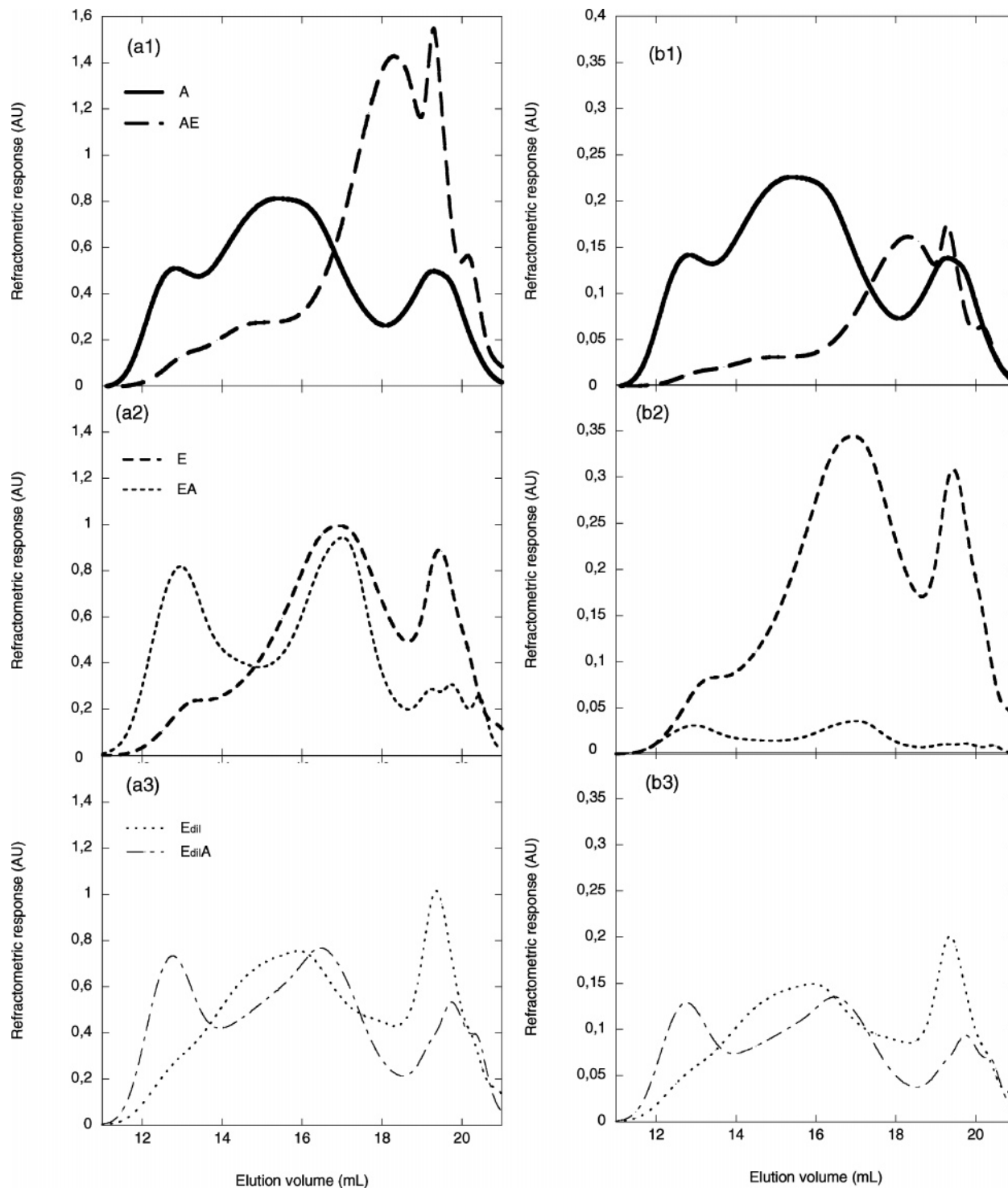
Figure 5. HPSEC profiles obtained for sequentially extracted pectins. (**a**) Raw profiles. (**b**) Profiles obtained after normalization by the extraction yield. (**1**) Extraction by acid, then enzymes (A and AE). (**2**) Extraction by enzymes, then acid (E and EA). (**3**) Extraction by diluted enzymes, then acid (E_{dil} and $E_{dil}A$).

Figure 5b illustrates the HPSEC patterns after normalization by extraction yields in order to show the relative proportion of each pectin fraction. For example, pectins EA seem to be interesting regarding their molar mass (**Figure 5a2**); however, their extraction yield is very low (less than 4%) and they represent a very small fraction of pectins (**Figure 5b2**).

Extraction from Other Raw Materials. To validate the enzymatic extraction method on other byproducts, preliminary studies were done on two different cauliflower byproducts. The first one is composed of small pieces of florets and leaves, and

the second one is composed of florets and stumps. AIR compositions are presented in **Table 2**. Cauliflower samples contain less galacturonic acid than chicory roots but a similar amount of other pectic sugars (rhamnose, arabinose, and galactose). Cauliflower is also richer in protein, especially sample 1, which contains leaves. Cauliflower pectins, extracted in acidic conditions, contain less galacturonic acid and more glucose than chicory root pectins.

Regarding results obtained on chicory roots, one enzymatic extraction condition has been selected: cauliflower AIRs were

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Figure 6. Solubilization yields from cauliflower AIRs for (**a**) glucose and (**b**) galacturonic acid. They are expressed as a percentage of the initial concentration in AIR. For each enzyme combination, the two bars describe the solubilization yields for cauliflower samples 1 and 2, respectively. Dark and clear bars describe the proportion of sugar released as polymers and oligomers, respectively. Enzymes codification: P1, Papain; P2, Neutrase; C1, Celluclast; C2, Cellulyve; C3, Maxazyme; X1, Pectinex AR.

incubated for 16 h at 40 °C with combinations of enzymes diluted 1/10. The proportions of glucose and galacturonic acid released by enzymes are described, respectively, in **Figure 6a,b**. Solubilization yields are very close for both cauliflower samples, but lower than those obtained for chicory roots. For example, the best enzyme combination (Neutrase + Celluclast + Cellulyve) releases around 50% of glucose and 40% of galacturonic acid from cauliflower samples instead of 70 and 60% from chicory roots. These results could be related to the higher protein concentration in cauliflower samples, decreasing the accessibility of pectins and/or cellulose. However, solubilization yields, obtained with the more efficient enzyme combinations for cauliflower galacturonic acid, are higher than the acidic solubilization yield.

Even if further experiments are required, the comparison of results obtained for chicory roots and cauliflower shows that this enzymatic method could be applied to other plant byproducts.

DISCUSSION

This study shows that the combination of commercial cellulases and proteases can be used to extract polymeric pectins from different plant byproducts. Other authors succeeded in pectin extraction with commercial cellulase preparation with no or very low pectinolytic activities. Shkodina et al. (*35*) extracted around 40% of galacturonic acid from pumpkin pulp with a purified cellulase preparation produced by *T. viride*. Matora et al. (*36*) isolated sugar beet and pumpkin pectins with a cellulolytic enzymatic preparation produced by *Bacillus polymyxa* in conditions known to minimize pectinolytic activities. In the two latter cases (*35*, *36*), pectins have much smaller molar mass and intrinsic viscosity than pectins extracted with acid.

With our conditions, enzymes extract pectins with a higher yield than acid. The yield of enzymatic extraction is 34.6% instead of 27.8% for acidic extraction, and the solubilization yield of galacturonic acid is 72.7% instead of 52.9%. The compositions of pectins E and A are quite similar, even if pectins E are richer in galacturonic acid and poorer in pectin side chain sugars (arabinose and galactose), probably because of hydrolysis of these side chains during extraction. The DM and DA are slightly higher for pectins E. The DM of both pectins is quite

low (around 50%); for comparison, industrial pectins extracted from citrus or apple have a DM higher than 70%. The DA of the chicory root pectins is around 15%, much higher than apple or citrus pectins (<1%) but smaller than sugar beet pectins (20%) (*2*). Pectins E have a smaller molar mass (300 000 g/mol) than pectins A (500 000 g/mol). Even if these values can be overestimated by the method used to evaluate molar mass, they remain high compared to commercial pectins (from 200 000 to 400 000 g/mol) (*2*). This mass difference between pectins A and E is probably not linked to the existence of two distinct populations of pectins because sequential extractions had similar global yields whatever the order of extracting agents. Difference in molar mass is likely to be related to differences in the mechanisms of extraction.

The mechanism of enzymatic extraction is not yet understood, and the implication of pectinolytic side activities remains unclear. The plant cell wall is constituted of different polymeric networks, mainly composed of cellulose/hemicellulose, pectin, and proteins. Recent studies showed the direct interaction between cellulose and pectin side chains (*18*, *19*). Hydrolysis of cellulose should therefore improve the isolation of pectin molecules. Cellulase preparations, used alone, solubilize on one side a large part of glucose (up to 50% for Cellulyve and 60% for Celluclast) and on the other side a significant quantity of pectins (more than 50% of galacturonic acid). The glucose solubilization rates are higher than those obtained with pure cellulases: 20% with endo-glucanase (*9*, *37*) or 30% with a combination of endo-glucanase and cellobiohydrolase (*11*). Concerning pectin solubilization, some authors showed that pectin extraction was almost impossible by using pure cellulases and that cellulose can be degraded only if the pectins have been previously removed. Endo-glucanases are unable to extract apple pectins on their own (*37*, *38*) and release only 14% of galacturonic acid from carrot cell wall material when associated with cellobiohydrolase (*11*). In our results, the higher solubilization yields for both cellulose and pectin could be partially explained by the pectinolytic side activities of Celluclast and Cellulyve, because cellulase activity is known to be hindered by pectin matrix (*11*, *39*). Pectinex AR, despite a low CMCase activity, is also able to extract 60% of glucose, which is in agreement with a synergy between cellulase and pectinase activities or with direct links between cellulose and pectin.

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Moreover, Renard et al. (*37*) suggested that the inability of endo-glucanase to extract galacturonic acid could be explained by a physical enmeshment, involving pectin side chains and/or glycoproteins. Hydrolysis of pectin side chains and proteins could improve the accessibility of cellulose and pectins and therefore favor pectin extraction. Implication of proteins in the cell wall network should be considered with interest, since in our study, HPSEC shows that high molar mass pectins A are associated with proteins. If used in combination with a cellulase, the protease preparations also improve pectin solubilization. This suggests that pectins interact with the cell wall protein network.

Further experiments, using enzymes totally devoid of pectinolytic activities, are required to fully understand the mechanism of enzymatic extraction of pectins. Macromolecular and functional properties of acidic and enzymatic pectins, such as gelling, thickening, or emulsifying properties, will be compared in a subsequent article.

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