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Enzymatic Extraction of Lemon Pectin by Endo-Polygalacturonase from *Aspergillus niger*

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Abstract Pectin was enzymatically extracted from industrial lemon pomace by using an endo-polygalacturonase from *Aspergillus niger* as a processing aid and compared to pectin extraction by hot hydrochloric acid. The yield of pectin was 17.6 and 20.2% with enzymatic and acidic treatments, respectively. The molecular weight distribution did not vary greatly between the samples extracted with enzyme or acid. Large differences in charge density were observed, however, when the samples were analyzed by anionic-exchange chromatography. Pectin extracted by the enzymatic treatment indicated higher charge density than that obtained by hydrochloric acid. The higher charge density could due to the presence of endogenous lemon pectinesterase, which was activated at low pH 4.5 *in situ* conditions during the process of enzymatic extraction, leading to low methoxylated pectin with a higher charge density.

Keywords: pectic substances, pectinesterase, pectinase

Introduction

Pectic substances are a heterogeneous group of high molecular weight acidic polysaccharides from the primary cell walls and intercellular regions of higher plants. Pectin is composed primarily of three polysaccharides: homogalacturonan consisting of (1 → 4)-α-linked D-galacturonan residues partially esterified by a methyl group; rhamnogalacturonan-I (RG-I) composed of a backbone of alternating (1→4)-α-linked D-galacturonan and (1→2)-α-linked rhamnopyranosyl residues to which linear and branched α-L-arabinofuranosyl and/or β-D-galactopyranosyl residues are connected through the rhamnose units; and substituted galacturonans (referred as RG-II) containing side chains of complex sugars connected to a backbone of linear (1 → 4)-α-linked D-galacturonan residues. Detailed information and elaborated models of pectin structure can be found in recent reviews (1-2).

Development of new technologies for the utilization of agricultural and domestic waste is important not only to preserve a clean environment, but also to harness value added products from natural resources. Key issues in pectin technology are: the extraction process (3) and the method of isolation and purification, which should depend on the end use of the pectin. Pectic substances can be extracted by chemical (4), physical (5-7), and biological methods (8). At the moment, industrial pectins are extracted by chemical methods, and then modified to suit to an end use. The chemical structure of acid-extracted pectins has been extensively investigated (9), however, pectin extraction by chemical methods can contaminate the environment and corrode the reactors used for the extraction process (8).

Biological (fermentation or enzyme) methods are considered as alternative procedure for releasing pectin because these processes are environmental friendly and the reaction conditions are less severe. For the enzymatic extraction of pectin, diverse enzymes such as polygalacturonases, xylanases, cellulases, and rhamnogalacturonases have been employed either in pure form or as crude extracts (8, 10). Endo-polygalacturonase (endo-PG) is an endo-acting hydrolytic enzyme capable of hydrolyzing the homogalacturonan-region on the α-(1→4)-glycosidic linkage between adjacent unesterified moieties. As the sole enzyme, endo-PG may attack such regions releasing polymeric pectin with high degree of methyl esterification (11). The pectin of lemon was extracted by crude extracts of endo-PG from *Klyuveromyces fragilis*, nevertheless physico-chemical characterization of pectin was not achieved (11). The objectives of this study were to evaluate the yield and molecular characteristics of the pectin released by enzymatic treatment with endo-PG from *Aspergillus niger* and to compare the physico-chemical characteristics of the pectin to those of a pectin extracted by a chemical method.

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Materials and Methods

Chemicals and biologicals Galacturonic acid was from

Sigma Chemicals (St. Louis, MO, USA). Endo-PG (E-PGALS, Lot MPG00301; 800 U/mg protein, 5000 U/mL) from *A. niger* was purchased from Megazyme, Ireland. All others chemicals were analytical grade.

Preparation of lemon peel pomace The lemon pomace of industrial grade was provided by Citrinor Co. (Tucumán, Argentina) of which only the lemon peel was used for the pectin extraction. The pomace free of seeds was ground by using a food processor, and was separated by sieving. The fraction that passed through a mesh 40 but retained by a mesh 50 was used for pectin extraction.

Enzymatic pectin extraction A 200 cm^3 glass flask with water jacket was used as the reactor for enzymatic pectin extraction. Five g of lemon pomace in 120 mL of acetate buffer (pH 4.5, 0.05 M) was treated with endo-PG (1.6 μg protein/g substrate, which is equal to a concentration of 0.07 μg/mL of protein in the final reaction mixture). The mixture was incubated under agitation (50 rpm) at 37°C for 12 hr followed by filtration through muslin cloth. The filtrate was boiled (100°C) for 5 min to inactivate the enzyme. After cooling at 4°C, the filtrate was mixed with 2 volumes of ethanol and left overnight under refrigeration to precipitate the polymeric material. Afterwards, pectin was recovered by centrifugation at 7,000×g for 30 min and the residue was dried in a ventilated oven at 40°C. The dry material was dissolved in water and then lyophilized to obtain the soluble material.

Chemical pectin extraction The chemical extraction of pectin was carried out by the procedure described by Royo-Iranzo *et al.* (12). Five g of lemon pomace was suspended in 120 mL of distilled water. The pH was adjusted to 2.0 with concentrated hydrochloric acid (HCl) and the mixture heated at 90°C for 60 min. The resulting macerate was filtered through a muslin cloth. The filtrate was mixed with 2 volumes of ethanol and left overnight under refrigeration to precipitate the polymeric material. Pectin was recovered by centrifugation, then the residue was dried, dissolved in water and lyophilized to obtain the soluble material.

High-performance ion-exchange chromatography (HPIEC) An anion-exchange column TSK DEAE 5PW (75×7.5 mm, Toyosoda, Japan) connected to an HPLC system (Waters 625 LC; Milford, MA, USA) was used for high-performance anion-exchange chromatography. The column was equilibrated with 0.01 M ammonium acetate buffer (pH 6). Three hundred μL of the extracts (2 mg/mL) were injected into the column and eluted with a linear gradient of 0.05-0.5 M ammonium acetate buffer (pH 6) at a flow rate of 0.6 mL/min. The eluate was continuously monitored using the *m*-hydroxydiphenyl (13) and orcinol (14) assays on an Alliance instruments (Méry/Oise, France) autoanalyzer.

Preparative ion-exchange chromatography An anion-exchange column of DEAE Sepharose CL-6B (40×2.5 cm) was connected to an HPLC system (Waters 625 LC; Milford). Fifty mL of extract in solution (2 mg/mL) were loaded to the column. The column was eluted in the following sequence with ammonium acetate buffer (pH 6): initially 200 mL of 0.05 M buffer was loaded, followed by 400 mL of a linear gradient of 0.05-0.5 M, 50 mL of 1 M buffer, and finally with 150 mL of 1 M buffer for washing the column prior to reconditioning. The flow rate was maintained at 2.5 mL/min. The eluate was continuously monitored as mentioned above. Peak-forming fractions were collected for further analysis.

High-performance size-exclusion chromatography (HPSEC) The molecular weight distribution of pectin was determined using an HPLC system involving a laboratory data control (LDC) programmable pump equipped with four Bio-Gel TSK columns (300×7.8 mm each) in series (50, 40, 30, and 25 PWXL; Tosohaas, Stuttgart, Germany), in combination with a TSK XL guard column (40×6 mm) at 35°C. Three hundred μL of the extracts (2 mg/mL) were injected into the column and they were eluted with 0.4 M sodium acetate buffer (pH 3.6) at 0.8 mL/min. The eluate was continuously monitored as described above. The system was calibrated with commercial pectins, whose molecular weights were previously determined by viscosimetric measurements using the equation of Owens *et al.* (15).

Other assays Galacturonic acid and neutral sugars (expressed as arabinose) were determined in the supernatants by the automated *m*-hydroxydiphenyl (13) and orcinol (14) assays, respectively, in which the baseline was corrected for interfering galacturonic acid on an Alliance instruments (Méry/Oise) autoanalyzer. The individual neutral sugars were analyzed by gas chromatography (capillary column of 30 m × 0.25 mm i.d. coated with DB225, 0.15 μm film thickness, J & W Scientific, Folsom, CA, USA) at 215°C, using hydrogen as the carrier gas, after sulphuric acid hydrolysis (16) and derivatization to alditol acetates (17). *Myo*-inositol was used as the internal standard.

Endogenous pectinesterase activity Lemon pomace (5 g) was incubated under agitation (50 rpm) for 4 hr at 4°C in 120 mL of 0.1M Tris-HCl buffer (pH 7.50) containing 0.25 M NaCl. At the end of the incubation, the suspension was filtered and the pectinesterase (PE) activity determined in the filtrate by the qualitative method of gellation. An appropriate volume of filtrate was mixed with an equal volume of pectin solution (10 g/L in 0.1 M Tris-HCl buffer, pH 7.5 containing 0.05 M of $CaCl_2$) and incubated at 37°C. The presence of PE activity was evidenced by gelation of the solution.

Results and Discussion

Pectin recuperation The pectin preparations were obtained by two different extraction methods, viz; with endo-PG from *A. niger* and with HCl. The chemical extraction gave higher yields (20.2%) of soluble pectin than that (17.6%) of enzymatic extraction. The results obtained for the enzymatic extraction were closely similar to the earlier reports of Donaghy and McKay (11) and Contreras-Esquivel *et al.* (18). They reported that crude extracts of endo-PG from *K. fragilis* and *A. kawachii* released pectin from lemon peel. The yield of pectin was 18.0 and 17.4%

with *K. fragilis* and *A. kawachii* respectively. Sakamoto *et al.* (19) extracted pectin from lemon using rhamnogalacturonase from *Trametes sanguinea* and reported a lower yield of 10.9%.

Analytical chromatography HPSEC profiles generated (Fig. 1) for the two extraction methods were quite similar. Both samples were composed of high molecular weight (Mw) polymers, however, the peak of the acid extract was narrower and had a slightly lower elution volume. This result indicated that the acid extract contained a higher molecular weight pectin which was demonstrated by HPSEC by yielding molecular weights of 70 and 52 kDa for acidic and enzymatic pectin, respectively. The profiles obtained for the orcinol (total sugars) and *m*-hydroxydiphenyl assays (uronic acids) were similar in both cases i.e. the distribution of the neutral sugars and uronic acids were homogeneous and were of high molecular weight. A minor amount of oligomers, however, could be detected in the enzyme extract, in spite of the purification process.

The analytical DEAE profiles were different (Fig. 2). The acid extract showed one main uronic acid peak eluting at a low ionic strength, which indicated the presence of highly esterified pectins (20). A neutral component (reaction with orcinol but not *m*-hydroxydiphenyl) was not retained on the column. The profile of acid extracted pectin was similar to the typical ion-exchange pattern for an acid pectin extract from citrus fruits and apples (21, 22). In addition, one noticeable feature was the presence of material with a lower degree of methoxylation. The presence of a lower methoxylated pectin might be due to the residual endogenous PE activity present in the lemon peel before drying or during the first steps of extraction. The fact that PE activity was eluted from industrial lemon pomace supported the results obtained with anion-exchange chromatography.

In the case of the enzyme-extracted pectin, elution was observed at high ionic strength. This pectin had a low degree of methylation which was confirmed by its high charge density. The charge density was also very heterogeneous. This result did not agree with the pattern that is generally reported for plant PEs. However Dénes *et al.* (23) recently reported that under low pH, apple PE deesterified pectins with an intermediary mechanism between mono- and multichain, resulting in similar DEAE patterns that are comparable to the results obtained in this study. A comparison of the orcinol and *m*-hydroxydiphenyl patterns revealed that the compound eluting earlier (elution volume between 10 and 20 mL) was richer in neutral sugars than that eluted at volume larger than 20 mL.

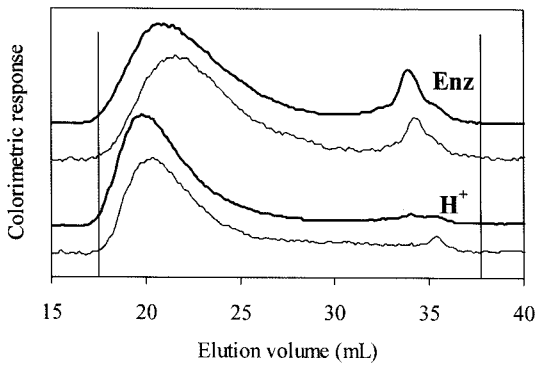

Fig. 1. Size-exclusion chromatography of the enzymic (Enz) and acidic (H^+) extracts from lemon peel. ▬, uronic acids (thick line); - , neutral sugars (thin line).

Preparative chromatography Preparative ion-exchange chromatography was carried out to purify and to further characterize the fractions identified by HPIEC. The patterns shown in Fig. 3 were in accordance with the analytical results. The acid extract was composed mostly of two populations such as a neutral fraction, being composed almost exclusively of galactose and some arabinose (peak A) and an acidic fraction containing minor amounts of galactose, arabinose, and rhamnose (peak B).

In the case of the enzyme extract, there were three retained pectin populations in addition to a very minor amount of non-retained material. The first fraction eluting at the lower ionic strength (Enz *A*) was richer in neutral sugars, mainly arabinose (Table 1). The composition of this fraction would be representative of a pectic fraction

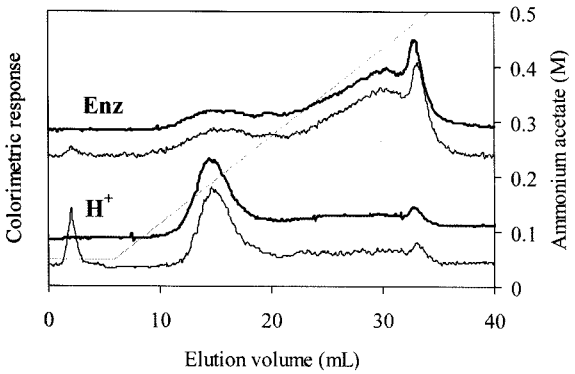

Fig. 2. Analytical ion-exchange chromatography of the enzymic (Enz) and acidic (H^+) extracts from lemon. ▬, uronic acids (thick line); - , neutral sugars (thin line); …., buffer molarity.

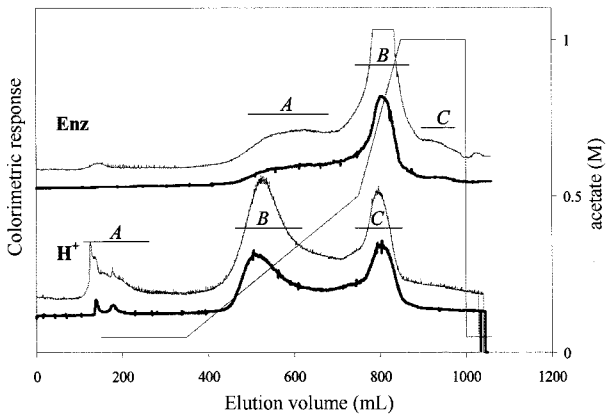

Fig. 3. Preparative ion-exchange chromatography on DEAE-sepharose, eluted with ammonium acetate buffer (pH 6) of the enzymic (Enz) and acidic (H^+) extracts from lemon. ▬, uronic acids (thick line); - , neutral sugars (thin line); …., buffer molarity.

Table 1. Yields (ratio to injected material) and sugar composition of the fractions from acid and enzyme extracted lemon pectins (in % mol)

	Acid extract				Enzyme extract			
Fraction	Total	*A*	*B*	*C*	Total	*A*	*B*	*C*
Yields		0.04	0.49	0.23		0.17	0.32	0.07
Sugars (% mol)								
Rhamnose	1	0	1	2	2	1	1	7
Fucose	0	0	0	0	0	0	0	0
Arabinose	4	10	3	4	14	10	4	32
Xylose	2	Tr.	Tr.	Tr.	2	1	0	4
Mannose	1	4	Tr.	1	1	1	1	17
Galactose	14	82	6	6	8	5	2	40
Glucose	1	4	Tr.	1	1	Tr.[2)]	Tr.	0
Uronic acid	76	0	89	84	72	82	92	ND[1)]

[1)]ND: not determined.
[2)]Tr: Trace.

rich in rhamnogalacturonan I. The second main fraction (Enz *B*) was very rich in uronic acid (> 90 mol%), and its main neutral sugar was arabinose (Table 1). The low yields (total of 56%) obtained for this extract might reflect the retention of very highly charged fractions (peak after 30 min elution in analytical ion-exchange) on the preparative column.

The chromatographic results we have obtained agree with the existence of pectin molecules in the cell walls with alternating homogalacturonic regions, and rhamnogalacturonan ones that are rich in side chains composed mostly of arabinose and galactose (23-25). During enzymic extraction, the residual endogenous PE in lemon peel acted slowly on the pectin, under low pH conditions, which led to a formation of pectin with different degrees of methylation (23). The added PG acted on demethylated areas with a random cleavage, which led to a production of high molecular weight polymers, including either more of rhamnogalacturonans (peak 1) or more of homogalacturonans (peak 2).

The arabinans that were originally present in high amounts were depolymerised during acid treatment, which demonstrated that galactose was the dominant neutral sugar and not arabinose. Some galactan side-chains were cleaved in a relatively intact condition, yielding neutral peak 1, while the rest of the pectin molecule including homogalacturonans, rhamnogalacturonans, and residual stubs of neutral sugars whose side-chains were extracted as relatively homogeneous material, quite representative of a typical 'industrial' pectin (9, 26, 27).

It can be concluded that the enzyme-extracted pectin is of high molecular weight and with varying degrees of methylation and could form gels with a divalent cation. However, it is unlikely to be able to gel under 'classical' conditions, i.e. under low pH and low water activity, due to its lesser degree of methoxylation. The use of this enzyme extracted pectin as a stabilizer in milk products, where the presence of high molecular weight polymers sensitive to calcium is characteristic, might be advantageous as a novel food additive (28).

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