PREPARATION OF PECTIC HYDROLYSATES FROM CUBAN COMMERCIAL PECTIN

J. C. Cabrera and A. Gutiérrez

ABSTRACT. Some oligogalacturonides released from the pectic polysaccharides which are present in the primary cell walls and middle lamella of plant tissues can regulate, at low concentrations, different processes associated with plant growth, development and metabolism. In this work, a procedure is proposed for the preparation of pectic hydrolysates from cuban commercial pectin concentrate. The procedure consists of an exhaüstive basic deesterification of pectin concentrate and further enzymic depolymerization of the pectic acid obtained with a commercial pectinolytic enzyme. A method is recommended to evaluate the degree of polymerization of these pectic hydrolysates.

Key words: oligopectates, cell wall, citrus pectins, pectinolytical enzymes

RESUMEN. Los oligopectatos son oligosacáridos liberados de los polisacáridos pécticos que componen la pared celular primaria de la mayoría de los vegetales y que regulan a muy bajas concentraciones diferentes procesos asociados al crecimiento, desarrollo y metabolismo de las plantas. En este trabajo se propone un procedimiento para la obtención de hidrolizados pécticos a partir del concentrado de pectina de producción nacional. El procedimiento comprende la desesterificación básica exhaustiva del concentrado de pectina y la ulterior depolimerización enzimática del ácido péctico con un preparado enzimático pectinolítico de uso comercial. Se recomienda, además, un método para evaluar el grado de polimerización promedio de los hidrolizados pécticos preparados según este procedimiento.

Palabras clave: oligopectatos, pared celular, pectinas cítricas, enzimas pectinolíticas

INTRODUCTION

Pectic substances are amorfous polysaccharides which are present in the primary cell walls and middle lamella of plant tissues. The pectin is its major component and pectin polymer backbone is based on (1-4) linked a -D-galacturonate residues, a large and varying proportion of which occurs as the methyl ester. Galacturonate sequence is interrupted by a small amount of rhamnose molecules within the main chain, and associated to it appear neutral sugar-rich segments forming side chains.

Pectic polysaccharides have an important structural function in plant cell walls and they can generate a group of signalling molecules, the oligosaccharins. An oligosaccharin is a soluble oligosaccharide having powerful effects in diverse aspects of plant growth, development and metabolism (Aldington, Mc Dougall and Fry, 1991).

Thus, either acid or enzymic hydrolysis of plant cell walls releases oligogalacturonides, acting as elicitors of phytoalexins, inducing lignification, activating proteinase inhibitor production, inhibiting auxin-induced growth and inducing morphogenetic changes in plants (Spiro *et al.*, 1993).

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The biological activity of oligogalacturonides depends on structural requirements; the main ones are oligomer size, its polyanionic character and the specific molecular shape of oligogalacturonide. Conversion of the reducing terminal D-galacturonic acid unit to the corresponding alditol diminishes its activity; nevertheless, the non-reducing terminal residue of a biologically-active Oligo-GaLA can be either D-galacturonic acid or its Δ-4,5-unsaturated derivative.

Many studies of oligogalacturonides have been conducted using heterogeneous mixtures which make the interpretation of results uncertain (Darvill *et al.*, 1992).

The broad spectrum of oligogalacturonide biological activities, the possibility of preparation from citrus residues, its biocompatible and biodegradable nature widen the possibility of using them in the agricultural field and crop biotechnology.

The aim of this work was to develop a methodology for the preparation of pectic hydrolysates from cuban commercial pectin.

MATERIALS AND METHODS

The pectin concentrate was obtained at IIIA (Research Institute of Food Industry), and prepared by extracting Persian lime peels with chlorhydric acid (Cabrera, 1994).

Pectin deesterification

Exhaustive basic deesterification of the pectin concentrate. Aqueous solution of pectin concentrate (0.5 % in AIS) was kept in a cold water bath at 4°C and pH was

adjusted to 12 with 1 M sodium hydroxide solution. After 1h, the solution was brought to pH 2.0 with 1 M hydrochloric acid and kept for 12 h under these conditions. The precipitate was gathered by filtration and desalted by successive washes with 70 % acidified ethyl alcohol (5 mL of HCl concentrate in 100 mL of 70 % ethyl alcohol) and 80 % ethyl alcohol.

Exhaustive basic deesterification of the citrus reactive pectin (SIGMA). Aqueous solution of citrus pectin (SIGMA) at 5 g/L was deesterified in the same way as pectin concentrate (as described above).

Enzymic hydrolysis

Enzymic hydrolysis of sodium polygalacturonate (SIGMA, P-1879). 225 mL of a 10 g/L solution of sodium polygalacturonate in 0.05 M Sodium phosphate buffer, pH 3.5 was treated with 25 mL of a diluted solution of a commercial enzyme preparation (Pectinex Ultra SPL, Novo Industri, Denmark, dilution factor 1:1000). Portions of the digest were removed in different times and then immediately autoclaved (15 min at 121°C) to inactivate the enzyme. The degree of polymerization in each enzymic digest was determined by the molar ratio of total uronic acid to reducing end groups and digest viscosity was measured with an Ostwald viscometer No. 509 (Schott Gerate).

Enzymic hydrolysis of the pectic acid obtained by EBD of the pectin concentrate. A pectic acid solution (3.5 g/L in uronic acid) in 0.05 M sodium phosphate buffer, pH 3.5 was hydrolized with 620 PGU of the Pectinex Ultra SPL. Portions of the hydrolysate were removed every hour and immediately autoclaved as described in the paragraph above. Undegraded macromolecules were separated by centrifugation (10 000 rpm x 15 min).

The enzymic reaction was also followed by the specific viscosity decrease of the solution, measured with a Cannon fenske No. 511 viscosimeter.

This assay was repeated using 1 400 and 2 020 PGU of enzymes.

Separation of pectic oligomers by ion exhange chromatography. Ion-exhange chromatography was performed on QAE-Sephadex A-25 (Pharmacia fine chemicals) columns (1.5 x 7.0 cm) equilibrated with Tris-HCl 0.06 M (pH 7.9) buffer. 15 mg of the pectic digest was loaded on to the column and the gel was washed with 60 mL of the buffer. The bound material was then eluted by a step Tris-HCl gradient at pH 4.8 (0.20, 0.40, 0.60 and 1.20 M; 50 mL of each one). The elution of each step was collected and the fraction with higher uronic acid content was desalted as previously described by Robertsen (1986); the degree of polymerization was calculated as in enzymic hydrolysis of sodium polygalacturonate.

Preparation and fractionation of alcohol-insoluble solids (AIS).

AIS from pectin and pectic acid were prepared and fractionated into water-soluble fraction (WSF), chelator soluble fraction (CSF) and acid-soluble fraction (HSF) by the extraction of AIS with distilled water, Ammonium oxalate (1 %, pH 4.5) and 0.05 M hydrochloric acid at 85°C as previously described by Cabrera, 1994.

Analytical Methods

Uronic acids (as galacturonic acid) were determined by m-hydroxydiphenyl method (Blumenkrantz and Hasen, 1973). Degrees of methylation were determined by Wood and Saddiqui's method (1971); reducing end groups were determined by Nelson's method (1944) and neutral sugars were quantified by Antrone's method (Dische, 1962).

RESULTS AND DISCUSSION

Pectic deesterification. Partially demethoxylated pectin concentrates produced by IIIA have a degree of esterification 20 % and a wide charge dispersion in the different pectic fractions composing it (Cabrera, 1995). This is a consequence of the acid deesterification method used to prepare such a concentrate. The IIIA made a comparative study between acid and basic deesterification methods using HCl and NH_4OH respectively, to obtain low methoxyl pectins. They found that working at pH 0.5-1 with hydrochloric acid at temperatures between -1 and 4°C, the lowest methoxyl pectins were obtained, however, a complete deesterification of pectic molecules was not obtained (Veliz, 1992).

The basic deesterification process of pectin with sodium hydroxide at pH≥12 garantees an exhaustive demethoxylation with minimum degradation if the reaction conditions are adjusted in such a manner as to reduce β–elimination reactions to a minimum (Thibault, 1984).

Taking into account those reports, the reaction conditions to obtain pectic acid by exhaustive basic demethoxylation with NaOH was studied resulting in the methodology presented here (see Materials and Methods).

Composition of raw material and the product obtained according the this methodology to demethoxylate pectic concentrate and citrus pectin (SIGMA) is shown in Table I. Furthermore, sodium polypectate (SIGMA) composition prepared from citrus pectin (SIGMA) by basic deesterification is also presented.

Table I. Chemical composition

	GUA	DM	DP	OSAS
Pectin concentrate (IIIA)*	0.85	20	n-d	0.40
Pectic acid prepared from pectin concentrate (IIIA)	47	5	n-d	13.3
Citrus pectin (SIGMA)	85	60	n-d	4
Pectic acid prepared by BD of citrus pectin (SIGMA)	100	0	34.9	0
Sodium polypectate (SIGMA)	85	6	35	1

GUA - Galacturonic acid
DM - Degree of methylation
DP - Degree of polymerization
OSAS - Neutral sugars
n-d - non determined
*... % of GUA and OSAS expressed in relation (w/v)

This table shows that degree of methylation of pectin concentrate decreases from 20 to 5 % with the methodology used while reactive pectin (SIGMA) is completely deesterified.

The degree of methylation was determined by Wood and Sadiqqui's method, which might not detect any methyl esterification because it used a similar deesterification approach to the one used here; therefore, to confirm the results with greater certainty, it is necessary to fraction by selective extraction the AIS composing pectin concentrate and pectic acid obtained from its EBD. Results are reported in Figure 1.

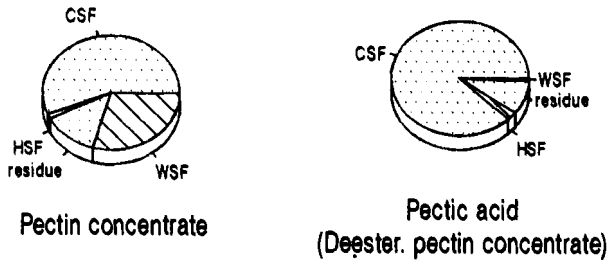

Figure 1. Fractionation of AIS from pectin concentrate and pectic acid obtained by its EBD

This result shows that oxalate soluble fraction increase proportional to low water soluble fraction is observed as a consequence of deesterification. High methoxyl pectins are water soluble while low methoxyl pectins and pectates are soluble in oxalate solutions (Saulnier, Brillouet and Moutounet, 1988). Thus, pectic substances present in the pectin concentrate, specially water soluble pectinic acids, are exhaustively deesterified through this methology.

The high content of neutral sugars in the pectic acid obtained suggests that associations between the pectic backbone and neutral sugar-rich segments are highly resistant to basic deesterification conditions. To obtain high purity pectic acid using this methodology, it is necessary to use high purity pectin or purify the pectic acid obtained.

Enzymic hidrolysis

The separation of oligogalacturonic acids is a prerequisite to study the action pattern of pectolytic enzymes and the isolated oligogalacturonides are very important to study the defense responses and morphogenetic changes in plants as induced by it. Consequently, in the latest years many investigations have been developed for the preparation and separation of oligogalacturonides.

The traditional proceeding consists of the polygalacturonic acid degradation by purifying endopolygalacturonase enzymes from *Aspergillus niger* extract and the isolation and purification of pectic hydrolysates by some chromatographic methods (Endred, Omran and Glerschner, 1991).

Commercial enzyme preparations are a convenient source of a wide variety of different enzymes and have frequently served as the starting material for their isolation. Pectinex Ultra SPL is a commercial pectic enzyme preparation derived from *Aspergillus niger* cultures. The endopolygalacturonase [poly (1,4-α-D-galacturonide) glycanhydrolase (EC 3.2.1.15)], exopolygalacturonase [poly (1,4-α-D-galacturonide) galacturonohydrolase (EC 3.2.1.67)], endo-pectin lyase [poly (methoxygalacturonide) lyase (EC4.2.2.210)], pectin esterase [pectin pectylhydrolase (EC 3.1.1.11) and endocellulases (Okai and Glerschner, 1991) have been identified in it.

The possibility of using this enzymic preparation without prior purification was evaluated. The pectic enzyme activity of Pectinex Ultra SPL under the conditions used by pectic acid hydrolysis is given in Table II.

Table II. Enzymic activity of the Pectinex Ultra SPL

Enzymes	Enzymic activity*
Polygalacturonase	804
Polymethylgalacturonase	963
Pectin lyase	< 10
Pectate lyase	n-d

n-d: non determined
*pectinolytic enzymatic activity was determined as described by Cabrera, Paz-Lago and Gutiérrez (1994)

As shown in Table II, the polygalactoronase activity of pectinex Ultra SPL is high while pectin and pectate lyase activity is low. Pectate lyase enzymes cleave α (1,4) bonds between deesterified galacturonate units in pectic acid by β-elimination releasing Δ4,5 unsaturated oligomers. The higher polymethylgalacturonase activity is not important during pectic acid hydrolysis.

To know the action pattern of polygalacturonase enzymes presented in the Ultra SPL, the enzymic hydrolysis of sodium polygalacturonate with this extract was studied.

As shown in Figure 2, 50 % loss in viscosity of polygalacturonate solution was observed when 5 % of the glycosidic bonds had been hydrolized.

A large decrease in viscosity accompanied by a small increase in the amount of aldehyde group indicates that the enzyme hydrolizes internal bonds of PGA. This fact confirms the endo-character of this enzyme. Nevertheless, in the last step of the hydrolysis reaction rate decreases and viscosity variation is very slow. This result suggests that in the last phase of degradation the enzyme proceeds by another mechanism (Thibault, 1983).

The viscosity of pectic acid (obtained by EBD of pectin concentrate) solutions during the enzymic hydrolysis with different enzyme/substrate relation as a function of reaction time is shown in Figure 3.

In general this parameter follows a similar performance to sodium polypectate. However, there is a lesser viscosity reduction of pectic acid solution compared to sodium polypectate for identical reaction times and an almost equal enzyme/substrate relation. It should be due to a lower pectic acid purity obtained by national pectin EBD. Neutral polysaccharides including these impurities contribute to solution viscosity and, seemingly, Pectinex Ultra SPL enzymic preparation does not contain enzymes capable of degrading these polysaccharides under the reaction conditions used; thus, a product viscosity decrease is mostly caused by pectic acid degradation.

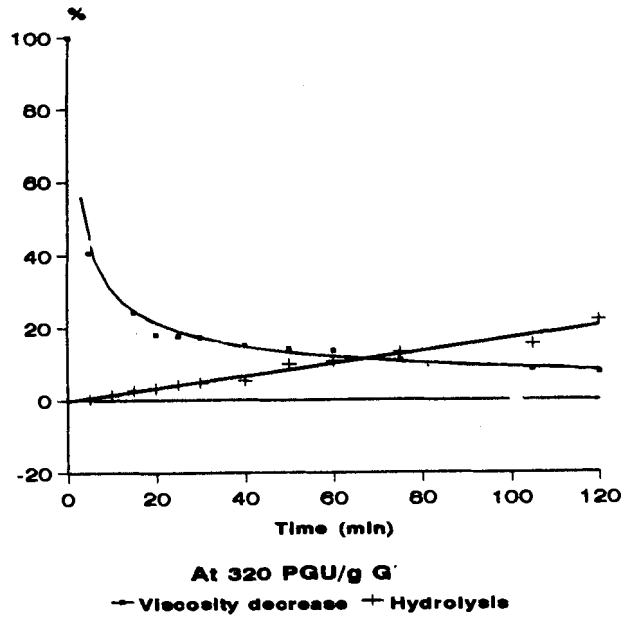

Figure 2. Sodium polypectate (SIGMA) hydrolysis with Pectinex Ultra SPL

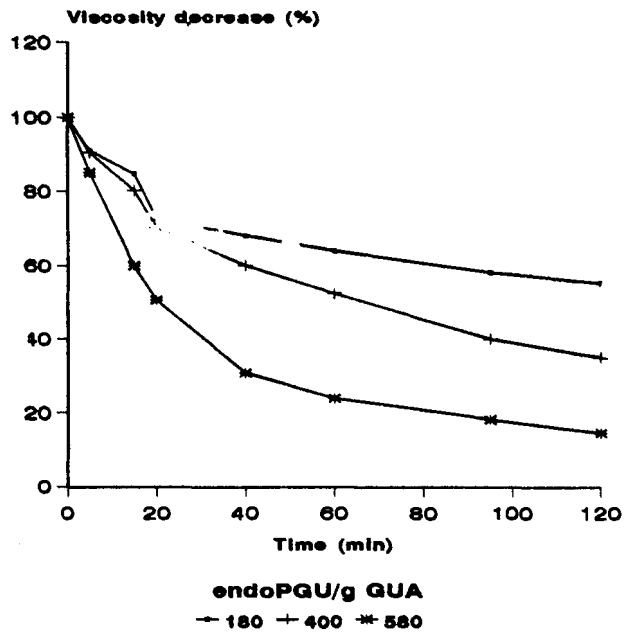

Figure 3. Enzymatic hydrolysis of pectic acid at different enzyme/substrate relations

Ion-exchange chromatography is an adequate way for eliminating these neutral polysaccharides and, in this sense, anionic resins are used (Jin and West, 1984; Robertsen, 1986) particularly QAE-Sephadex A-25 (Hahn, 1992).

Figure 4 shows batch elution profiles for pectic acid hydrolysates obtained by EBD.

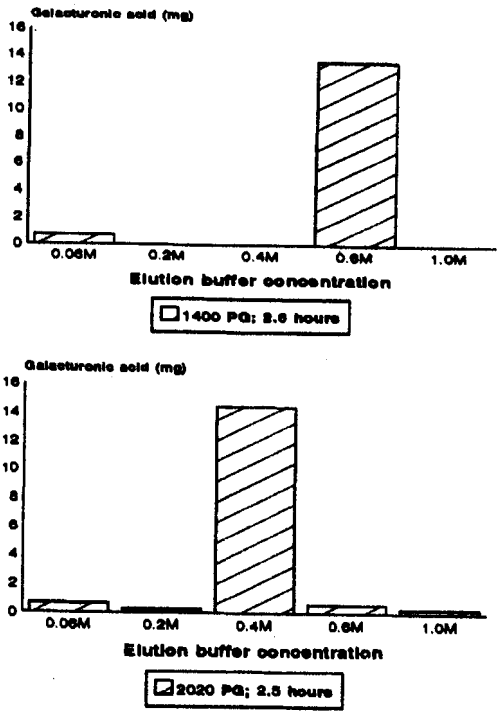

Figure 4. Hydrolysate chromatography on QAE-Sephadex A-25 for a hydrolysis time of 2.6 hours by using 1 400 and 2020 UPG

A higher retention of greater-molecular weight pectic oligomers is evident. At the same time, chromatography on QAE-Sephadex enables to follow reaction course regarding the average polymerization degree of oligomers obtained. No neutral sugars are detected in eluates having different ion strength (data not shown). Batch elution methodology is feasible to take advantage of column capacity for oligomer purification and yields of 14-15 mg galacturonic acid, according to the working conditions used.

In brief, this methodology is quite interesting as a relatively quick and cheap alternative to obtain oligogalacturonides in the laboratory. On one hand, all techniques employed permit to study raw materials and products obtained; on the other hand, results show the need to get high-purity pectic acid as raw material to obtain pure oligomers from galacturonic acid. In this sense, investigations are under way.

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