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Research Article Open Access

# Evaluation of Enzymatic Pectin Extraction by a Recombinant Polygalacturonase (PGI) From Apples and Pears Pomace of Argentinean Production and Characterization of the Extracted Pectin

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## Abstract

Argentina produces, annually, 1.8 million tons of pome fruit, distributed almost equally between apple and pear. These fruits, produced in the High Valley of Río Negro are already a registered brand in the world of fruit. *Aspergillus kawachii* produces an acidic polygalacturonase (PGase) called PGI that has attracted considerable interest because of its potential use in the food industry, particularly those involving fruit production in this region (such as pectin extraction). The enzyme was cloned and expressed in *Saccharomyces cerevisiae*. The objective of this work was to evaluate the use of PG1 in the enzymatic pectin extraction from apples and pears pomace. Characterization of the extracted pectin was also done. The performance of PGI extraction process was compared to the traditional chemical extraction process and to the enzymatic extraction with commercial enzymes. The esterification degree and the content of uronic acids from the pectin obtained were determined. In all cases, the extraction with PGI had higher yields than the chemical extraction process. Enzymatic extracted pectins exhibited an esterification degree> 50% so that they can be considered belonging to the group of high methoxylpectins. According to these results, PGI could be used to produce pectins from fruit pomaces converting these materials, currently considered wastes, into by-products of the fruit industry.

**Keywords:** Polygalacturonase; Pectin extraction; Apples; Pears

## Introduction

Among the major constituents of plants cell walls there are pectic substances, a mixture of acidic and neutral polymers, highly branched, and generically called pectins. Pectin is described, in a very simplified manner, as galacturonic acid linear segments linked by β-(1,4) with a polymerization degree of 70-100 (homogalacturonan, HG), periodically interrupted by segments of rhamnogalacturonan (RG) that are branched [1].To determine the number of groups esterified with methanol two different criteria can be use alternately: the Esterification Degree (ED) and the content of methoxyl. The ED is defined as the amount of carboxyl groups belonging to the remains of AGA esterified, in relation to their total number. Methoxyl content is calculated by relating the total mass of the methoxyl groups to the total mass of residues AGA (esterified or not). An ED 100% (all the carboxyl groups neutralized with methanol) corresponds to a methoxyl content of 16.32%. There are two different categories of pectins in relation to this property: the ones that possess a methoxyl content greater than 8% (ED> 50%) are called high methoxyl pectin (HM) and those that have methoxyl content less than 8 % (ED <50%), are called low methoxylpectins (LM) [2]. Pectins have some properties such as gelation, stabilization of emulsions and contribution of nutritional fiber; because of this they have a broad application in the food and pharmaceutical industries, among others. For its production are used some byproducts of fruit juice industries, mainly cítric peels and apple pomace [3].

The industries widely used physical-chemical processes, to produce pectin from plant tissues. However, physical-chemical methods have the disadvantage of handling both strong acids and high temperatures [4]. The pectinolytic enzymes have the ability to degrade pectins; these could be used as an alternative method of pectin extraction from by-products of the fruit industry [5].

*Aspergillus kawachii* IFO 4308 is a filamentous fungus that produces several acidic depolymerases such as a-amylases and glucoamylases [6], xylanases [7], and acidophilic proteinases [8]. An acidophilic polygalacturonase (PGase) woke up a great interest because it showed a maximum activity at pH 2.0 and also has PPase activity, it was called PG1 [9]. This enzyme was purified and characterized biochemically, and turned out to be a PGasabe a PGasa of an endo-type with MW 60 kDa. Its most important property is the ability to hydrolyze substrates in the pH range 2.0-3.0. Moreover, it was determined the N-terminus sequence of the mature polypeptide (STCTFTDAATASESK) [10]. As a continuation of that work, PG1 was cloned and expressed in an inducible vector (pYES2) for *Saccharomyces cerevisiae* [11].

The industrial production of pectin is concentrated in some European countries, mainly Germany and England, where there are plants that produce more than 8,000 tons annually [12]. In Argentina there is no significant production of pectin, being mostly imported, making this a strong component that increases the production costs. The High Valley of Río Negro is a fruit zone by excellence, where these fruits are already a registered brand in the world of fruit. There are many related industries that produce large quantities of by-products during processing of the fruit, which may be reused for a subsequent extraction of pectin. In previous studies it was determined the pectin content in different varieties of apples and pears produced in the High Valley of Río Negro, demonstrating with the result that these fruit have high values compared to the contents reported for other fruits [13]. Given this background it becomes relevant in this country the study of

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systems that allow the enzymatic extraction of pectin, offering in this way an alternative for the utilization of fruit waste through innovative biotech treatments and low environmental impact.

The objective of this work was to evaluate the use of PG1 in the enzymatic pectin extraction from apples and pears pomace, thus increasing the range of value-added products offered by local production. Subsequently it was possible to evaluate the performance of PGI extraction process comparing it with the traditional chemical extraction process and with the enzymatic extraction using commercial enzymes. Finally, the extracted pectin was partially characterized by determining the content of uronic acids (expressed as AGA) and the esterification degree.

## Materials and Methods

### Chemicals

Polygalacturonic acid (PGA), D-Galacturonic acid monohydrate (GALA), and m-hydroxydiphenyl were obtained from SIGMA CHEMICAL CO. (St. Louis, MO, EE.UU). All of these products were used as recommended by the manufacturers. All other chemicals were of analytical grade.

### Enzyme sources

PGI of *Aspergillus kawachii* was obtained from *Saccharomyces cerevisiae* clone cultures containing the inducible vector pYES2.These cultures were carried out in a 1.5 l Bio Flo 310 Benchtop Bioreactor (New Brunswick Scientific Corp., Edison, NJ, USA) through the use of a fed-batch culture system [14] with a synthetic medium containing glucose as carbon and energy source (FCE), urea as a nitrogen source (FN), salts, and micronutrients defined in previous work [15]. The culture pH was measured with a glass electrode (Mettler-Toledo). The dissolved oxygen was measured with a polarographic-type electrode (Mettler Toledo).The biomass was removed by centrifugation(10 min, 4°C) at 16,000×g, and the supernatant was concentrated by evaporation under reduced pressure to reduce total volume and then stored at -20°C.This solution had a polygalacturonase activity of 40 U/ml.

A commercial highly concentrated polygalacturonase (BA) by NUTRING S.A, and two enzymatic pools with high activity poly galacturonasa ($J_1$and $J_2$) by JUGOS S.A. company were obtained.

### Buffer composition

Citric phosphate buffer (CPB): 50 mM citric acid and 25 mM $Na_2HPO_3$, pH=4. In all cases, it was utilized a dilution of this buffer (BCP½).

### Enzyme activities

PGase activities (PGI, BA, J1, J2) were determined as described by Contreras Esquivel [16] and Cavalitto [17] with a few modifications. These assays were based on measuring the releasing rate of reducing groups from PGA by the method of Somogyi-Nelson with GALA as standard. One unit of PGase activity was defined as the activity that releases a reducing power equivalent to 1 mmol of GALA per min. The enzyme activities were measured at pH=4with 2.0g/L of PGA in CPB ½ as the substrate. The concentrate enzyme solutions were diluted with CPB ½ buffer to measuring correct of its enzyme activities.

### Pectin extraction

For the tests it was used pomace of the varieties Granny Smith in apples and Winter Bartlett in pears. These varieties were chosen because they had high pectin content in previous studies [13].The samples came from the fruit production of region of High Valley of Río Negro.

PGI was used for enzymatic pectin extraction and the performance of PGI extraction process was compared to the traditional chemical extraction process and to the enzymatic extraction with commercial enzymes.

The applied methodology was previously described by Zapata-Zapata [12] with a few modifications. The technique for pectin enzymatic extraction consisted of mixing pomace (1 g) with the BCP ½ buffer (1,5 ml) at pH 4 and each enzyme diluted solutions (2 ml). These dilutions were suitably made with all enzymes to achieve a polygalacturonase activity in the final solution of 35 U. System was agitated in a reciprocant shaker water bath Jeio Tech BS-11at 150 strokes/min for 6 hs at 37°C.

Pomace chemical extraction was effected by hydrolyzing in acid medium, with a final pH of 1.5. The solution was allowed to boil for 40 minutes.

Pectin obtained in both methods was separated from the solution by filtration. Then, ethanol 96% (v/v) was added to the permeate. The gel formed was centrifuged and then dried in an oven at 40 °C during 4 hours. It was cooled, weighted and this operation was repeated every hour until constant weight. With this procedure, the measure of pectin corresponds to the dry weight of ethanol insoluble material (EIM) [10].

### Characterization of the extracted pectin

**Determination of uronic acids:** The colorimetric method of m-hydroxy diphenyl (mhdf) was employed to quantification of pectin. This tecnic uses the determination of uronic acids content applying a pattern AGA monohydrate curve (0-150 mg / L). Measurements were made in a spectrophotometer Metrolab330 [18].

**Determination of esterification degree (ED):** A titrimetric method it was used to determinate the amount of carboxyl groups present in the remains of AGA sterified. Fifty mg of pectin was dissolved in 10 mL of boiled distilled $H_2O$ and was titrated with 0.1 N NaOH ($V_1$) using phenolphthalein as indicator. Subsequently, was added 20 mL of NaOH (0.5 M) and allowed to stand for 30 min. Then the solution was neutralized with 20 mL HCl (0.5 M) and stirred vigorously. It was titrated again with NaOH (0.1 N) ($V_2$). Degree esterification (DE) was calculated using this equation [19]:

$$DE(\%) = \frac{V_2}{V_1 + V_2} \times 100$$

Values of ED were obtained as average from three replicates.

### Statistical method

One-way ANOVA F-Test was performed to compare the yields of different methods pectin extraction used in this study essay, and the ED and uronic groups content of extracted pectins. It was used a significance level of 95% (p-value = 0.05). Statistical analysis was done using software Matlab (version R2009A).

## Results and Discussion

The results of pectin extraction are shown in the Table 1.

In all cases, the results of the pectin extraction from apple and pear pomace allowed to observe a great performance when using PGI. This would indicate a higher affinity of the PGI enzyme for this substrate.

| | Apples (85% Humidity) | Pears (90% Humidity) |
|---|---|---|
| | **Yield (g EIM/100 g material)** | |
| **PGI** | 26,00 – 20,60* | 68,40 – 42,00* |
| | 3,90 – 3,09** | 6,84 – 4,20** |
| **BA** | 13,60 – 9,87* | 28,00 – 18,00* |
| | 2,04 – 1,48** | 2,81 – 1,80** |
| **J1** | 4,33 – 2,93* | 12,60 – 6,80* |
| | 0,65 – 0,44** | 1,26 – 0,68** |
| **J2** | 5,00 – 2,13* | 12,90 – 6,70* |
| | 0,75 – 0,32** | 1,29 – 0,67** |
| **Chemical** | 20,88 – 15,30* | 25,10 – 5,94* |
| | 3,13 – 2,29** | 2,51 – 0,59** |

*Dry basis of material processed, **Wet base of material processed. EIM: ethanol insoluble material

**Table 1:** Yields pectin extraction (ethanol insoluble material).Yield: g pectin/100 g tissue.

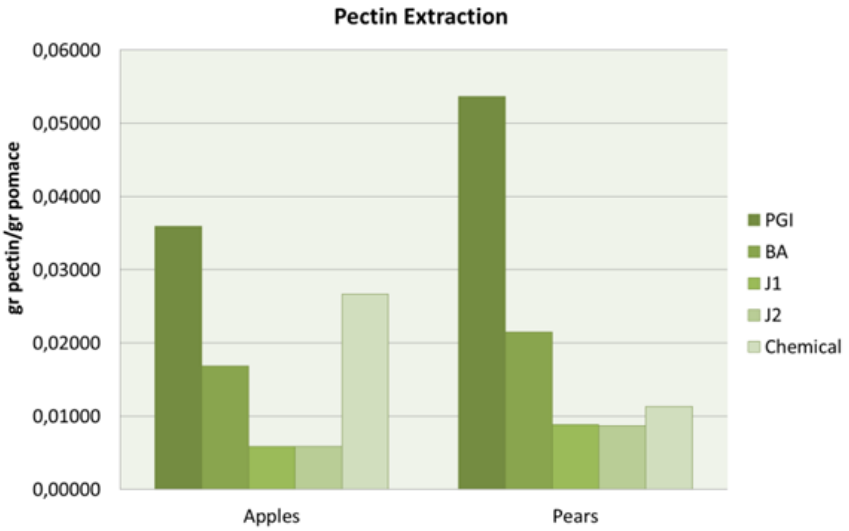


**Figure 1:** Pectin enzymatic extraction from pear and apple pomace (g pectin /g pomace).

Particularly for apples, the pectin extraction by PGI was approximately 20% higher than with chemical extraction, 50% higher than with BA enzyme, and 80% higher than with $J_1$ and $J_2$enzymes. And for pears the pectin extraction by PGI was approximately 60% more than with BA enzyme and chemical extraction, and 80% higher than with $J_1$ and $J_2$ enzymes. The BA commercial enzyme and chemical extraction showed a lower yield than PGI. However, the yield of the $J_1$ and $J_2$ enzymatic pools was much lower. Therefore, PGI enzyme presented optimal conditions to pectin extraction from fruit residues. One-way Analysis of Variance (ANOVA) indicated a p-value less than 0.05 in both cases (apples and pears); this showed that there is statistically significant difference between the yields pectin extraction by different methods. Through analysis of multiple comparisons, it was found that the yields pectin extraction by PGI have average different significantly and superior about the yields pectin extraction by the other methods.

On the other hand, it was observed also that pears showed a higher content of pectin than apples (Figure 1). These results indicate that pear pomace seems to be a better substrate for pectin enzymatic extraction rather than apple pomace (Figure 1).

In apples the ED of the enzymatically extracted pectins was for PGI 80%; BA 84%; J1 85% and J2 80% since the ED of the chemically extracted pectin was 61%. In pears the ED was for PGI 84%; BA 86%; J1 87% and J2 89%. And the ED of the chemically extracted pectin was 63%. All samples showed an ED > 50% so they can be considered belonging to the group of HM pectins. These pectins only gelled in the presence of relatively high concentrations of sugar, in acid medium. Conversely the gelation of pectins LM occurs in the absence of sugar, but in the presence of concentrations of ions, particularly divalent (for example $Ca^{+2}$). About 80 % of the world production of highly methoxylated pectin is used in the manufacture of jams and jellies, in order to correct the natural deficiency pectin of some fruits [2,20,21]. One-way Analysis of Variance (ANOVA) indicated a p-value less than 0.05 in both cases; showing that there is statistically significant difference between the ED of extracted pectins by different methods. Through analysis of multiple comparisons, it was found that the ED of enzymatically extracted pectins has averages different significantly and higher about chemically extracted pectins. This decrease in the ED of the pectin is due to chemical extraction is more aggressive and damages the molecular structure and release the methoxil groups from the sterified AGA residues.

Uronic groups content (principally AGA) in EIM was about 60% in both fruits.

## Conclusions

According to these results, PGI could be used to produce pectins

from fruit pomaces converting these materials, currently considered wastes, into by-products of the fruit industry.

At the same time it was observed that these results are within the ranges reported for other cultivars elsewhere.

Both, fruit production elected from High Valley of Río Negro and the PGI enzyme could be good agents for the pectin extraction with different purposes, which would give added-value to the local production.

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