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Production of an Acidic Polygalacturonase from *Aspergillus kawachii* by Solid State Fermentation and Their Application for Pectin Extraction

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Abstract An acidic polygalacturonase (PG) from *Aspergillus kawachii* was produced by solid state fermentation employing a polyurethane foam support. The conditions used for the production of acidic PG were particle size of support (0.6 or 500 mm^3) and fermentation time. From the factors studied, the particle size had important influence on enzyme production. The best conditions for acidic PG production were 0.6 mm^3 particle size, 18 hr at 30°C and initial pH of 5.0. In addition, pectin was extracted from citrus pomaces (grapefruit, lime, and tangerine) by acidic PG at 50°C for 24 hr with citric acid solution. Infrared spectroscopy showed that lime pomace had more high-methoxylated (65%) endogenous pectin than was obtained than from grapefruit or tangerine pomaces. The enzymatically extracted pectin yield in dry basis (d.b.) for grapefruit and lime pectins were 6.95 and 4.25%, respectively. The citric acid solution alone also contributed to pectin extraction from citrus pomaces (7-9%, d.b.). Limited pectin extraction by acidic PG from tangerine pomace was most likely due to the presence of low-methoxylated endogenous pectin. The enzymatic method for pectin extraction using acidic PG from *A. kawachii* is a promising technique for releasing highly polymerized pectic substances from high-methoxylated lime or grapefruit pomaces.

Keywords: pectinase, pectic substance, citrus pomace, pectin-releasing enzyme, solid state culture

Introduction

Citrus fruit constitutes one of the most important horticultural commodities in Mexico and it is cultivated in the tropical and subtropical regions of the country. Among them, Hidalgo State is an important region (Huasteca Hidalguense) with more than 6,000 ha used for citrus production (1). The citrus industry in Hidalgo State makes substantial contributions to the Mexican economy through fresh fruit sales at local and country markets and some processed products. During citrus juice production, the peel waste accounts for approximately 35-45% of the total fruit weight (2). The term pomace refers to citrus peel which has been carefully dried after leaching with water to lower the concentration of soluble sugars and acids (3,4).

Most the industrial procedures adapted for pectin extraction are based on thermo-chemical hydrolysis of the citrus pomaces with mineral acids (5). With these methods there is environmental pollution due to discharge of waste water from the acid extraction process. In addition, corrosion of the equipment used during extraction is enhanced. Other methods for pectin extraction from pomaces include extrusion (6), microwave (7,8), high pressure (9), or ultrasound (10). Since these methods consume a lot of energy, attempts have been made to develop a procedure on the basis of a biotechnological principle able to use for pectin extraction (11). Biotechnological methods are based on the limited pectin-degradation of insoluble pectic substances (protopectin) for subsequent releasing of water soluble pectin under mild conditions by action of restricted enzymatic degradation.

Chemically, pectin is constituted mainly of galacturonic acid units linked by α-(1$\rightarrow$4) bonds (12). Detailed information, new insights and elaborated models of pectin structure can be found in recent reviews (13,14). Polygalacturonase (PG) from fungi (15) or yeast (16) has been used for pectin extraction from citrus (17-19), apple (20,21), or pear pomaces (22). PGs are hydrolytic pectic-degrading enzymes capable of catalyzing the hydrolysis of α-(1$\rightarrow$4) glycosidic bonds between adjacent unesterified galacturonic acid moieties present in homogalacturonan region of pectic substances (16).

Acidic enzymes are considered as potential catalysts with specific application for development of clean-technology processing. *Aspergillus kawachii* Kitahara, a white-*koji* mold, is commonly used in Asia to make alcoholic beverages. In Japan, white-*koji* mold is used for preparation of traditional *soju* (23), meanwhile *takju (makgeoli)* is prepared in Korea (24). Other products have been developed using this fungus in China (25). Production of acidic PG from *A. kawachii* by liquid state culture using glucose or complex raw materials as source carbohydrate has been studied (26,27). Productions of PGs from *Aspergillus niger* by solid state fermentation (SSF) have been studied using polyurethane foam (PUF) support (28).

To date, however, there has been no report on the production of acidic PG from *A. kawachii* using PUF. In this paper, a research work was carried out to determine the conditions for production of acidic PG from *A. kawachii* by

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SSF employing PUF support. Finally, we investigated the process for pectin extraction from citrus pomaces using acidic PG.

Materials and Methods

Materials Polygalacturonic acid, pectins with 30, 60, and 90% degree of methyl-esterification, galacturonic acid monohydrate, and Tween® 80 were purchased from Sigma-Aldrich (Mexico City, Mexico). Blue dextran 2000 and 30 kDa cutoff membrane were purchased from Amersham Biosciences/GE Healthcare (Uppsala, Sweden). Pectinex Ultra SP-L® was provided from Novozymes (Bagsvaerd, Denmark). Polyurethane foam (PUF) was procured from Mexican Polyurethanes Co. (Ramos Arizpe City, Coahuila State, Mexico). PUF was cut in cubes of 500 mm^3 or powder ground through a turbo mill (Pulvex® 100 Mini, Mexico City, Mexico) to pass 0.6 mm^3-mesh screen. Both materials were washed with distilled water at 90ºC twice and dried at 60ºC until constant weight. Grapefruit (*Citrus paradasi*), lime (*Citrus aurantifolia*), and tangerine (*Citrus reticulata*) pomaces were obtained from our Food Research Department Collection (Saltillo City, Coahuila State, Mexico) (29). Pomaces were ground using a Wareing blender (Osterizer, Mexico City, Mexico) to pass 50-mesh screens and stored at room temperature in sealed plastic bags. Cobalt chloride, isopropanol, and glucose were purchased from Jalmek Scientific Co. (San Nicolas de los Garza City, Nuevo Leon State, Mexico). Other chemicals were analytical grade.

Microorganism The strain of *Aspergillus kawachii* NBRC 4308 used in this study was purchased from NITE Biological Resource Center (NBRC), Biotechnology Department (Chiba, Japan). The fungal strain was provided from Coyotefoods Biopolymer and Biotechnology Co. (Saltillo City, Coahuila State, Mexico) and maintained on skim milk-glycerol cryoprotectant at –20ºC until its use. Inocula were prepared from 6 days *A. kawachii* cultured on potato dextrose agar by suspending of the spores in 0.01%(v/v) Tween 80®.

Culture conditions and processing of fermented medium Culture experiments were done in Erlenmeyer flasks (250-mL) containing a mixture of 3 g of PUF and 7 mL of culture medium with the following composition (g/L): glucose (10), tryptone (5), K_2HPO_4 (1.0), KCl (0.5), $MgSO_4 \cdot 7H_2O$ (0.5), and $FeSO_4 \cdot 7H_2O$ (0.01). Before inoculation, flasks were sterilized at 121ºC for 15 min. Sterilized flasks were inoculated with 2×10^7 spores/g of support. SSF was carried out at 30°C for 42 hr. For PG recovery, the content of each flask was mixed with 10 mL of distilled water. The fermented medium from SSF was recovered at room temperature by pressing the PUF using a muslin cloth. Extracts were adjusted at pH 5.0 and passed by fiber microfilters (cellulose acetate, 0.45-μm; Whatman International Ltd., Kent, UK) and filtered again through acetate cellulose microfilters of 0.45-μm (Whatman International Ltd.). Samples were frozen at –20ºC until use. Residual glucose from filtrates was evaluated by the glucose-oxidase colorimetric enzymatic assay (Randox Laboratories Ltd., Antrim, UK). Fungal biomass was estimated by colorimetric method after acid hydrolysis with 0.25 M phosphoric acid during 7 min (30). All experiments were done on triplicate and average values being reported.

Preparative scale production of acidic PG Preparative scale production and recovery of acidic PG was done basically in the same manner as was described above. SSF was done in aluminum trays (39×21 cm, 4.5-cm depth) containing a mixture of 300 g PUF and 700 mL of culture medium. Before inoculation, PUF and culture medium were sterilized separately at 121ºC for 15 min. Extract was processed as described above. The filtered extract was concentrated by ultrafiltration with a 30 kDa cutoff membrane on a QuixStand system (GE Healthcare) and diafiltered 3 times with 500 mL of 20 mM sodium acetate buffer (pH 4.5) at 4ºC. Concentrated extract with acidic PG (1.4 mL) was subjected to fractionation on fast protein liquid chromatography (FPLC) system (Akta Prime®; Amersham Biosciences, Uppsala, Sweden). For this test, a gel permeation column Sephadex™ G-25 Hi-Trap Desalting (5 mL, GE Healthcare) was used and equilibrated with 20 mM sodium acetate buffer (pH 4.5) with NaN_3 0.02%(w/v). The enzyme was eluted (10 mL) with the same buffer at a flow rate of 1 mL/min and 2 mL fractions were collected. The obtained fractions were analyzed for PG activity.

Acidic PG activity Enzymatic activity was determined by measuring the release of reducing sugars from 500 mg/L polygalacturonic acid solution with 50 mM sodium acetate buffer (pH 3.0) incubated for 5 hr at 37ºC. Reducing sugars were assayed with the Somogyi-Nelson reagent (31,32) using D-galacturonic acid as standard. The activity was expressed in units (U)/L where 1 U is defined as: enzyme activity that converts 1 mmol of substrate in 1 min.

Up- and down-stream processing for pectin by acidic PG Enzymatic extraction of citrus pectins were carried out in duplicate in a 1-L vacuum flask (FoodSaver, Tila, NY, USA) containing 300 mL 0.25%(w/v) citric acid, 15 g of correspondent citrus pomace, and 2 mL of concentrated acidic PG solution (0.74 U). A vacuum of −1.5 bars was then applied to close the flasks and incubated at 50ºC, on a rotary shaker at 150 rpm for 24 hr. A blank was performed in the same manner except that distilled water was used instead of enzyme. After extraction time, the whole content of the flasks was collected and filtered through muslin cloth and filter paper (Whatman 41). Filtered samples were precipitated by addition of 2 volumes of 98% isopropanol. The precipitates were collected, dried at 55ºC to a constant weight, powdered with a mortar, and stored in Eppendorf tubes for further analysis. The pectin yields are reported in dry weight of extracted material/dry weight of citrus pomace.

Fourier transform infrared (FTIR) spectroscopy Citrus pomaces and extracted pectins were investigated by FTIR attenuated total reflectance (FTIR/ATR) spectroscopy, which was performed by Perkin-Elmer (Waltham, MA, USA) equipment operating at 4/cm resolution. The mirror velocity was 0.08/cm and 35 interferograms were co-added before Fourier transformation. Spectra were collected from 4,000 to 650/cm and normalized that the absorption band at

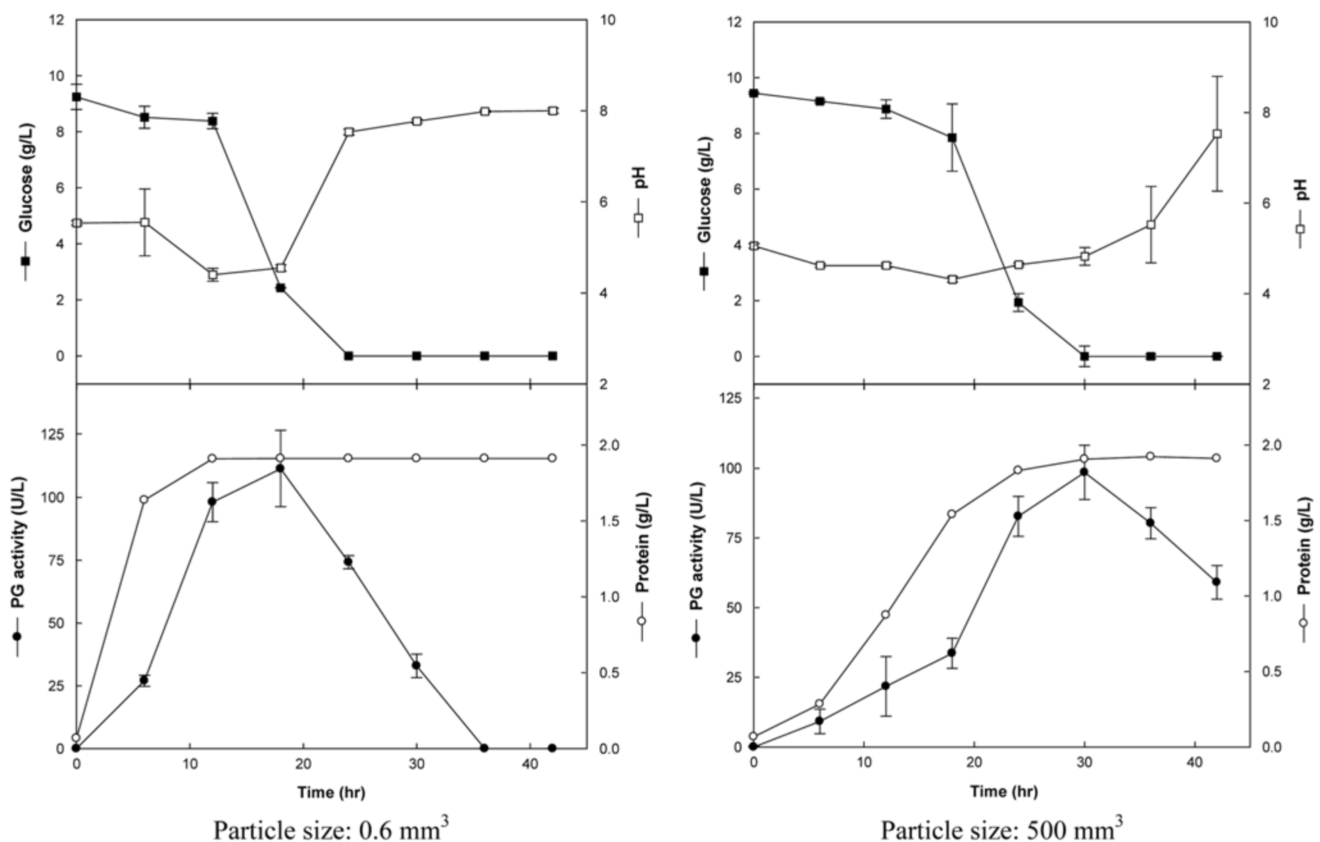

Fig. 1. Effects of particle size of polyurethane foam (PUF) and fermentation time on fungal growth, glucose consume, pH, and synthesis of acidic PG by solid state fermentation with *A. kawachii* NRBC 4308.

ca.1,008/cm equaled 1. Normalization did not alter the proportion of signals in the original spectra.

Pectin characterization by gel filtration chromatography Gel filtration chromatography of extracted pectin was carried out on a XK 50/60 column (GE Healthcare). Pectin extract (4 mg/mL) was filtered through 0.45-μm cellulose microfilters and loaded (500 mL) to fractionate on FPLC system (Akta Prime®; Amersham Biosciences). Column was packed with Sephacryl S-300 High Resolution (GE Healthcare) and equilibrated with 50 mM sodium acetate buffer (pH 4.5) with NaN_3 0.02%(w/v). The sample was eluted (90 mL) with the same buffer at a flow rate of 1 mL/min and 2 mL fractions were collected. Void and dead volume were determined using Dextran blue 2000 and cobalt chloride, respectively. The obtained fractions were analyzed for total reducing sugars by a colorimetric/enzymatic method using Pectinex Ultra SPL diluted 1:50. The method consist in hydrolyze 200 μL of each fraction with 50 μL of diluted Pectinex Ultra SPL. Samples were incubated during 18 hr at 37ºC and then assayed for total reducing sugars by Somogyi-Nelson method (31,32). Galacturonic acid was used as standard in the assay.

Results and Discussion

Production of acidic PG by SSF *A. kawachii* was cultured in glucose-tryptone media by SSF supported in PUF with the particle size of 0.6 or 500 mm^3. The growth, acidic PG activity, pH, and glucose consumed were followed over a 42 hr period (Fig. 1). During growth of *A. kawachii* 70% initial water content was used. The fermentation used in these experiments allowed a good regulation of the relative humidity which did not produce a drying of the media supported on PUF. For support size of 0.6 mm^3, the maximum peak of acidic PG activity was seen at 18 hr of fermentation with 110 U/L, meanwhile 500 mm^3 particle sizes showed maximum PG activity at 30 hr. Figure 1 also shows the influence of particle size on pH profile during fermentation of *A. kawachii* on SSF. In both fermentations, the initial pH was about 5.50, after 12 hr mycelia growth promoted rapid acidification until pH 4.30 was obtained on 0.6 mm^3 particle PUF. Meanwhile 18 hr was necessary for a large particle size. *A. kawachii* is recognized as a citric acid hyper producer for this (23), the use of SSF system presented here could be used for simultaneously production of citric acid and acidic PG. A rapid consumption of glucose by *A. kawachii* also was promoted when smaller particle size was used. On the smallest particle size of support, the fungal growth was faster for 12 hr than larger particle size.

Acidic PG from *A. kawachii* has been produced by liquid state fermentation using glucose-tryptone media, however a 24 hr period was necessary to consume glucose and obtain activity (26). Kojima *et al*. (27) also studied these enzymes by liquid state fermentation using soybean flour-polypeptone media, and the recommended time for enzyme production was 28 hr. In this study, time had a

profound influence on the rate of acidic PG production. Our studies indicate that fermentation on synthetic medium supported on 0.6 mm^3 PUF reduced the time of maxiumum acidic PG activity to 12 hr. Previous results carried out by Diaz-Godinez *et al.* (28) have shown maximum pectinase production on polyurethane foam within 18 hr, albeit at 35ºC. Hart *et al.* (33) investigated the production of PG from *Rhizopus oryzae* via SSF on orange finisher pulp. Optimization of the medium culture components for the production of pectin releasing enzymes from *Bacillus subtilis* has been recently studied with soy flour (34). In general, it seems that the particle size of the PUF affect the rate of microorganism growth, glucose consumption, and pH and therefore the production of acidic PG. The concentrated extract obtained by ultrafiltration and fractionated through gel filtration chromatography show an acidic PG of 370 U/L.

Characterization of citrus pomaces by IR spectroscopy Citrus pomaces were analyzed by FTIR in the region 4,000-650/cm to determine whether IR spectroscopy could differentiate the chemical structure and composition of the materials. The polysaccharides and proteins are the main components in citrus pomaces. The IR spectra of the dried citrus pomaces are shown in Fig. 2. The major peaks detected in citrus pomaces could be observed around 3,296 (O-H stretching), 2,920 (C-H stretching), 1,733 (C=O stretching), 1,645 (C=O stretching and C-C ring stretching), 1,466 (C-H bending), 1,234 (C-O-H bending), and 1,008/cm (C-O-C stretching). The IR spectra of citrus pomaces mainly composed of the absorption band corresponding to polysaccharides. The assignments of peaks in the IR spectra for citrus pomaces were consistent as previously reported by Contreras-Esquivel *et al.* (35). The FTIR spectra of the 3 kind of citrus pomaces have small differences especially in the range of 950-1,200/cm corresponding to carbohydrate fingerprint feature (Fig. 2). However, the spectral features between citrus pomaces vary mainly in the region from 1,185 to 1,780/cm. Small ester (1,736/cm) peaks were observed for grapefruit and tangerine pomaces indicating the presence of endogenous low methoxyl pectins. Meanwhile lime pomace show major peak at the same wavenumber corresponding to high methoxyl pectins. The observed differences between citrus pomaces FTIR profiles are attributed to pectinesterase, a demethoxylating pectin enzyme, which convert high methoxyl pectin into low methoxyl pectin (36). Also, the presence of naringin was identified at a wavenumber of 1,515/cm. The spectroscopic studies were performed in order to understand the relationship between endogenous pectic substances and its degree of methyl-esterification.

Pectin extraction by acidic PG The acidic PG produced by SSF was used to extract pectin from citrus pomaces. The yields of citrus pectins prepared by enzymatic methods and controls are displayed in Fig. 3. Major pectin yields by enzymatic treatment with acidic PG were observed from grapefruit and lime pomace. The enzymatically extracted pectin yield in dry basis for grapefruit and lime pectins were 6.95 and 4.25%, respectively. The pectin yield obtained from tangerine pomace treated with enzyme was the lowest because this substrate contains endogenous low methoxyl

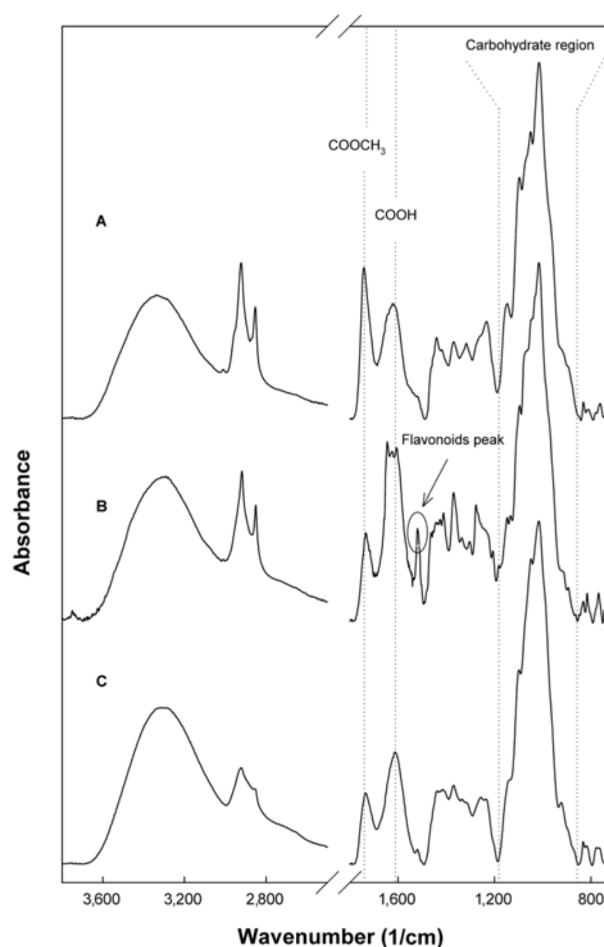

Fig. 2. IR spectra of citrus pomaces from lime (A), tangerine (B), and grapefruit (C).

pectins. The pectin yield for controls in dry basis from grapefruit, lime, and tangerine pomaces were 9.65, 8.45, and 7.16, respectively. The data show that enzymatic extraction with acidic PG from grapefruit and lime pomaces gave increase in the yield of pectic substances as compared to controls. The real contribution in the increase of enzymatically extracted pectins yields from grapefruit and lime pomace compared to controls were 41.8 and 33.4%, respectively. The pectin extracted enzymatically from tangerine pomace increase only 4.5%(d.b.) compared to control. Endogenous low methoxylated pectins are able to interact with calcium to form insoluble structures that reducing the enzymatic hydrolysis. The organic acids did not dissolve calcium-pectate, nor did PG increase pectin hydrolysis (37). The results obtained for enzymatic extraction were similar to earlier reports by Donaghy and Mckay (16) and Contreras-Esquivel *et al.* (23,26) from citrus pomaces. Apple pectin from cell walls extracted enzymatically with pectinlyase showed a pectin yield of 17.4%(d.b.) (38). Little attention has been given to evaluate the pomaces previous to enzymatic treatments for pectin recovery. In this research, pomaces were evaluated by FITR and we found significant differences in methoxyl content, while a difference in sugar content was not significant. The PG is a dependent enzyme of methoxyl content in pectic substances; furthermore the enzyme could be affected in

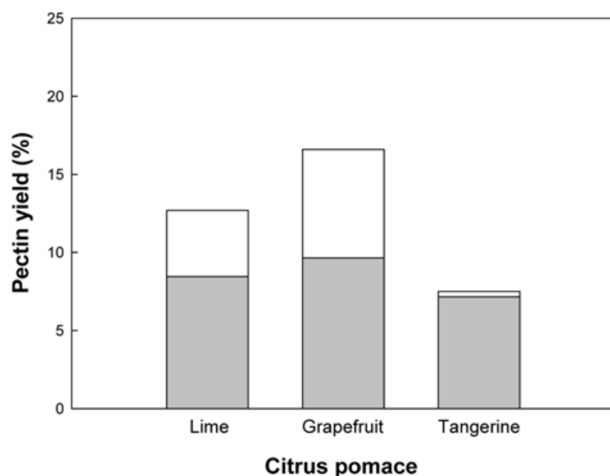

Fig. 3. Enzymatic pectin extraction yields from citrus pomaces using acidic PG from *A. kawachii*. Gray areas denote pectin extracted by acidic conditions (control) and white areas denote enzymatically pectin extracted.

their activity by insoluble structures present in pomaces. The most efficient extractions of pomaces with acidic PG were obtained with grapefruit and lime which show endogenous medium and high methoxylated pectins, respectively.

FTIR spectra of enzymatically extracted pectins The IR spectra of the enzymatically extracted pectins and its controls are presented in Fig. 4. Signals around 1,650 and 1,750/cm are indicative of free and esterified carboxyl groups which are useful in identifying pectin samples. The FTIR-ATR citrus pectins obtained by acidic PG had features similar to the control, except the 1,750/cm stretch was larger in the case of the pectin extracted with acidic PG. The FTIR was performed in order to study the relationship between endogenous pectic substances and its degree of methyl-esterification present into citrus pomaces to establish the performance for pectin extraction with acidic PG. The polysaccharides and proteins are the main components in citrus pomaces. FTIR of standard pectic polysaccharides with 0, 30, 60, and 90% of degree of methoxylation were used for comparison with extracted pectin in this study. The lime pectins show a degree of methylesterification of about 65%, while grapefruit of about 60%. Pectins from tangerine showed the lowest degree of methyl-esterification. This observation emphasizes the importance of evaluation of endogenous content of methyl-esterification present in pectins to be released with pectin releasing capacity of PG. This process involves a restricted hydrolysis of endogenous pectic substances through the α-1-4-glycosidic bond of non-methylated galacturonic acid, and the subsequent release of water-soluble pectin to extractive phase. Enzymatic pretreatment of soybean hull with mannanase increased the extractability of pectin, however by FTIR spectroscopy was evidenced recovery of low methoxylated pectins (39). Kamnev *et al.* (40) reported that FTIR spectra of pumpkin pectin extracted by HCl showed significant ester bands at 1,750/cm, while enzymatically extracted showed lower intensity corresponding to a low degree of methoxylation. The

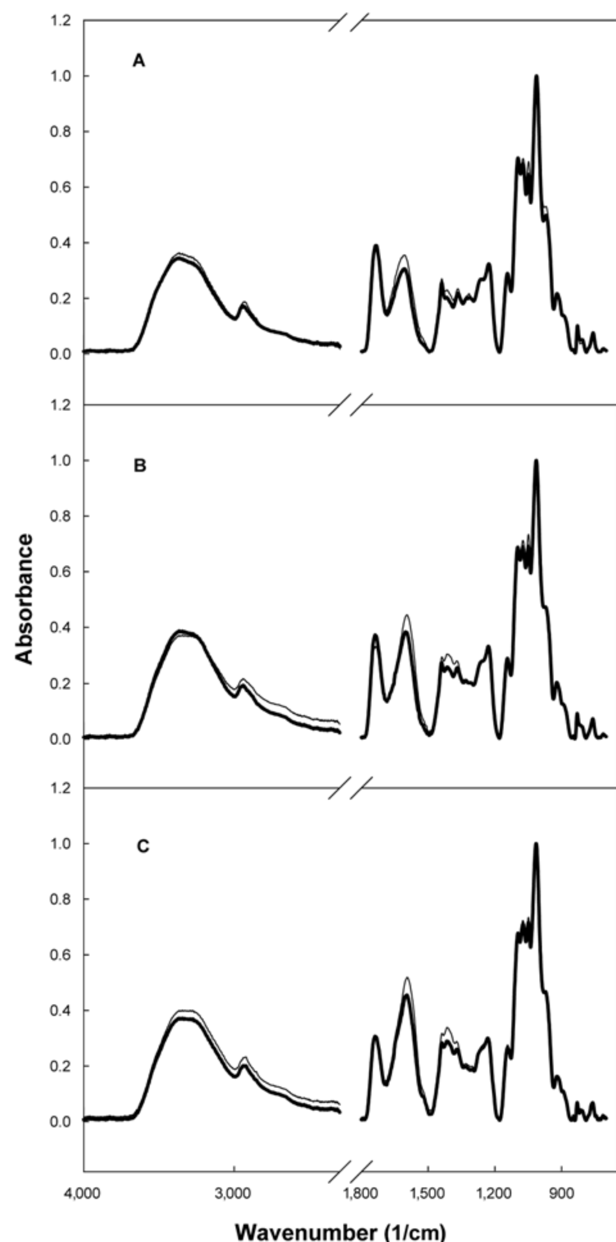

Fig. 4. IR spectroscopy of enzymatically extracted pectins from citrus pomaces by acidic PG. (A) lime, (B) grapefruit, and (C) tangerine. Thick line (enzyme) and thin line (control).

results of FTIR spectroscopy in this study indicate that the citrus pectin extracted by enzymatic method has a medium degree of methyl-esterification. The observed limitations for pectin extraction from tangerine pomace will be explained due to presence of unesterified domains of pectin which can self-associate to form aggregates with calcium-dependent mechanism in which the mechanical strength of the cell walls is increased and reduce the release of the pectin molecule by acidic PG.

Pectin characterization by gel filtration chromatography The gel filtration chromatographic profiles of the enzymatically extracted pectins and controls are show in Fig. 5. Analyzed samples showed narrow and well separated peaks. The lime pectin extracted by enzyme had the major peak at

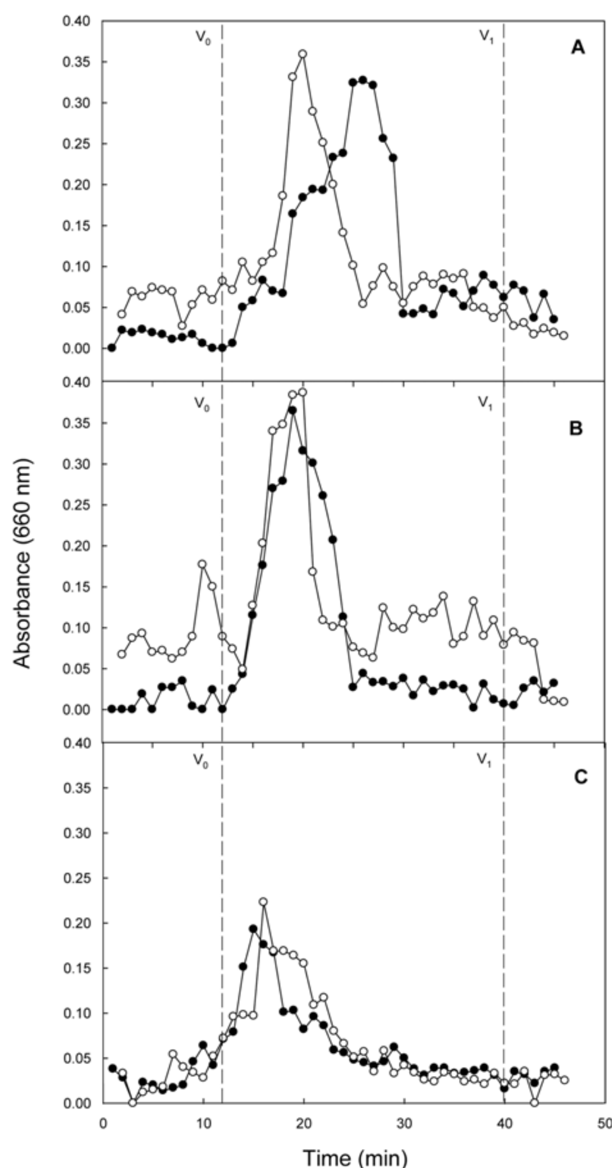

Fig. 5. Gel filtration profiles of extracted pectins from citrus pomaces by acidic PG from *A. kawachii*. (A) lime, (B) grapefruit, and (C) tangerine. Open circles, controls; black circles, enzymatic extraction.

elution times around 25 min. The enzymatic pectin-releasing activity on lime pomace gave high molecular weight and oligomeric reaction products. The blank sample for lime pomace was eluted near to void volume of the column indicating high molecular weight of the pectin. The grapefruit pectin and blank gave similar patterns for compounds of high molecular weight. The enzymatically extracted lime pectin has low apparent molecular weight than blank due to the substrate degradation by enzyme producing pectin-oligosaccharides. Renard *et al*. (38) and Choi and Lee (21) have been observed the presence of low molecular weight molecules due to partial degradation during enzymatic extraction process. The pectin extracted from the grapefruit pomace by acidic PG-treatment was higher both in apparent molecular weight than pectin extracted from lime pomace. The use of non-pectolytic enzymes for pectin extraction has been showed lower gelling strength than pectin extracted by chemical methods due to their low molecular weight (41).

The SSF have a tremendous potential for enzyme production of industrial enzymes (42) using PUF as support. The developed process is a suitable system for growth *A. kawachii* into PUF containing 10 g/L of glucose for production of acidic PG. The acidic enzyme is able to release pectin from citrus pomaces; however a limiting extrinsic factor for enzymatically pectin extraction is the endogenous degree of methyl-esterification. Citrus pomaces with high and medium methoxyl content are the best substrate for pectin extraction using acidic PG from *A. kawachii*.

Acknowledgments

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