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Isolation, characterization and modification of citrus pectins

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ABSTRACT

Orange and lemon peels were used for obtaining pectic polysaccharides. Citrus peels were previously treated with 96% ethanol, and the obtained alcohol-insoluble solids (AIS) were subjected to a sequential extraction with hot distilled water and hot 0.5% HCl. Water- and acid-extracted orange (WEOP and AEOP) and lemon (WELP and AELP) pectins were obtained. Acid extraction gave higher yields of pectin than water extraction and lemon peels were richer in pectin. Comparative investigations were carried out with chromatographically purified commercial citrus pectin (CPCP). Chemical and physicochemical characterization of all pectins was accomplished. It was found that pectins were similar in anhydrouronic acid content (AUAC, 69-81%), but differed in their degree of methylesterification (DM, 55-81%). Generally water-extracted pectins were with higher DM. Both orange pectins were with higher DM and degree of acetylation (DA, 2%), in comparison with the corresponding lemon pectins. Water-extracted pectins were with higher degree of feruloylation (DF, 0.12-0.34%). To our knowledge this is the first report on the estimation of ester-linked ferulic acid in orange and lemon peel pectins. Pectic polysaccharides differed in molecular weight and homogeneity. WELP was with the highest molecular weight and homogeneity. The pectins contained D-galacturonic and D-glucuronic acids, L-arabinose, D-galactose, L-fucose, L-rhamnose and D-xylose. All investigated pectins showed immunostimulating activity by complement activation in the classical pathway at 1.25 and 2.5 mg/mL. Pectic polysaccharides were modified with endopolygalacturonase. Enzyme-modified CPCP and WEOP had higher anti-complementary activity than the corresponding initial pectins.

Key words: citrus pectin, monosaccharide composition, ferulic acid content, immunostimulating polysaccharides

Introduction

Pectic substances in fruits were discovered by the French chemist Louis Nicolas Vauquelin in 1790 in tamarind fruit (Vauquelin, 1790). The term "pectin" was introduced by Henri Braconnot because of the gelling properties of these substances (Braconnot, 1825). Pectic polysaccharides are localized in the primary cell wall and middle lamella in all higher plants, where they are responsible for different physiological processes (Knox, 2002). In the cell walls they serve as one of the main agents cementing the cellulose fibrils and may be linked covalently to other polymers. Intracellular pectins provide the channels for passage of nutrients and water (Tamaki et al., 2008). Apples, citrus fruits, sugar beet and sunflower heads are very rich in pectins and their by-products are the most important sources for pectin industry (Kertezs, 1951). Commercial pectins are extracted at low pH and high temperature. Pectins are widely used as food additives (E440) with gelling and stabilizing properties in jams, jellies, marmalades, milks and confectionery products (Sakai et al., 1993).

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Pectic substances are complex plant heteropolysaccharides. In 1917, Ehrlich announced that the basic building block of these substances is D-galacturonic acid (D-GalA) (Ehrlich, 1917) and later Henglein and Schneider (1936) proposed that the linkages between the building units in polygalacturonic acid are 1→4. Nowadays it is well-known that pectic polysaccharides are composed of several structural regions. The most studied and simply arranged structural fragment is called homogalacturonan. It is composed of (1→4)-linked α-D-GalA residues. The carboxyl groups are methylesterified to different extent, which results in low methoxylated (< 50%) and high methoxylated (> 50 %) pectins. Some hydroxyl groups are partly acetylated at O-2 and/or O-3 (Schols & Voragen, 2003). This mode of esterification is critical for pectic functional properties. The second fragment is rhamnogalacturonan I, constituted of linear repeating structure [→2)-α-L-Rhap-(1→4)-α-D-GalpA-(1→]. Some of α-L-rhamnose (Rha) residues are substituted at O-4 and rarely at O-3 with neutral side chains, such as β-D-galactans, (1→5)-α-L-arabinans and arabinogalactans (Voragen et al., 2009). There are arabinogalactan type I (arabino-1,4-β-D-galactans) and II (arabino-3,6-β-D-galactans), which are among the most important pectic fragments responsible for different biological activities (Morris, 2009). Some α-L-arabinose (Ara) and D-galactose (Gal) residues could be feruloylated (Ralet et al., 2005). The third famous reported structure is rhamnogalacturonan II. It represents a homogalacturonan with four side chains at O-2 or O-3, composed of rare sugars like D-apiose, L-fucose, 2-keto-3-deoxy-D-manno-2-octulosonic acid (Kdo), 3-deoxy-D-lyxo-2-heptulosaric acid (Dha), aceric acid, their methoxylated analogs and others (Yapo, 2011).

It is found that pectins have several biological and physiological functions in human nutrition and health (Yamada, 1996). As dietary fibres pectic polysaccharides are able to regulate the lipid metabolism (Groudeva et al., 1997), to reduce the absorption of glucose in the serum of diabetics (Schwartz et al., 1988) and to intensify the detoxification from heavy metals (Stantschev et al., 1979). Pectic polysaccharides can prevent the adherence of pathogens on the intestinal mucosa and are fermented by probiotic bacteria into short-chain fatty acids, which inhibit necrotic processes in the colon (Wang & Friedman, 1998; Jun, 2006). Furthermore some pectins have immune-regulatory effects in the gastric mucosal immune system through Peyer's patch cells, stimulate lymphocyte and macrophage proliferation (Yamada & Kiyohara, 2007), and have general anti-complementary activity (Kratchanova et al., 2000; Kratchanova et al., 2010). In particular citrus pectins and their modified derivatives (modified citrus pectins, MCP) are reported to have preventive effect on cancer growth and metastasis (Eliaz, 2001; Ramachandran et al., 2011). Both molecular weight and structure influence the binding properties of the polysaccharide. MCP can be obtained by physicochemical or enzyme modification.

The aims of this study were to investigate commercial citrus pectin and isolated from orange and lemon peels pectins, in order to characterize their chemical composition and to evaluate their anti-complementary activity, before and after modification with endopolygalacturonase (EC 3.2.1.15).

Materials and Methods

Materials

Highly esterified citrus pectin was purchased from Danisco® (Czech Republic) with degree of esterification (DE) 74.20% and AUAC 56.13%. Orange Valencia fruits and lemon Argentine fruits were bought from the local market in Plovdiv (Bulgaria). All chemicals were of analytical grade and purchased from the local representatives of Merck (Darmstadt, Germany) and Sigma (St. Louis, USA). Alcohol oxidase (*Pichia pastoris*) 10-40 IU/mg protein was purchased from Sigma (St. Louis, USA). Endopolygalacturonase (PGI, *Aspergillus aculeatus*) 5000 IU/mL was purchased from Megazyme (Ireland).

Preparative methods

Preparation of CPCP

Commercial citrus pectin was purified by column chromatography, according to the method of Kratchanov & Popova (1990).

Preparation of AIS from albedo and flavedo tissue

The orange and lemon peels were separated from the fruit, washed with deionized water (dH_2O), diced into fine pieces. One kilogram orange and lemon peels were separately treated with 2500 mL 96% ethanol preheated to 65ºC. The mixtures were kept for 1 h at 65ºC and additionally for 15 h at room temperature. Then they were filtered by hands through two cheese cloths, after which the insoluble materials were washed twice with warm 96% ethanol. The alcohol-insoluble solids (AIS) from orange and lemon peels, thus obtained were dried at 60ºC and stored at room temperature until use.

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Sequential fractionating extraction

Fifty grams AIS from orange and lemon peels were separately treated with 1250 mL dH_2O at 82ºC for 1 h with continuous stirring and then filtered. Each retentate was treated in the same way with 500 mL dH_2O for 10 min and then filtered. The crude filtrates with water-extracted pectins (WEP) were obtained. Both residues (R_1) were separately further extracted with 1000 mL 0.5% HCl for 50 min at 82ºC and continuous stirred at pH 1.7. Each retentate was treated in the same way with 400 mL 0.5% HCl for 10 min at 82ºC and then filtered. The crude filtrates with acid-extracted pectins (AEP) were obtained. The crude extracts were precipitated with two volumes cold 96% ethanol and left for an hour. WEP were coagulated with acidic ethanol (0.5% HCl). The precipitated crude pectins were separated by filtration, washed once with 100 mL of 70% acidic ethanol, then with 70% ethanol to a neutral pH and finally with 100 mL of 96% ethanol. Pectins were dried at 60ºC in a laboratory dryer.

Scheme for preparation of AIS and pectin extraction from orange and lemon peels is presented on Figure 1.

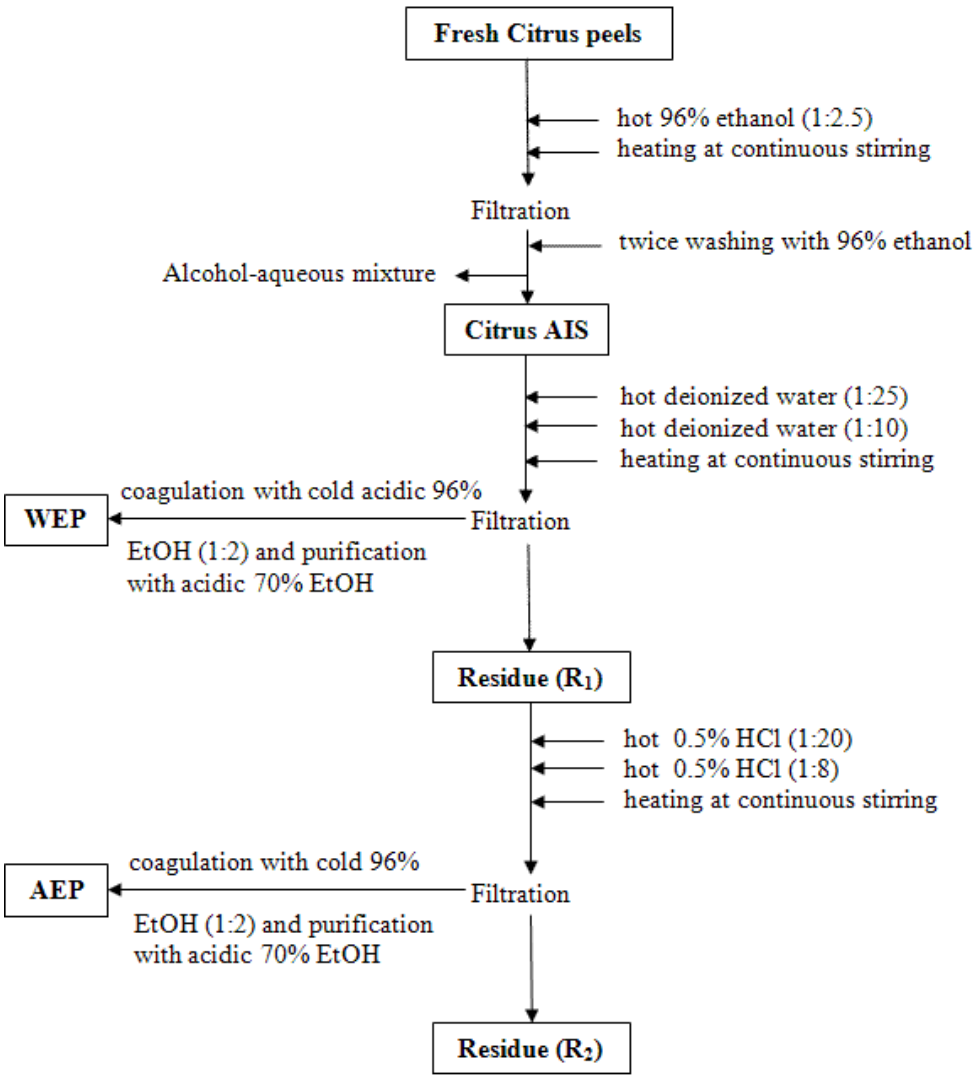

Figure 1. *Extraction and purification of WEP and AEP from orange and lemon peels.*

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Analytical procedures

General methods

Total carbohydrates were determined by microplate variant of phenol-sulfuric acid method (Fox & Robyt, 1991), using D-Gal as a standard. AUAC was determined by *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973), using D-GalA as a standard. DM was determined by quantification of methanol after saponification of pectin samples. The released methanol was determined by combined enzymatic and colorimetric method with alcohol oxidase and Purpald® reagent (Anthon & Barrett, 2008). DA was determined by the hydroxamic acid reaction, using β-D-glucose pentaacetate as a standard (McComb & McCready, 1957). DF was determined spectrophotometrically at 345 nm and 375 nm on freshly prepared solutions of citrus pectins in 0.05 M glycine-NaOH buffer (pH 10.0), using a molar absorption coefficients according to Micard et al. (1994). DF was calculated on the basis of L-Ara and D-Gal content. Protein content was assessed by Bradford's method with bovine serum albumin as a standard (Bradford, 1976). Moisture content was determined by drying the sample (0.3-0.5 g) for 5 h at 94ºC.

Determination of monosaccharide composition and molecular weight

The crude polysaccharides (20 mg) were hydrolyzed with 2 M trifluoroacetic acid TFA (10 mL) for 1 h and 3 h for neutral sugar and uronic acid determination. Hydrolyzates were analyzed for neutral sugars on HPLC system (Agilent LC 1220, USA) with Zorbax Carbohydrate column (4.6x150 mm, 5 μm), and Zorbax Reliance Cartridge guard-column. The mobile phase was AcN/H_2O (80/20) at 1.0 mL/min. The eluate was monitored at 35ºC, using a refractive index detector (RID 1260). Uronic acids were determined on HPLC (Waters, Milford, MA, USA) with Aminex HPX-87H column and 4 mM H_2SO_4 as eluent at 30ºC. The elution rate was 0.8 mL/min and the eluate was monitored using a refractive index detector (R401, Waters). Monosaccharides were identified by their retention times.

Molecular weights of the polysaccharides (2 mg/mL) were determined on HPSEC (Waters) with Ultrahydrogel® 120 and Ultrahydrogel® 500 columns (7.8x300 mm, Waters). Elution was carried out at 25ºC with 0.1 M $NaNO_3$ at 0.6 mL/min. The columns were calibrated, using Shodex standard P-82 (Showa DENKO, Tokyo, Japan). The standard kit contains eight pullulan standards with molecular weights in the range $0.59x10^4$ – $78.8x10^4$.

Endopolygalacturonase modification of citrus pectins

All citrus pectins (10 mg/mL) were saponified with 0.1 M NaOH for 24 h at 4ºC. Then the samples were neutralized with 3 M HCl and precipitated with two volumes 96% ethanol for 2 h. The coagulated pectins were centrifuged at 6000 rpm for 20 min, suspended in 80% ethanol, centrifuged again and further lyophilized. The supernatants were used for total polyphenol determination (data not published). Deesterified pectins (500 mg) were dissolved in 0.05 M acetate buffer at pH 5.0 and modified with endo-polygalacturonase (endo-PG, 0.83 IU/5 mg pectin) at 40ºC for 24 h. Endo-PG digests were collected and fractionated with ethanol, as mentioned above and modified citrus pectins were obtained.

Complement fixation activity

Complement activity was measured using a micro-assay method, according to Klerx et al. (1983, 1985). The test is based on the colorimetric measurement of hemoglobin released from target erythrocytes after incubation with normal human serum. In the classical pathway assay veronal buffered saline (25 mM, pH 7.4), containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ served as diluents. Mutton red blood cells sensitized with rabbit anti-sheep red blood cell (SRBC) antibodies at a concentration of $2x10^8$ cells/mL were used as targets. The effect of polysaccharides on complement activity was determined after pre-incubation for 45 min at 37ºC of equal volumes of normal human serum (dilution giving about 50% lysis of the target cells) and appropriate diluted samples (1.25 and 2.5 mg/mL). After incubation (1.5 h at 37ºC) of target erythrocytes and treated sera, hemolysis in cell supernatants was measured at 541 nm. The anti-complementary activity of the samples was expressed as a percentage inhibition of the total complement hemolysis (TCH_{50}) of the control serum given by the formula:

$$\text{Inhibition of } TC_{50}(\%) = \frac{TCH_{50} \text{ of control serum} - TCH_{50} \text{ treated with pectic serum}}{TCH_{50} \text{ of control}} x100$$

Results and Discussion

Obtaining and chemical features of pectins

Orange and lemon peels were preliminary treated with 96% ethanol at 65ºC for removing of alcohol-soluble low molecular weight substances and for inactivation of endogenous enzyme systems (Figure 1). All this resulted into a good starting material for further pectin extraction. It was obtained 16.0 g AIS/100 g orange fresh peels and 17.5 g

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AIS/100 g lemon fresh peels. Sequential fractionating extraction with hot water and hot 0.5% HCl was used. The obtained pectins were yellowish because extraction was made from albedo and flavedo tissue. Commercial citrus pectin from Danisco® was purified with acidic 70% ethanol by column chromatography for removing of sugar fillers. As a consequence of this purification with 70% acidic ethanol AUAC was increased.

In Table 1 are shown the yields and general characterizations of investigated pectins.

Table 1. *Yield and characterization of CPCP, and pectins obtained from orange and lemon peels (% dry matter).*

Pectin	Yield[a]	AUAC	DM	DA	DF	Protein
CPCP	-	77.45	59.93	0.58	0.07	0.69
WEOP	1.75	69.23	81.26	2.29	0.34	1.72
AEOP	2.95	74.81	70.81	2.20	0.04	3.09
WELP	2.85	81.17	75.36	1.59	0.12	1.02
AELP	3.15	74.60	55.61	1.44	0.06	0.85

[a] The yield is expressed on the base of fresh peels.

All values (except yields) presented below and in the other tables were calculated on a moisture free basis. The dry matter was between 87-92%. As seen in Table 1 lemon peels were richer in pectin 6.00 g/100 g fresh material than the orange peels 4.70 g/100 g. Acid extraction was more appropriate according to yields. It can be calculated from Table 1 that after acid extraction were recovered 62.77% and 52.50% from the available pectin in orange and lemon peels, respectively.

Yapo et al. (2007) carried out sequential fractionating extraction from AIS of industrial citrus peels (1:30 AIS:extractant) with dH_2O, oxalate, hot 0.05 M HCl and cold 0.05 M NaOH. They obtained 5.80 g/100 g AIS WEP and 27.30 g/100 g AIS AEP. In our study we obtained 10.94 g and 16.3 g water-extracted orange and lemon pectins per 100 g AIS, and 18.44 g and 18.00 g acid-extracted orange and lemon pectins per 100 g AIS. Ralet & Thibault (1994) conducted interesting water extraction from industrial lemon fibres, after extrusion cooking and direct acid extraction with 0.05 M HCl at 85ºC. They obtained 12.5-29.4 g WELP/100 g dried fibres and 34.1 g AELP/100 g dried fibres, which were higher than our yields – 11.4 g WELP and 12.6 AELP. DA of the extracted pectins was from 3.0-5.0%. Liu et al. (2006) carried out water-based extraction of orange flavedo and albedo separately under different conditions. They concluded that 1:12.5 ratio peels:water had given them the best yields. Comparing above presented results with these in Table 1, it could be concluded that besides material source and pretreatment procedure the extractant type, temperature, concentration and extraction sequence are critical for best extraction conditions.

AUAC were relatively high for all pectins between 69.23% for WEOP and 81.17% for WELP (Table 1). From these data can be found that neutral fraction in each polysaccharide was low no matter the extraction method. After extraction the solid residual materials from orange and lemon AIS were retained and further analyzed for AUAC and DE. The orange residual material was with AUAC=18.21% and DE=64.14%, and lemon with AUAC=20.72% and DE=60.88%. From results for DM, DA and DF can be seen that there was a correlation between the extractant behavior and DE with methanol, acetic acid and ferulic acid. Maybe as much aggressive is the extractant as lower is DE of the obtained polysaccharide. For example DM of WEOP was higher from AEOP. This was also observed in our earlier publication (Kratchanova et al., 2000) and was in good correlation with the values reported by Yapo et al. (2007). The results for AUAC, DM and DA for AEOP were in agreement with these reported by Kar & Arslan (1999). Authors obtained acid-extracted orange peel pectin from fruit albedo and flavedo tissue with 0.11% HCl (1:25 material:0.11% HCl) at 90ºC for 90 min.

DF was higher in WEOP (0.34%) and WELP (0.12%) than CPCP, AEOP and WEOP. To our knowledge this is the first report on the estimation of ester-linked ferulic acid (FA) in orange and lemon peel pectins. Generally feruloylated pectic polysaccharides have been isolated from sugar beet, spinach, glasswort and quinoa (Mathew & Abraham, 2004; Ralet et al., 2005). Wang et al. (2008) investigated flavonoids in citrus peels and they reported 45.3 μg/g and 59.1 μg/g total FA in dried peels of *C. sinensis* and *C. limon*, respectively. In the current study we calculated that ester-linked FA in the extracted pectins from orange and lemon peels was 840 μg/g in WEOP, 160 μg/g in AEOP, 480 μg/g in WELP and 180 μg/g in AELP. Total ester-linked FA in dried orange and lemon peels differed depending on the extraction procedure. We calculated 53.06 μg/g and 17.04 μg/g, after water and acid extraction for dried orange peels, and 54.67 μg/g and 22.67 μg/g for dried lemon peels, respectively. Ester-linked FA was less after acid extraction probably because of some kind of acid de-feruloylation. Therefore, calculated after water extraction values per gram dried peels could give us

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approximately idea for ester-linked FA in the peels, since the structure of extracted pectin is closer to the native structure. Above calculations showed that most FA in dried citrus peels was predominantly ester-linked in pectic polysaccharides. But Gorinstein et al. (2001) found 39.2 mg/100 g total FA in fresh orange peels and 44.9 mg/100 g FA in fresh lemon peels. In this study we calculated 1.47 mg/100 g total ester-linked FA in fresh orange peels and 1.37 mg/100 g in fresh lemon peels, both on water extraction base.

The acidic ethanol purification of commercial citrus pectin let to lower DE in CPCP, which was in agreement with the results obtained by Kirtchev et al. (1989) (Table 1).

Sugar composition and physicochemical features

Sugar composition

The general chemical composition of CPCP and laboratory obtained pectins was determined with impact on the sugar content. The results are summarized in Table 2 and Table 3, respectively. Except WEOP all other pectins had >80% total sugar content, which simply corresponded for their purity. For most pectins protein content was a minor part of their composition.

Table 2. *Chemical composition of CPCP and pectic polysaccharides isolated from orange and lemon peels.*

Chemical composition	CPCP	WEOP	AEOP	WELP	AELP
Total sugars % (dry matter)	86.80	78.59	92.10	98.70	85.90
Uronic acids[b]	20.65	24.30	16.15	19.60	18.50
Oligomers[a]	78.60	76.95	77.25	76.85	74.30
Oligomers[b]	65.70	63.45	59.35	66.85	65.50
Neutral sugars[a]	15.95	15.45	15.90	19.40	19.35

Data for uronic acids, oligomers and neutral sugars are directly extracted from chromatograms as % Area. Values are the average of two replicates.
[a] Acid hydrolysis with 2 M TFA for 1 h at 121ºC.
[b] Acid hydrolysis with 2 M TFA for 3 h at 121ºC.

Detailed HPLC analysis in two independent chromatographic systems (Agilent LC 1220 and Waters Milford, MA, USA) was performed after acid hydrolysis of pectin samples with 2 M TFA for 1 h and 3 h at 121ºC for neutral sugar and uronic acid release. The experimental conditions were adapted and modified from the works of Jones & Albersheim (1972), Quemener & Thibault (1990) and Schols et al. (1991). The data evaluation showed that citrus pectin macromolecule was very resistant to high hydrolysis with 2 M TFA in the current conditions.

Table 3. *Monosaccharide composition of CPCP and pectic polysaccharides extracted from orange and lemon peels (% dry matter).*

Monosaccharide	CPCP	WEOP	AEOP	WELP	AELP
Ara	12.84	6.97	25.86	28.81	19.05
Gal	7.43	17.91	17.72	11.85	14.39
Rha	6.25	8.05	7.70	11.68	4.44
Xyl + Fuc	0.34	0.98	0.88	0.73	0.39
GalA[a]	1.34	4.52	0.83	1.68	1.41
GalA[b]	9.86	11.49	8.61	11.97	10.87
GlcA[a]	1.28	0.82	3.08	0.17	2.19
GlcA[b]	0.67	0.33	0.77	0.47	1.25
Gal/Ara	0.58	2.57	0.69	0.41	0.76

Neutral sugars were determined on Agilent LC 1220 (USA) and uronic acids on Waters (Milford, MA, USA). Values are the average of two replicates.
[a] Acid hydrolysis with 2 M TFA for 1 h at 121ºC.
[b] Acid hydrolysis with 2 M TFA for 3 h at 121ºC.

Thibault et al. (1993) studied the length of homogalacturonan region in commercial citrus pectin (0.1%) after hydrolysis with 0.1 M HCl up to 72 h at 80ºC. In this prolonged hydrolysis they obtained predominantly D-GalA (83.2%) and a minor neutral sugar fraction. It seems that most of neutral sugars were degraded during the acid treatment. BeMiller (1967) reported that at pH <2.0 linkages between uronic acid residues are notably more stable than linkage between an uronic acid and a neutral sugar. Comparing the results for total neutral sugars released after 1 h and 3 h, and the low total uronic acid content from Table 2 it could be assumed that the resulted oligomers were predominantly composed of D-GalA and a minor amount of neutral sugars. This assumption was also supported from the chromatogram data, since oligomer's peaks were closer to uronic acid peaks. Kurita et al. (2008) determined 34.3% D-Gal and 44.6% neutral sugars in mandarin peel pectin after acid hydrolysis with 4 M TFA for 16 h at 100ºC on HPLC system after derivatization. Ralet & Thibault (1994) determined 42.4-50.6% D-GalA and <40% neutral sugars in WELP after hydrolysis with 1 M TFA for 2 h at 121ºC, and further the resulted insoluble fraction was hydrolyzed with 0.7-1.0 M H_2SO_4 for 2 h at 100ºC. Emaga et al. (2012) carried out chemical (0.2, 1.0 and 2 M H_2SO_4, 2 M TFA, 2 M HCl), enzymatic (Pectinex® Ultra SP-L) and combined enzymatic and chemical kinetic hydrolysis (0.2 M TFA or H_2SO_4 then Pectinex® Ultra SP-L) of pectin from flaxseed mucilage at 80-100ºC for different times. They observed that 2 M TFA caused less damage to sugars than 2 M H_2SO_4 and 2 M HCl, but technical combined enzymatic preparation

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released more D-GalA than 2 M TFA. The combination of moderate chemical hydrolysis (0.2 M H_2SO_4 at 80ºC) with enzymatic hydrolysis was less effective than chemical hydrolysis in drastic conditions (2 M H_2SO_4 at 100ºC). Authors concluded that 2 M H_2SO_4 for 4 h at 100ºC was the best choice for quantifying total monosaccharide content in flaxseed mucilage. Generally in acid hydrolysis of pectins a compromise must be made between conditions for acid treatment with minimal destructive action on neutral side chains, and effective action on 1→4 linkages in the homogalacturonan. Maybe this could be overcome by prior saponification and further successive hydrolysis with pectinolytic enzymes and TFA. Therefore additional studies must be performed for elucidation of these observations.

In Table 3 are shown the detected neutral sugars in the investigated pectins after 1 h hydrolysis with 2 M TFA at 121ºC. D-GalA and D-GlcA contents released after 1 h and 3 h hydrolysis with 2 M TFA at 121ºC are also included in the table. L-Ara and D-Gal were predominant from the neutral sugars in all citrus pectins. WEOP and AEOP characterized with higher D-Gal content than WELP and AELP. WEOP had the highest D-Gal and lowest L-Ara content. WELP and AELP had higher L-Ara than D-Gal content. This was in agreement with the results of Ralet & Thibault (1994), but their pectins had lower D-Gal content (3.0-4.7%) and very low L-Rha (1.1-2.3 %). D-Xyl content was between 2.6-5.4%, which was higher than our results. These differences could be associated with the source material, extraction and hydrolysis. Yapo et al. (2007) and Kratchanova et al. (2000) also detected D-Man and D-Glu in citrus pectin, but they worked in more severe acid hydrolysis conditions.

Gal/Ara ratio is proposed to be a critical parameter for the immunomodulating activity of pectic polysaccharides. Only WEOP distinguished with Gal/Ara ratio 2.57. AELP characterized with the lowest L-Rha. AEOP and WELP had the highest neutral sugar contents. Neutral sugar composition was in good agreement with AUAC and other determined non-sugar sample components. D-GlcA content decreased after 3 h hydrolysis with 2 M TFA at 121ºC. Maybe this was connected with the fact that CPCP and AEP had higher D-GlcA content than WEP. The results for D-GlcA were in good agreement with those obtained for mandarin peel pectin by Kurita et al. (2008).

Physicochemical features

All water-extracted pectins were more viscous than acid-extracted ones after dissolution prior saponification with 0.1 M NaOH. CPCP had the best swelling properties during neutralization with 3 M HCl and coagulation with 96% ethanol. All enzyme-modified citrus pectins lost their gelling properties.

In Table 4 are presented molecular weights and molecular polydispersity of the investigated pectins. All pectic polysaccharides were with relatively high molecular weights. WEOP and AEOP were between 700-960 kDa with small oligomeric fractions. WELP was the only homogeneous pectin with the highest molecular weight 4300 kDa. AELP was very heterogeneous with predominant fraction of 860 kDa.

Table 4. *Molecular weight and homogeneity of CPCP, and pectins isolated from orange and lemon peels.*

Pectin	Molecular weight [Da]	%
CPCP[a]	6.5×10^4	-
WEOP	9.6×10^5	83.8
	2.6×10^2	16.2
AEOP	7.0×10^5	91.0
	8.0×10^3	9.0
WELP	4.3×10^6	100
AELP	4.1×10^6	5.2
	8.6×10^5	79.2
	4.6×10^2	15.6

[a] Molecular weight was determined on Pharmacia LC (HiLoad® Pump P-50 and GradiFracTM Collector) on Sephacryl-S300 column and dextran standards in the range 35 – 200 kDa. The elution rate was 1.0 mL/min.

Corredig et al. (2000) determined the molecular weight of four different commercial citrus pectins between $8x10^3$-$1x10^6$ by HPSEC MALLS. They concluded that the determinations were not affected by the eluent composition and its flow rate. Berth (1988) determined the molecular weight of commercial citrus pectin (DE=60% and AUAC=70%) by gel permeation chromatography. She calculated the molecular weight of crude pectin at about $3x10^6$ Da, and after purification by ultracentrifugation at $1.54x10^5$ Da, and ion-exchange chromatography at $1.65x10^5$ Da. The molecular weight is important physicochemical parameter for pectin gelling properties and absorption in the intestinal tract. The second property is critical for immunostimulating activity of pectic polysaccharides.

Complement fixation activity

The complement system is an important effector pathway of the nonspecific humoral immune response, and the

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complement fixation test has been widely used for location of possible immunomodulating compounds of polysaccharide nature. The complement system is associated with various important biological systems, such as the thymus-dependent anti-body response, regulation of specific cyclical anti-body production, regulation of IgM - IgG switch, modulation of T and B cell proliferation, induction of suppressor or helper T cells and modulation of monokine or lymphokine release (Nergard et al., 2005).

In vitro experiments with mutton red blood cells sensitized with rabbit anti-SRBC antibodies were carried out in search of anti-complementary polysaccharides. Investigations on the relationship between the extraction method, modification with endopolygalacturonase and immunostimulating activity were made. The results are summarized on Figure 2.

CPCP and all extracted polysaccharides showed dose-dependent complement fixation activity in the classical pathway, expressed as a percentage inhibition of hemolysis at 1.25 and 2.5 mg/mL sample concentrations. CPCP had the highest anti-complementary activity at 1.25 mg/mL. AEOP had the highest anti-complementary activity at 2.5 mg/mL, followed by CPCP and AELP. Interestingly AEOP had the highest neutral sugar content 52.16% and Gal/Ara <1.0, which could be in good correlation with its anti-complementary activity (Tables 2 and 3). CPCP had the lowest neutral sugar content 26.86%, including the lowest D-Gal content, but it expressed >70% hemolysis inhibition at 2.5 mg/mL. As was mentioned earlier D-GlcA was in relatively high concentrations in CPCP and AEP, and that could also contribute to its biological activity. For example Sakurai et al. (1998) reported an antigenic epitope in biologically active pectic polysaccharides from *Bupleurum falcatum* (L.), containing D-GlcA in the side chain. Kiyohara et al. (1988) isolated anti-complementary pectins which were composed of 90% homogalacturonan and a small amount of ramified regions.

All investigated pectins were modified by endo-polygalacturonase, and their alcohol-precipitated fractions were analyzed for anti-complementary potential. Endo-PG is an endo-acting depolymerase that cleaves glycosidic linkages between two non-esterified α-D-galacturonic acid residues inside homogalacturonan fragment. The enzyme is not active on rhamnogalacturonans, therefore it modifies initial pectins to products, containing rhamnogalacturonans rich in neutral sugars like L-Ara and D-Gal. Modified CPCP and WEOP had higher immunostimulating activity than the initial polysaccharides at both investigated concentrations. Actually WEOP had the highest D-Gal, L-Rha and D-Xyl content and Gal/Ara ratio >2.0. Modified CPCP expressed 90% hemolysis inhibition at 2.5 mg/mL.

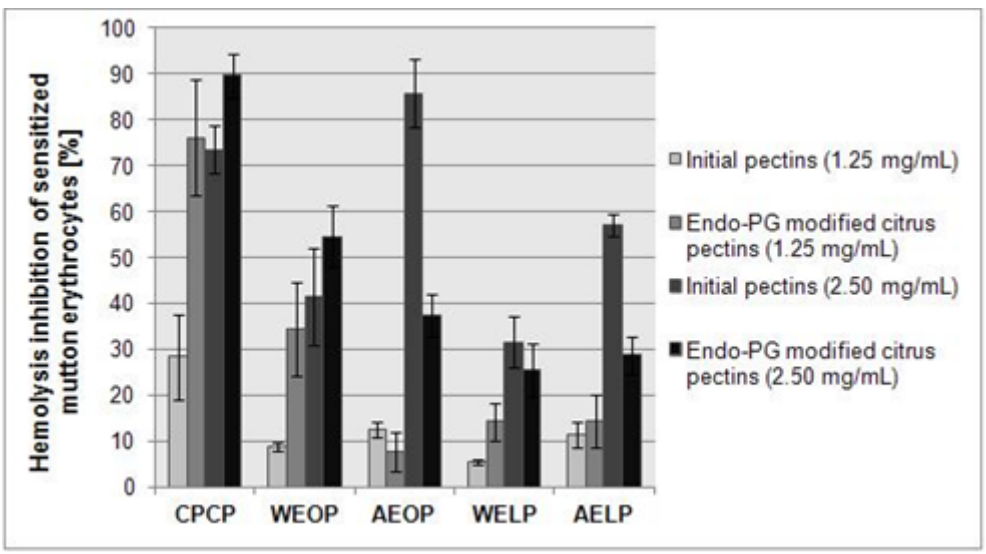

Figure 2. *Immunomodulating activity of initial pectins and their endo-PG modified fractions, by complement activation in the classical pathway at 1.25 and 2.5 mg/mL.*

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Enzyme modification for AEOP, WELP and AELP did not show clear positive or negative effect. This was with agreement with the results of Kiyohara et al. (1988). They isolated and further purified four low-methoxylated pectic polysaccharide fractions from hot-water extracts of the roots of *Angelica acutiloba* Kitagawa. All fractions had molecular weights between 4.2-24.5x10^4 Da, high AUAC (91.7-98.6%), L-Ara (23.1-39.3 mol%), D-Gal (15.4-38.4 mol%) and L-Rha (15.4-30.9 mol%) contents, and DA between 5.0-10.0%. All polysaccharides showed anti-complementary activity, via the classical pathway. It increased or decreased for different fractions after endo-PG modification.

Results for complement fixation test showed that for better understanding of immunomodulating activity polysaccharide samples should be chromatographically purified and further dialyzed prior to analysis. Actually this is one of our present tasks. Biochemists should search immunostimulating activity in polysaccharides not only in the elementary sugar composition, but also in the way that different sugars bind to each other and their accessibility to biological receptors. This could be achieved by carbohydrate analysis and enzyme modification.

Conclusion

We obtained four different pectic polysaccharides from citrus fruit albedo and flavedo tissue, after sequential fractionating extraction with hot water and hot diluted HCl. Polysaccharides were chemically characterized in AUAC, DM, DA and DF by reproducible and fast well-known spectrophotometric methods.

To our knowledge this is the first report on the estimation of ester-linked ferulic acid in orange and lemon peel pectins. An effort was made in the quantification of individual monosaccharides in the extracted citrus pectins. It was shown that effective neutral sugar and uronic acid determination by HPLC without derivatization could be obtained after acid hydrolysis with 2 M TFA. The ratio D-GalA/D-GlcA was in the range 1:8 to 1:35.

Gal/Ara ratio is proposed to be a critical parameter for the immunostimulating activity of pectins. We obtained anti-complementary pectic polysaccharides and modified their activity by treatment with endo-polygalacturonase. Initial AEOP, CPCP and its modified alcohol-insoluble product showed the highest anti-complementary activity at 2.5 mg/mL determined by the classical pathway.

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