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Action pattern of Valencia orange PME de-esterification of high methoxyl pectin and characterization of modified pectins

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Abstract—Valencia pectinmethylesterase (PME) fractions, B-PME, containing 36 and 13 kDa protein bands and U-PME, containing a 36 and 27 kDa protein bands, were used to de-esterify original pectin (O-Pec) from 73% degree of esterification (%DE) to 63% (B-Pec) and 61% DE (U-Pec), respectively. Most O-Pec eluted from ion exchange chromatography at low salt concentration and a smaller component eluted at higher ionic strength. B-Pec and U-Pec eluted as one broad peak at higher ionic strength. PME modification did not change molecular weight: O-pectin (134,000 g/mol), U-Pec (133,850 g/mol), and B-Pec (132,250 g/mol). The NMR signal of GG and GGG increased after modification, whereas the signal of EE and EEE decreased. The negative ζ-potential increased with pH for all pectins. U-PME and B-PME created differently modified pectins that vary in degree and length of multiple attacks and fraction of the pectin population that was modified.

Keywords: PME; Modified pectin; Charge and charge distribution; NMR; IEX; Molecular weight; ζ-Potential

1. Introduction

Pectins are a complex group of structural polysaccharides with an important role as primary constituents in the cell walls of plants and also as gelling agents in food systems.[1] Pectin methylesterases (PME, E.C. 3.1.1.11) from different sources have different action patterns with respect to the removal of methoxyl esters.[2–7] Some plant PMEs have the capacity to remove a limited number of methyl esters, yielding short un-esterified blocks[7,8] that are pH dependent.[7]

In orange, individual isozymes can be distinguished by expression patterns, physical and biochemical properties.[9] Valencia orange PMEs differ in activity and clarification of orange juice,[10] which is increased by cations.[11] Rapid clarification is associated with a thermolabile PME that contains 36 and 27 kDa protein bands and PME extracts that contain 36 and 13 kDa protein bands do not rapidly clarify juices.[12] The potential for PME induced juice clarification is correlated to pectin modification by Valencia PME isozymes.[13] Recently, three protein bands were identified[6] with PME activity with estimated molecular weight values of 34, 27, and 8 kDa. The immunohistology[14] and gene expression[15] of Valencia PMEs have been characterized. A salt-independent orange PME produces calcium sensitive pectin without influence on molecular weight (MW).[16] Functional properties of pectins may be related to differences in the extent and pattern of de-esterification by different PMEs. Based on the hypothesis that PME fractions containing the 36 and 27 kDa protein bands will yield differently modified pectins than PME fractions containing the 36 and 13 kDa protein bands, the objective of this study is to use two Valencia PME fractions to de-esterify pectin to a target DE value and characterize the resultant pectin product for charge and charge distribution. Ultimately, the availability of unique PMEs could enhance the structural

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characterization of pectins and correlation with functional properties.

2. Results and discussion

2.1. PME characterization

There was no significant difference in protein content between the two PMEs fractions but crude PME (0.22 mg/mL) had 5-fold higher protein content than U-PME (0.04 mg/mL) or B-PME (0.05 mg/mL) ($p < 0.05$). IEX chromatography of crude PME resulted in B-PME and U-PME to obtain different enzyme activities and protein bands. For PME activity, U-PME showed higher value (4.87 U/mL) than B-PME (1.21 U/mL). The specific activities were significantly ($p < 0.05$) different for U-PME (101.31 PEU/mg protein), crude PME (68.29 PEU/mg protein), and B-PME (25.55 PEU/mg protein), respectively. Based on SDS-PAGE analysis (data not shown), separation of PME after two different columns, resulted in PME fractions with different protein bands. Crude PME extract indicated protein bands at 36, 27, and 13 kDa. B-PME had dominant bands at 36 and at 13 kDa. U-PME had 36 and 27 kDa protein bands. These three bands were similar to those from the purified commercial orange PME, which has a dominant band estimated 34, and secondary bands at 27, and about 8 kDa.[6] Cameron et al.[17] isolated two peaks, peak 2 (33.5 kDa and pI 9.2) and peak 4 (33.4 kDa and pI 10.1) from two salt-dependent orange PMEs by heparin and CM-Sepharose chromatography. The N and C terminus of the 34 kDa protein bands is nearly identical to the N and C terminus of the 27 and 8 kDa, respectively.[6]

2.2. Properties of modified pectins and fractions

2.2.1. Charge distribution of pectins by IEX. The distribution of methoxyl groups on pectin was studied by analytical IEX, using gradient elution. The elution profile of the O-Pec is polydisperse (Fig. 1). The main component eluted at lower salt concentrations and a smaller fraction eluted at higher ionic strength. The main peak of B-Pec and U-Pec eluted as one broad peak near the same ionic strength as the second, smaller fraction of the O-Pec. This elution of B-Pec or U-Pec shifted to higher salt concentration which suggested increased charge density. That is, de-esterification by Valencia PMEs produced B-Pec and U-Pec with a small change in the DE, but a completely different pattern towards IEX from O-Pec because of the change in charge density.

As observed by IEX,[18,19] pectins eluted in a relatively broad peak. A blockwise distribution might result in zones of higher charge density, which bind strongly to the ion exchanger. The broadness of the peaks indicated

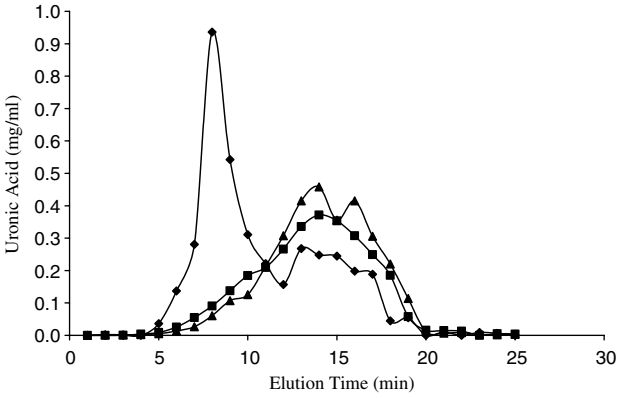

Figure 1. Analytical ion exchange chromatography elution of O-Pec (◆), B-Pec (■), U-Pec (▲).

that pectin charge was distributed over a wide range of DE. Schols et al.[20] reported pectins with a random and blockwise distribution of methoxyl groups that elute in more narrow and broad distribution curves, respectively. The broad elution of citrus PME demethylated pectins was explained by the separation based on intermolecular charge density and not the total charge of the molecules. Anger and Dongowski[21] also showed the difference in the distribution of the free carboxyl groups along the pectin backbone in IEX elution.

2.2.2. Degree of methyl esterification. Based on elution of uronic acid content into fractions, O-Pec was pooled into five fractions. U-Pec and B-Pec were pooled into four fractions. Fractionation of B-Pec and U-Pec by IEX allowed the collection of pectin populations with similar charge and charge density. Usually, high DE values and less charge dense pectins elute first and as elution volume increases, %DE decreases.[22] Kravtchenko et al.[23] reported that structural features other than average DE govern the strength of binding to an anion exchange column. Kravtchenko et al.[24] reported that the DE of the fractions by IEX of three samples decreased regularly from fractions 2 to 8, but pectin fractions with a DE different from that expected had a higher phenolic content. In chromatography of O-Pec, B-Pec, and U-Pec, the %DE decreased with increase in fraction number. Moreover, the first eluting pectin fraction had higher DE value than unfractionated pectin. Because the DE of unfractionated pectin is an average of DE values, the pectin population may have higher or lower DE value. U-Pec eluted over a more narrow range of DE values (between 69% and 49% DE) than O-Pec (76–45% DE) or B-Pec (65–33% DE). The more homogeneous charge and charge density of U-Pec relative to B-Pec suggests a different mechanism of action by PMEs.

2.2.3. Molecular weight and particle size. All unfractionated and fractionated pectins are polydisperse with

M_w/M_n ratios ranging from 1.30 to 2.11. For unfractionated pectin, the MW of O-Pec, B-Pec, U-Pec was 134,000, 132,000, and 134,00 g/mol, respectively, and there was no significant difference after modification ($p > 0.05$). After IEX fractionation, MW of fractionated pectins was significantly lower than unfractionated pectins and the yield of pectin recovered from the column was between 60% and 80%. Some highly de-esterified pectin aggregates may be irreversibly bound on the column. Among fractionated pectins, there was no significant difference in MW regardless of modification except for the latest eluting fractions of O-Pec and U-Pec (Fig. 2).

In a study via SEC and light scattering, fractions of a given hydrodynamic volume within one pectin sample remained highly heterogeneous on the basis of their molecular weight, indicating the coexistence in pectins of particles of very different shapes and DE.[25] Accurate determination of the MW distribution is extremely difficult because of the heterogeneous nature of pectin such as the presence of smooth and hairy regions, and the varying inter-and intramolecular distribution of methyl esters.[25] Several studies[5,6,16,17] observed the same MW before and after orange PME de-esterification regardless of PME isozymes used to modify the charge of pectin. The impact of %DE on hydrodynamic properties of de-esterified pectins was reported for a range of %DE between 78% and 28%.[26] The authors reported that the 65% DE pectin had the greatest flexibility, highest sedimentation coefficient, lowest intrinsic viscosity, and these parameters were the least with the 38% DE pectin. In a comparison of the 65% DE and 78% DE of their study, the pectins with most similar DE of pectins in this study, little difference was observed by Morris and

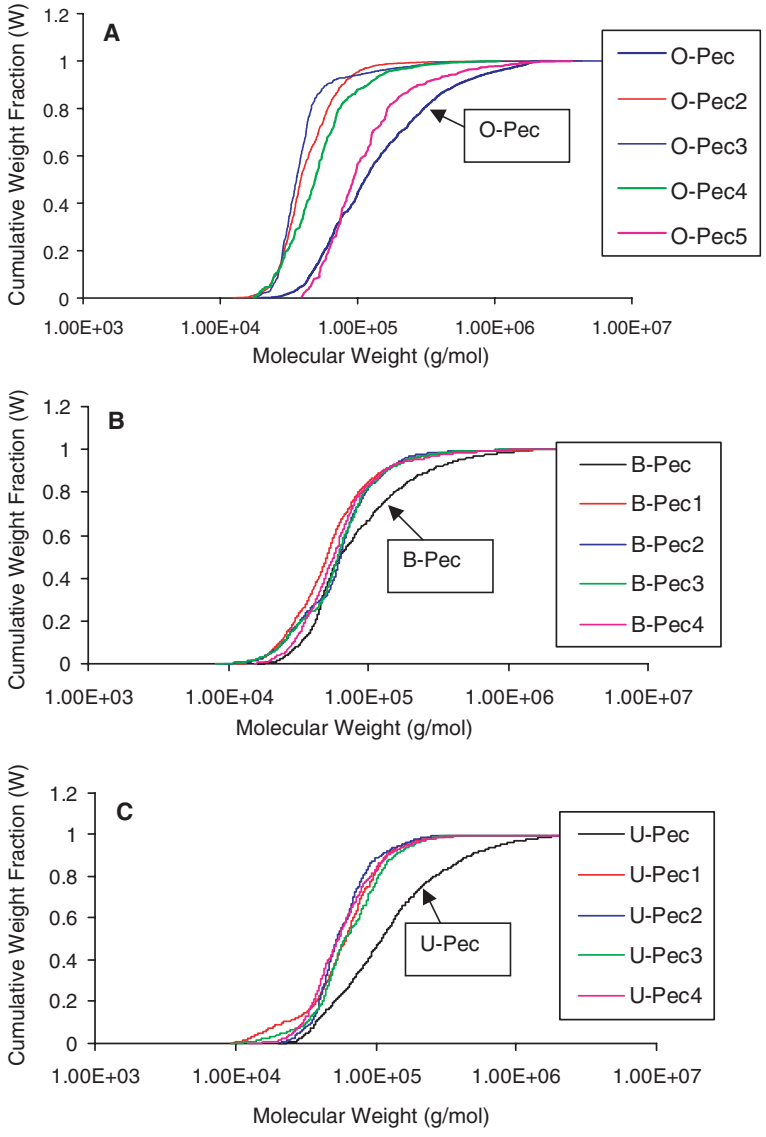

Figure 2. SEC of unfractionated and fractionated pectin samples. Each number after sample means the pooled IEX fractionated number.

co-workers in hydrodynamic properties, including Wales–van Holde ratio, frictional ratio, sedimentation coefficient, MW by sedimentation equilibrium and SEC-MALLS. Intrinsic viscosity differences were the only property that varied between 78% and 65% DE pectins in their study.

2.2.4. ^{1}H NMR analysis. ^{1}H NMR spectra of unfractionated and fractionated pectins, which differentiate between dyads, and triads in partly esterified galacturonic acid are shown in Table 1. There were three main signal groups, the protons H-1, H-4, and H-5 in the G and E residues of ester galacturonans. The protons were shifted to slightly higher parts per million value compared with other researchers. Most likely this is due to a chemical shift of protons of E (esterified galacturonic acid) and G (de-esterified galacturonic acid) residues were dependent on the nature of neighboring units.[7] The spectra of unfractionated pectins are depicted in Figure 3. Since there was a 10% DE decrease from O-Pec to U-Pec or B-Pec, the intensity of G increased and the intensity of E decreased in order of O-Pec, B-Pec, and U-Pec. At the dyads and triads in partly esterified galacturonic acid, the Valencia PME modification showed different spectra among pectin samples. The signal of GG and GGG of B-Pec and U-Pec increased compared to O-Pec. B-Pec and U-Pec showed similar frequency at GG and GGG. In contrast,

Table 1. Monad, dyad, and triad frequencies of Valencia PME modified pectins and pectic fractions at 50 mM phosphate buffer, pH 7.0 in D_2O

	Monad (H-4)		Dyad (H-1)				Triad (H-5)				
	G (4.34)	E (4.39)	GG (5.01)	GE (5.06)	EG (4.84)	EE (4.89)	GGG (4.61)	GGE (4.58)	EGG (4.56)	EGE (4.54)	EEE (4.97)
O-Pec	0.27	0.73	0.49	0.24	0.27	0.59	0.12	0.13	0.20	0.13	0.69
O-Pec2	0.24	0.76	0.44	0.26	0.31	0.65	0.05	0.11	0.15	0.05	0.66
O-Pec3	0.35	0.65	0.60	0.29	0.37	0.51	0.13	0.18	0.20	0.07	0.59
O-Pec4	0.46	0.54	0.71	0.33	0.32	0.30	0.25	0.25	0.14	0.07	0.42
O-Pec5	0.55	0.45	0.83	0.35	0.31	0.25	0.40	0.26	0.13	0.12	0.31
B-Pec	0.37	0.63	0.67	0.29	0.28	0.58	0.25	0.12	0.21	0.22	0.65
B-Pec1	0.35	0.65	0.63	0.31	0.36	0.42	0.14	0.20	0.17	0.06	0.45
B-Pec2	0.38	0.62	0.62	0.29	0.35	0.46	0.15	0.18	0.17	0.05	0.50
B-Pec3	0.44	0.56	0.71	0.26	0.32	0.39	0.27	0.16	0.14	0.03	0.45
B-Pec4	0.67	0.33	1.02	0.28	0.28	0.17	0.60	0.27	0.09	0.05	0.18
U-Pec	0.39	0.61	0.65	0.28	0.29	0.50	0.27	0.13	0.16	0.18	0.53
U-Pec1	0.31	0.69	0.57	0.30	0.33	0.48	0.10	0.19	0.18	0.04	0.52
U-Pec2	0.33	0.67	0.59	0.29	0.35	0.47	0.12	0.16	0.17	0.06	0.52
U-Pec3	0.35	0.65	0.62	0.31	0.32	0.46	0.17	0.17	0.15	0.05	0.51
U-Pec4	0.51	0.49	0.81	0.26	0.33	0.32	0.30	0.22	0.14	0.03	0.36

( ) means chemical shift, unit, ppm. O-Pec, B-Pec, and U-Pec were repeated 2–4 times and coefficient of variation ranged from 0.23% to 3.96%. Values reported are from a single run.

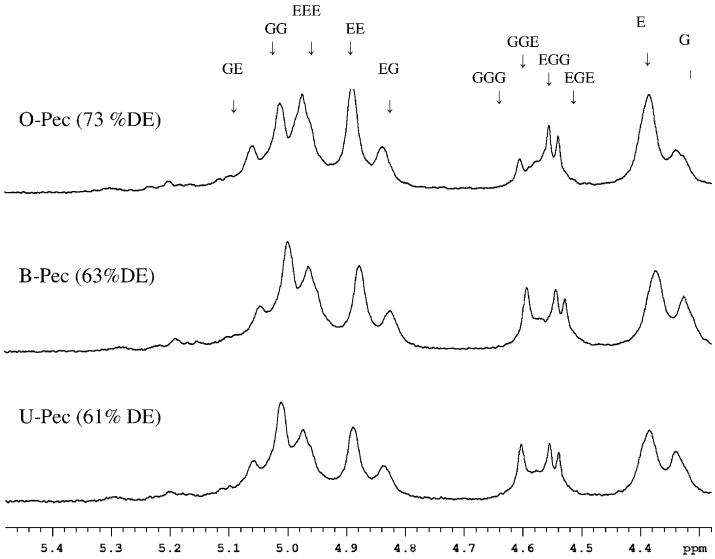

Figure 3. ^{1}H NMR spectra of unfractionated pectins.

the signal of EE, EGG, and EEE of B-Pec and U-Pec decreased. However, the signal associated with F_{EE}, F_{EEE}, and F_{EGG} in B-Pec and U-Pec was slightly different. B-Pec exhibited closer frequency to O-Pec than U-Pec in those signals. For the signal of GE, EG, and GGE, there was no difference among O-Pec, B-Pec, and U-Pec.

In the fractionated pectins (Figs. 4–6), the signals of peaks show greater differences compared to peaks from unfractionated pectins, especially protons H-1, which represents triads GGG, EGG, GGE, and EGE. Later eluting IEX fractions showed higher F_{GG} and F_{GGG}, but lower F_{EE} and F_{EEE}. Comparing among fractionated O-Pec, B-Pec, and U-Pec, the spectra from protons H-1 and H-5 were different. In O-Pec (Fig. 4) and B-Pec (Fig. 5), the frequencies in fractions are different for H-1 and H-5 protons. However, in U-Pec (Fig. 6), the frequencies of H-1 and H-5 protons do not change between Fractions 1–3. Fractions of O-Pec and B-Pec were variable in frequency of F_{GG} and F_{GGG} while U-Pec had more consistent F_{GG} and F_{GGG} among fractions. For the signal of EEE, all fractionated pectins had lower frequency than unfractionated pectins. This indicates that there were less contiguous blocks of ester in fractionated pectins than in unfractionated pectins. For U-Pec, the F_{EEE} frequency among fractions of U-Pec was the same except for U-Pec4.

Distinguishable line patterns and the intensity of signal arise in the NMR spectra and result from different DE values and sequential arrangements of free and methyl esterified carboxylic groups along the polymer chains.[27] Denes et al.[7] described the behavior of purified apple PME at pH 7.0 and 4.5 by a combination of indirect (IEX) and direct (^{1}H NMR spectra) methods. They evaluated the frequency of F_{GGG} and F_{EEE} as a function of final DE following action of PME. The average number of successive E residues estimated the degree of multiple attack of PME. The frequencies F_{GGG}, which had higher than the Bernouillian probabilities, were considered as blockwise distribution. Andersen et al.[28] reported that a block-type distribution in enzyme treated samples is indicated by stronger lines in the spectra corresponding to contiguous arrangement of esterified and de-esterified units denoted by EE, EEEE, and GG, and corresponding weaker lines from residues characterizing block transitions, EG and GE. Grasdalen et al.[27] also reported the enzymatic reaction resulted in a high content of homogeneous triads (GGG and EEE) that demonstrated the production of a sequential structure. Especially, production of a block structure enhanced the F_{GGG} fraction.

2.2.5. Surface charge and mobility by zeta (ζ)-potential. The ζ-potential of O-Pec, B-Pec, and U-Pec and IEX fractions in a pH range from 3 to 7 are shown in

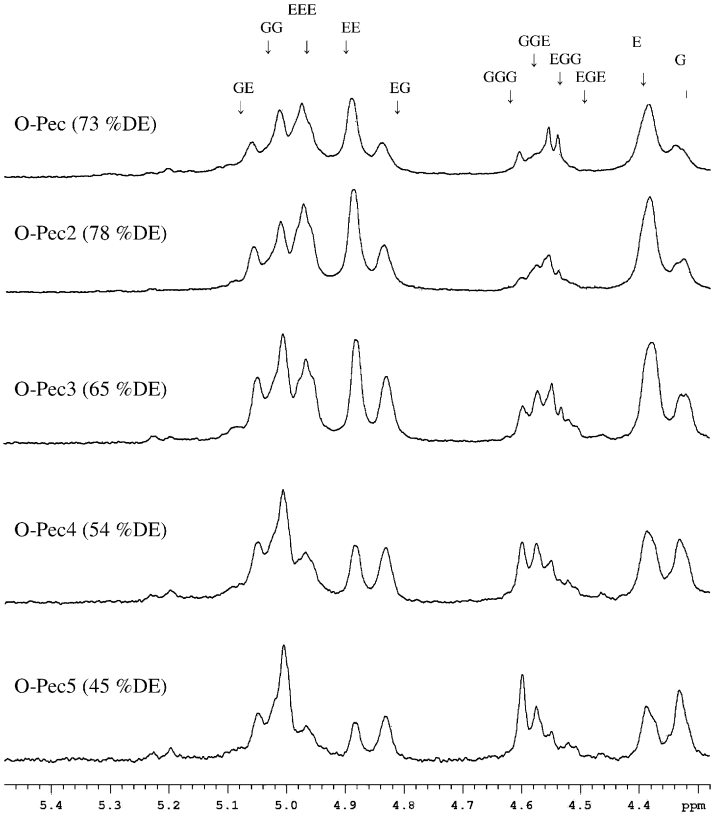

Figure 4. ^{1}H NMR spectra of O-Pec and pooled pectic fractions. (O-Pec): unfractionated, O-Pec2 (#90–126), O-Pec3 (#127–180), O-Pec4 (#181–216), O-Pec5 (#217–300).

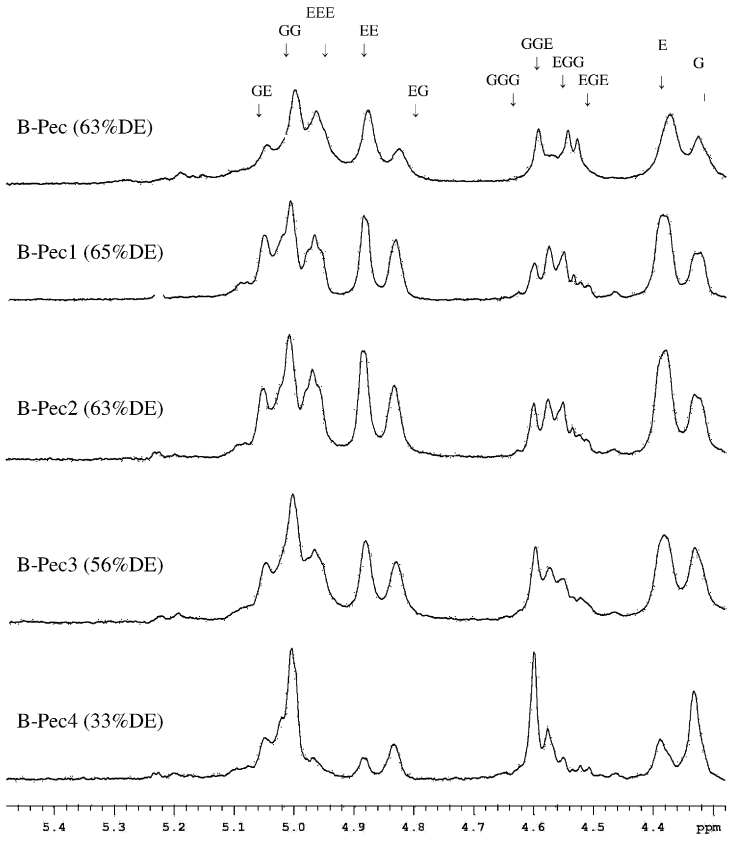

Figure 5. [1]H NMR spectra of B-Pec and pooled pectic fractions. B-Pec1 (#50–116), B-Pec2 (#117–178), B-Pec3 (#179–251), B-Pec4 (#252–310).

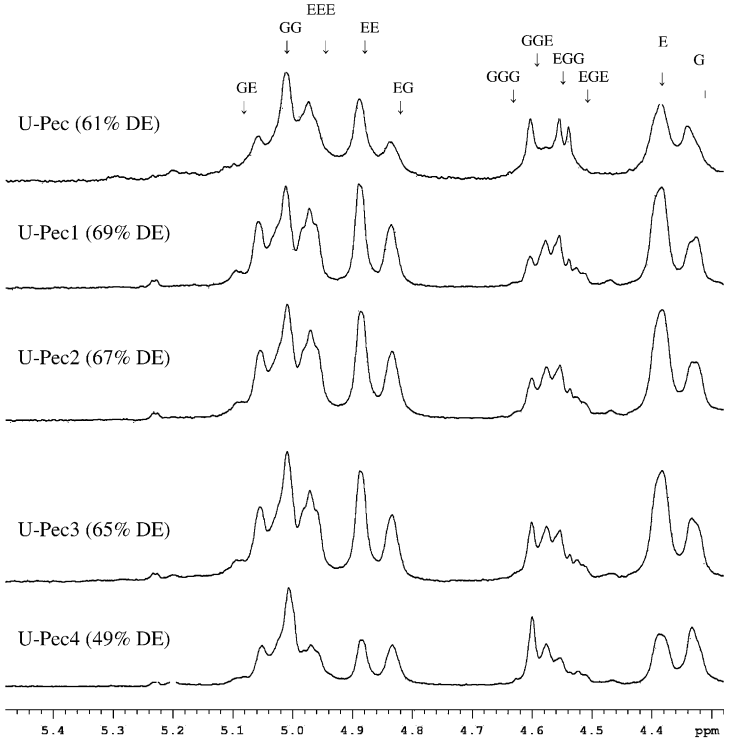

Figure 6. [1]H NMR spectra of U-Pec and pooled pectic fractions. U-Pec1 (#60–128), U-Pec2 (#129–156), U-Pec3 (#157–203), U-Pec4 (#204–313).

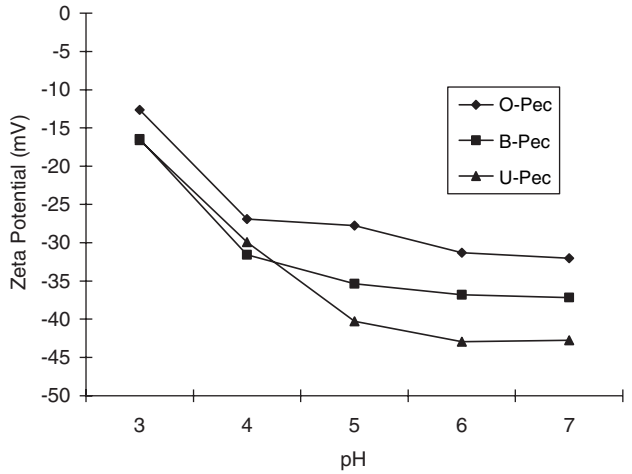

Figure 7. The zeta (ζ)-potential of 0.4% unfractionated pectin dispersion in deionized water.

Figure 7. The negative ζ-potential decreased with an increase in pH for all pectins regardless of modification. At any pH value between pH 3 and 7, O-Pec had less negative ζ-potential than U-Pec or B-Pec. The negative ζ-potential of O-Pec and B-Pec changed greatly from pH 3 to 4 and less between pH 4 and 5. For U-Pec, the extent of negative ζ-potential change was greatest between pH 3 and 5 followed by slow decline. In unfractionated pectins, at any pH, the order of change in ζ-potential was O-Pec, B-Pec, and U-Pec. The surface charge of colloids is often estimated by the ζ-potential, which can be derived from the electrophoretic mobility of the particles.[29,30] The measurement of the ζ-potentials yields information on the surface charge of pectin in solution at a specific pH as well as the change of the electrophoretic mobility. As pectin is an acidic polysaccharide having galacturonic acid as a component sugar, the negative ζ-potential increases with pH. The ζ-potential of soybean soluble polysaccharide (SSPS), which has a pectin like structure is negative and the negative ζ-potential of SSPS is smaller than that of pectin.[29] Enzyme treatments increased the negative ζ-potential because the galacturonic acids, which were not methylesterified were digested and lost from the main backbone.

3. Conclusions

Valencia U-PME and P-PME de-esterify pectins, which retain high MW, have more negative ζ-potential, and have different charge distributions. Based on elution of IEX, chemical shift in NMR, and ζ-potential, we observed a blockwise de-esterification pattern following a 10% decrease in DE. From elution of IEX, the peaks of B-Pec and U-Pec widened and shifted to a higher ionic strength, due to increased charge and charge density, indicating blockwise action. In addition, the negative ζ-potential of B-Pec and U-Pec was greater in magnitude than O-Pec at the same pH. Negative ζ-potential is enhanced by blockwise charge distribution. Based on the 2-fold increase F_{GGG} fraction, both B-PME and U-PME created blockwise de-esterification pattern. Then, based on results from NMR, IEX, and ζ-potential, B-PME and U-PME have multi-attack and multi-chain pattern for modifying pectin. However, U-PME produces shorter attacks and affects more chains than B-PME.

4. Experimental

4.1. Materials

Crude Valencia PME extract was prepared from Valencia orange pulp (donated by Citrus World, Lake Wales, FL) and commercial, unstandardized high methoxyl pectin (Citrus pectin type 104, high methoxyl, CP Kelco, Lille Skensved, Denmark) was used as pectin source.

4.2. Valencia PME preparation and characterization

PME extract was prepared as described earlier.[12] Crude extract was extracted from frozen pulp with 0.1 M NaCl, 0.25 M Tris buffer, pH 8 at a ratio of buffer to pulp of 4:1. The extract was homogenized (Pro 300A, Proscientific Inc., Monroe, CT) for 1 min at 4 °C. After adjustment of pH to 8.0, the homogenate was filtered through Miracloth (CalBiochem, La Jolla, CA). The filtrate was concentrated by 30% ammonium sulfate precipitation overnight at 4 °C, and centrifuged (Sorvall RC-5B centrifuge, Dupont Instruments, Doraville, GA) at 8000*g*, 4 °C for 20 min. The supernatant was dialyzed overnight against 50 mM Na phosphate, pH 7. The dialysis tubing (Spectra/Por, MWCO 6000, Fisher Scientific, Atlanta, GA) was boiled in 10% acetic acid and rinsed in deionized water to minimize loss of PME activity. After dialysis, the enzyme extract was filtered through Miracloth.

To prepare PMEs, chromatography was performed using an Äkta Prime system (Amersham Pharmacia Biotech, Uppsala, Sweden) following the modified method.[31] All buffers were degassed and filtered through an 0.45 μm filter (Whatman, Clifton, NJ) before use in chromatography. The crude PME extract was first loaded onto a 5 mL Hi-Trap SP cation exchange column (Amersham Pharmacia Biotech, Uppsala, Sweden) at 5 mL/min. PME that did not bind Hi-Trap SP column was loaded onto a 5 mL Heparin (HP) affinity column. PMEs were eluted with 10 mM Na phosphate, pH 7 and 10 mM Na phosphate/1 M NaCl, pH 7 gradient. The PME activity in fractions was qualitatively identified using a pH sensitive dye to detect pectin methylester hydrolysis.[32] PME active fractions were quantified and

pooled. Bound PME that eluted from the Heparin column after applying a salt gradient was denoted as bound PME (B-PME). PME that did not bind the Hi-Trap SP, nor the heparin column was denoted as unbound PME (U-PME).

The PME activity of the purified Valencia PME was determined by a pH stat titrator (Brinkmann, Westbury, NY) at 30 °C in 1% high methoxyl pectin (Citrus pectin type CC104, Citrus Colloid Ltd, Hereford, UK) and 0.1 M NaCl at a set point pH of 7.5. A unit of PME activity was defined as the microequivalent of ester hydrolyzed/min at 30 °C.

The amount of protein was quantified by Bradford protein assay[33] using a Microplate Reader (MPR Model 550, Bio-Rad Inc., Hercules, CA). Protein subunit composition of fractions was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Amersham Pharmacia Co., Uppsala, Sweden). Samples were diluted to a constant protein concentration and a 5 μl aliquot was run on a PhastGel gradient gel, 8–25%. The gel was stained with silver stain according to manufacturer specifications (Amersham Pharmacia Co., Uppsala, Sweden). The staining intensity of the bands on SDS-PAGE was measured by densitometry with a scanner (Model GS-670, Bio-Rad Inc., Hercules, CA).

4.3. Pectin modification and fractionation

Pectin (2% w/v) was hydrated in 10 mM EDTA solution and washed with ethanol and acetone. Washed pectin was placed in glass pans and left to dry in the fume hood overnight. Washed pectins were ground using an ultra centrifugal mill (ZM 100, Retsch, Haan, Germany) with 0.25 mm sieve and labeled as original pectin (O-Pec). To modify pectins, O-Pec was de-esterified by U-PME or B-PME to a targeted %DE value. A 1% pectin dispersion in 0.1 M NaCl was equilibrated to 30 °C and adjusted to pH 7.5 with 2 M NaOH. Valencia U-PME or B-PME was added at 3.0 units/g pectin, and the pH was maintained at pH 7.5 with 0.5 M NaOH for the calculated time to achieve the target DE. The PME activity in the dispersion was stopped by the addition of 95% ethanol and boiling for 10 min. After cooling to room temperature, the modified pectin was washed with ethanol and acetone. After drying and grinding, modified pectins were labeled as U-Pec and B-Pec, respectively, according to U-PME or B-PME, respectively, used to make the modification.

To fractionate O-Pec, B-Pec, and U-Pec, chromatography was performed with an Äkta Prime (Amersham Pharmacia Biotech, Uppsala, Sweden) with an XK-50 column (Amersham Pharmacia Biotech, Uppsala, Sweden), 500 mL column volume, Macro-Prep High Q Support (Bio-Rad, Hercules, CA). A 2% (w/v) pectin dispersion in 0.5 M acetate buffer, pH 5.0 was filtered through two layers of Miracloth (Calbiochem, LaJolla, CA). After degassing, the pectin dispersion was gently mixed into the equilibrated anionic exchanger, and equilibrated for 1 h at room temperature. Using a flow rate of 20 mL/min, the pectin sample and packing material were packed into the column, washed with column volumes of 0.5 M acetate buffer and eluted with gradient of 0.5–1.3 M acetate buffer, pH 5.0 at flow rate of 12 mL/min. Based on galacturonic acid assay,[34] fractions were pooled into four large fractions, dialyzed against deionized water, freeze dried (Unitop 600L, Freeze Mobile 25SL, VisTris, Gardiner, NY), and ground (ZM 100, Retsch, Haan, Germany) and stored at −20 °C until further study.

4.4. Characterization of modified pectin

4.4.1. Ion exchange chromatography and ^{1}H NMR spectroscopy. Analytical ion exchange chromatography (IEX) was used to estimate charge distribution. Using a flow rate of 2 mL/min, the pectin samples were loaded onto a 5 mL Q column (Bio Rad, Hercules, CA, USA), equilibrated in 0.5 M acetate, pH 5.0, and then eluted through the gradient of 0.5–1.3 M acetate buffer, pH 5.0. After the IEX elution, uronic acid content was analyzed using the *m*-hydroxydiphenyl method.[35]

To determine the %DE of pectins, ^{1}H NMR spectroscopy was performed using the modified method of Andersen et al.[28] Pectin samples for NMR analysis were lyophilized five times with ^2H_2O (6 mg pectin in 0.7 mL 50 mM phosphate buffer, pH 7.0 in ^2H_2O and four times in 1 mL ^2H_2O) to remove most solvent protons. Then the samples were dissolved in 0.75 mL 99.96% ^2H_2O. ^{1}H spectra were acquired with a Varian Inova 500 spectrometer (Varian, Inc., Palo Alto, CA) at 80 °C using the presaturation experiment to suppress residual water signal in the sample. The concentration of single proton from 0.04% water is about 45 mM. Without water suppression, the water signal severely overlapped with the ^{1}H resonances for the measurements. The data were processed and analyzed using VNMR 6.1C software of the NMR spectrometer. The ^{1}H chemical shift was internally referenced to the water resonance of 4.26 ppm.[28] The NMR spectra were measured at 80 °C with dilute pectin sample with phosphate buffer made in ^2H_2O, at pH 7.0 in order to decrease the aggregation and increase the solubility of the sample. In preliminary trials, 1 M NaOD (55 μL) added in the NMR tubes before measuring NMR gave a poor spectral peak. ^{1}H NMR resonances were assigned according to the published assignments.[7,27] The values of DE and the probabilities of dyads and triads fractions are quantitatively determined from the integration volumes of the assigned spectral peaks based on the relationships.[27]

$$\mathrm{DE} = I_{\mathrm{E}}(\text{H-4})/I_{\mathrm{E}}(\text{H-4}) + I_{\mathrm{G}}(\text{H-4})$$

$$\mathrm{F_{GGG}} = I_{\mathrm{GGG}}(\text{H-5})/I_{\mathrm{E}}(\text{H-4}) + I_{\mathrm{G}}(\text{H-4})$$

where I represents the integration volumes, E and G are denoted as esterified and de-esterified resonances, respectively. The overlapped peaks were deconvoluted using the VNMR software.

4.4.2. HPSEC-multi-angle light scattering. Molecular weights (MW) were determined as described by Corredig et al.[35] using an HPSEC-multi-angle light scattering system consisting of a Waters P515 pump with an in-line degasser (Waters, Milford, MA) and two in-line filters (0.22 and 0.10 mm pore size, Millipore, Bedford, MA). Dispersions of pectin in 50 mM sodium nitrate (3 mg/mL) were filtered through 0.8 μm (polypropylene, 25 mm, Whatman, Maidstone, England). The mobile phase was 50 mM sodium nitrate, sequentially filtered through 0.2, 0.1, and 0.1 μm filters (47 mm, Gelman Sciences, Ann Arbor, MI). Separation was achieved by using a guard column and two PL-Aquagel-OH linear mix columns (8 μm pore size, Polymer Laboratories, Inc., Amherst, MA) connected in series. A multi-angle light scattering detector and a refractive index detector were connected in series (Wyatt Technologies, Santa Barbara, CA). The multi-angle light scattering detector (DAWN DSP-F) was equipped with a P10 flow cell and a He–Ne laser-light source (633 nm). The refractive index detector was an Optilab DSP interferometric refractometer operating at 633 nm. Data was processed using the ASTRA/Easi SEC software (vs. 4.74.03). Molecular weight as a number average (M_n), weight average (M_w), and z-average (M_z) was calculated for each sample. Data presented are the average of three replications. Specific refractive index increment (dn/dc) values were determined with the Optilab using a syringe pump (Razel Scientific, Stamford, CT). Serial dilutions were made (ranging from 0.06 to 1.2 mg/mL) to determine the slope of the increment. Results were processed using the software (vs. 5.2) provided by the manufacturer (Wyatt Technologies, Santa Barbara, CA).

4.4.3. Zeta (ζ)-potential. Measurement of ζ-potential was performed by a modified procedure[29] using a Particle Size Analyzer adding the BI-Zeta option (90 Plus, Brookhaven Inst., Holtsville, NY) with a 50 mV diode laser (90 angle) and a BI-9000AT correlator. A 0.4% (w/w) pectin solution was adjusted to the pH range of 3.0–7.0 with HCl and NaOH. All experiments were carried out at 25 °C with the laser beam operation at 659.0 nm and 1.330 as the refractive index. The measurements were carried out in triplicate with three runs of 2 min each and 5 s between each run. The ζ-potential was determined subsequently after the particle size determination for the same sample of pectin solution. The effective diameter of the particles in solution was calculated from a cumulative fit of the intensity autocorrelation function obtained by the intensity fluctuation of the scattered light[30] with 90-Plus particle sizing software (version 3.37, Brookhaven Instruments, Worcestershire, UK). The measurements were carried out in triplicate with five runs of 2 min between each run.

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