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Enzymatic Modification of Pectin To Increase Its Calcium Sensitivity while Preserving Its Molecular Weight

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A commercial high-methoxy citrus pectin was treated with a purified salt-independent pectin methylesterase (PME) isozyme isolated from Valencia orange peel to prepare a series of deesterified pectins. A series of alkali-deesterified pectins was also prepared at pH 10 under conditions permitting β-elimination. Analysis of these pectins using high-performance size exclusion chromatography (HPSEC) with on-line multiangle laser light-scattering, differential viscometer, and refractive index (RI) detectors revealed no reduction in weight-average molecular weight (M_w; 150000) in the PME-treated pectin series, whereas a 16% reduction in intrinsic viscosity (IV) occurred below a degree of esterification (DE) of 47%. In contrast, alkali deesterification rapidly reduced both M_w and IV to less than half of that observed for untreated pectin. PME treatment of a non-calcium-sensitive citrus pectin introduced calcium sensitivity with only a 6% reduction in the DE. Triad blocks of unesterified galacturonic acid were observed in ^{1}H nuclear magnetic resonance spectra of this calcium-sensitive pectin (CSP). These results demonstrate that the orange salt-independent PME isozyme utilizes a blockwise mode of action. This is the first report of the preparation of a CSP by PME treatment without significant loss of the pectin's M_w due to depolymerization.

KEYWORDS: Pectin methylesterase; pectin deesterification; molecular weight; intrinsic viscosity; calcium sensitivity; HPSEC; NMR

INTRODUCTION

Commercial pectin is an acidic hydrocolloid extracted from the plant cell wall; it is important as a food ingredient and in a growing number of industrial, personal care, and pharmaceutical applications (*1*−*3*). Pectin is primarily homogalacturonan [minimum anhydrogalacturonic acid (AGA) content of 65%] with a high (65−80%) degree of methyl esterification (DE) (*1*). To alter its functional properties, pectin can be chemically deesterified with acid, alkali, or ammonia (*1*, *4*). Chemical deesterification of pectin is a random process that can result in decreased molecular weight due to depolymerization of the pectin backbone α-(1→4)-galacturonosyl glycosidic bonds by β-elimination (*5*−*7*). Because preservation of pectin's molecular weight is desirable for functional properties such as gelation (*8*), enzymatic deesterification with pectin methylesterase (PME) represents a attractive alternative to chemical deesterification. Although enzymatic deesterification of pectin should be less degradative than chemical deesterification, only two previous studies (*9*, *10*) were able to modify pectin's DE with a plant PME without significantly lowering pectin's molecular weight (*5*, *11*−*14*).

Pectin gelation occurs by two general mechanisms depending on its DE (*5*, *8*). High-methoxy (HM) pectins (DE > 50%) require low pH (<3.5) and the addition of a water soluble solute, typically sucrose (65%), for gelation involving hydrogen bonds and hydrophobic interactions (*8*). Low-methoxy (LM) pectins (DE < 50%) gel through the ionic interactions of polyvalent cations, such as calcium, with free carboxyl groups in the pectin backbone, in the absence of sucrose (*5*, *8*). Therefore, LM pectins can be used in low-calorie jams and jellies. Calcium-sensitive pectins (CSP) also have been described in which HM pectins can gel in the presence of calcium without the addition of sucrose as long as blocks of deesterified pectin are present (*15*). CSPs retain more water than LM pectins (*15*, *16*), making them softer and more commercially desirable for dietetic food

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and many other acidic food applications (*15*−*17*). A calcium-sensitive pectin, produced by limited treatment with a salt-dependent PME from orange peel, was able to stabilize casein in acidic milk-based beverage products (*18*−*20*). The gel strength of HM pectin gels is directly dependent upon molecular weight with all other conditions constant (*5*). In addition to molecular weight, LM pectin gel strength is also determined by the method of deesterification used. Gels produced by blockwise deesterification are weaker than those produced by random deesterification (*4*, *5*, *11*). Therefore, modification of DE and molecular weight are the principal means to alter pectin's functional properties, which include gelling agent, viscosity modifier, thickener, protein stabilizer, emulsifier, flavor releaser, binder, and film former (*1*, *2*).

Plant PMEs have been reported to act in a blockwise pattern. These PMEs proceed linearly along the pectin backbone, starting with a free carboxyl group or the chain reducing end, producing a block of deesterified pectin (*6*, *21*−*24*). On the basis of nuclear magnetic resonance (NMR) evidence, modes of action reported for plant PMEs now include single-chain, where the enzyme acts in a blockwise fashion moving along one pectin chain, and multiple-attack, where the enzyme attaches and moves along the pectin chain hydrolyzing a limited number of methyl ester groups, producing a shorter block of deesterified pectin than by the single-chain mechanism (*25*). Plant PMEs can also exhibit different modes of action depending on the pH (*26*−*28*). Two types of plant PMEs have been distinguished on the basis of their biochemical properties, including activation by cations (salt-independent or salt-dependent) and tissue expression patterns (*29*−*31* and references cited therein). Savary et al. (*31*) established that the major PME purified from Valencia orange peel was a salt-independent isozyme.

Several methods have been used to determine differences in the pattern of methyl ester distribution in pectin produced by PME including electrophoresis (*5*), gel stability constants (*6*), high-performance ion-exchange chromatography (*27*, *32*), size exclusion and ion-exchange chromatography (*12*), NMR (*25*−*27*, *33*, *34*), and calcium sensitivity (*13*). We used NMR and changes in calcium sensitivity to determine the mode of action for purified Valencia orange salt-independent PME. Finally, this enzyme was examined for its ability to deesterify pectin without significantly reducing its molecular weight due to depolymerization.

MATERIALS AND METHODS

Orange PME and Citrus Pectin. The salt-independent PME isozyme from Valencia orange peel was purified from a commercial preparation (Sigma Chemical Co.; P5400) (*16*) or directly from fresh peel as previously described (*35*). Both preparations of this enzyme were free of polygalacturonase (PG), acetylesterase, and salt-dependent PME isozyme activities (*31*).

GENU BB Rapid Set citrus pectin and a non-calcium-sensitive pectin (NCSP) were from Hercules, Inc. (Wilmington, DE). The BB Rapid Set pectin had 69% DE and 74% AGA contents. NCSP (76% DE) was prepared from lemon peel as described by Joye and Luzio (*15*). Pectin solutions (1%) were prepared by dissolving pectin powder into 150 mM NaCl (containing 0.02% sodium azide) with stirring overnight at room temperature.

Pectin Deesterification. BB Rapid Set pectin solutions were deesterified by PME or alkali treatment in a water-jacketed reaction beaker at constant temperature (30 °C) and at designated pH with NaOH (5.0 M) delivery controlled by a Radiometer autotitration system. Following treatment, solutions were adjusted to pH 5.5 with HCl, and 3 volumes of warm ethanol (30 °C) was added to solutions to inactivate the enzyme and precipitate pectin. After chilling to 4 °C, the pectin was dialyzed [1000 molecular weight cutoff (MWCO), Spectrum] in deionized water at 4 °C and lyophilized prior to further analysis. Batch treatments of 5 g were prepared with the change in pectin DE being calibrated to the amount of NaOH consumed. Enzyme treatments were performed at pH 7.0, using ∼30 units of enzyme per gram of pectin. Alkaline deesterification was performed at pH 10 with no enzyme added. β-Elimination was measured by absorbance increase at 234 nm. The limit of enzymatic deesterification was measured to be ∼74% of total saponifiable esters. A series of deesterified pectins were produced at quartile intervals relative to the approximate limit of enzymatic deesterification and analyzed by high-performance size exclusion chromatography (HPSEC).

NCSP was similarly enzymatically deesterified as described above, but with hydrolysis limited to 6% of the total saponifiable methyl ester groups. Sufficient enzyme was added to obtain the calculated amount of base consumption in ∼1 h. The pectin solution was then titrated to pH 4.0 and processed as described above. The 6% deesterified pectins were subsequently analyzed by the calcium-sensitive pectin ratio (CSPR) assay and by ^{1}H NMR spectroscopy.

Pectin Molecular Weight and Intrinsic Viscosity. HPSEC of pectin samples was performed according to the method of Fishman et al. (*36*) with minor modifications noted here. Samples (5.5 mg) were dissolved in 5 mL of equimolar 0.15 M lithium acetate/acetic acid buffer (pH 4.60) and then filtered (0.22 μm) prior to injection (200 μL). HPSEC separation utilized two PL-Aquagel OH-60 columns and one OH-40 column (Polymer Labs) in series and a equimolar 0.15 M lithium acetate/acetic acid (pH 4.6) mobile phase, 45 °C, 0.7 mL/min. Detection was with a Dawn DSP multiangle laser light scattering (MALLS) photometer (Wyatt Technologies), a model 100 differential pressure viscometer (Viscotek Corp.), and an RI-SE 61 differential refractive index detector (Shodex). A value of 0.146 mL/g was used for the concentration dependence of the differential refractive index (dn/dc) of pectin (*37*). The weight-average molecular weight (M_w) and intrinsic viscosity (IV) were determined for triplicate HPSEC runs for each deesterified pectin sample. The maximum standard deviation values for M_w and IV were 0.1×10^5 and 0.3 dL/g, respectively. The mean and standard deviation were then calculated for the mean M_w and IV of two completely independent sets of deesterified pectins.

CSPR Assay. This assay was modified slightly from that published previously (*17*). Pectin samples (0.2 g) were weighed into duplicate tubes to the nearest milligram and dissolved in 10 g of deionized H_2O by heating to 70 °C. The solutions were cooled to 25 °C, and the pH was adjusted to 4.0. Cold (4 °C) 80% isopropyl alcohol (IPA; 20 mL) was added to one of the duplicate tubes for each sample to precipitate the total pectin. The precipitate was collected by centrifugation at 8000g for 30 min (10 °C) and was washed twice with 60% IPA, collecting the precipitate during each wash step by centrifugation. These tubes were then dried under vacuum and weighed to the nearest milligram. The weight of pectin precipitated was divided by the initial weight of pectin added to the tube, and this value was designated *A*. Ten milliliters of 30 mM $CaCl_2$ in 8% IPA was added to the second of the duplicate tubes for each sample. The suspension was allowed to gel for 24 h at 25 °C with occasional vortexing. The gel was collected by centrifugation (8000g, 30 min) and washed twice with 10 mL of 30 mM $CaCl_2$ in 8% IPA, collecting the gel during each wash step by centrifugation. The gel was then weighed to the second decimal, and twice this weight of 80% IPA was added; the suspension was collected by centrifugation and washed twice with 60% IPA, collecting the suspension each time by centrifugation. These tubes were then dried under vacuum and weighed. The *B* value was determined by dividing this weight by the original amount weighed into the tube. The CSPR was determined by dividing the amount of pectin that gels in 30 mM calcium and 8% IPA (*B*) by the total amount of pectin which precipitates in 60% IPA (*A*). Therefore, CSPR values close to 1.0 indicate a CSP, whereas a NCSP has low CSPR values. The assay was repeated so that the values reported are means of triplicate assays.

NMR Spectroscopy. Pectins were prepared for ^{1}H NMR spectroscopy by dissolving 10 mg of pectin in 0.7 mL of D_2O (99.96%; Cambridge Isotopes, Andover, MA), lyophilizing, and then repeating these steps before final dissolution in D_2O. ^{1}H spectra were acquired with a 400 MHz Varian Unity Plus spectrometer at 80 °C using the instrumental acquisition parameters reported by Neiss et al. (*34*) (sample

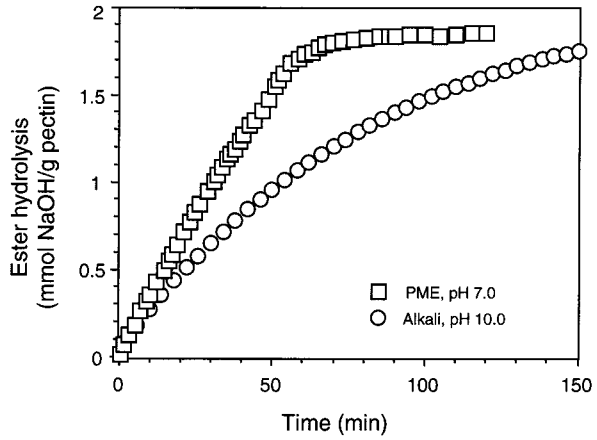

Figure 1. Deesterification of commercial citrus pectin. The time course of salt-independent orange PME deesterification is compared with that produced by alkali (pH 10).

pH was 4.2 following analysis). ^{1}H NMR shifts were assigned according to the guidelines of Neiss et al. (*34*), Grasdalen et al. (*25*), and Andersen et al. (*33*) for an untreated NCSP and this pectin following its treatment with PME. The H_5 chemical shift of a GGG (G = unesterified galacturonic acid) block is 4.75 ppm, whereas 4.71 and 4.67 ppm H_5 chemical shifts were reported for GGE/EGG (E = esterified galacturonic acid) and EGE triads, respectively (*34*).

RESULTS AND DISCUSSION

With an automatic titration system, large quantities of commercial citrus pectin (DE = 69%) were deesterified by controlled enzymatic and chemical hydrolysis producing pectins with defined DE (**Figure 1**). Up to 10 g of pectin was deesterified in batches at constant pH and temperature. Using 30 units of purified salt-independent orange PME (*31*) per gram of pectin, the limit of enzymatic deesterification was approached in ~2 h. The PME- and alkali-treated pectin series (DE = 58, 47, 37, and 26%) were prepared using the absolute quantities of base equivalents consumed to determine the final DE. Although the salt-independent orange PME's exact substrate specificity is not known, inactivity for rhamnogalacturonan methyl ester or acetyl ester groups or the inability to deesterify pectin with less than a critical number of contiguous methyl ester groups may explain its incomplete deesterification of pectin. At pH 10, chemically deesterified pectins were prepared in a time frame comparable to the that of the enzymatic reaction (**Figure 1**). Complete alkaline deesterification was calculated from the curve in **Figure 1** to occur after the addition of 2.86 mmol of NaOH/g of pectin. This value was close to the theoretical amount of saponifiable methanol (3 mmol/g of pectin) for this commercial citrus pectin based on its DE and AGA. The alkaline deesterification rate we observed using the automatic titration system was comparable with previous results using a buffer-controlled system (*7*). Alkali treatments were not performed at 1 °C (*9*), which permitted pectin depolymerization by β-elimination. At pH 10 and 30 °C the rate of β-elimination was much faster than that of methyl ester hydrolysis, and it approached a plateau well before the later reaction (**Figure 2**). These results are consistent with those reported by Renard and Thibault (*7*), who offered a possible explanation: the necessity for two adjacent methyl-esterified galacturonic acid residues for β-elimination to occur.

Analysis of pectins deesterified with the salt-independent orange PME by HPSEC equipped with MALLS, differential viscometer, and RI detectors revealed very little change in the

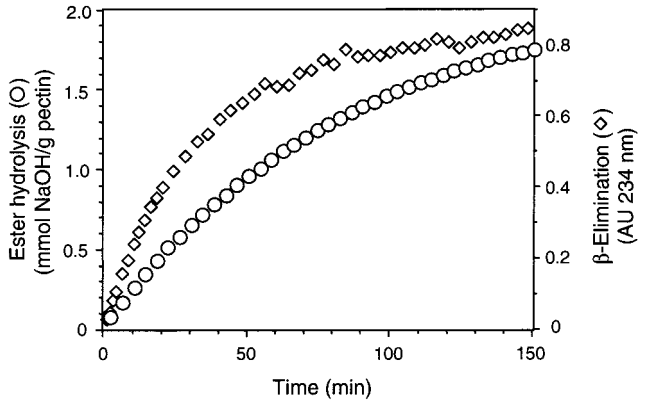

Figure 2. Time course of deesterification and β-elimination for citrus pectin treated with alkali (pH 10).

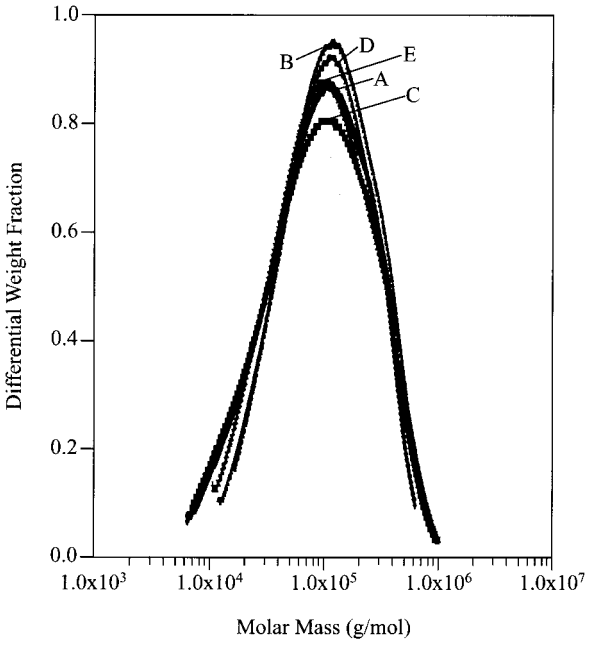

Figure 3. HPSEC analysis with three detectors (MALLS, differential viscometer, and RI) of pectins deesterified with a salt-independent orange PME. Overlaid differential weight fraction versus molar mass curves are shown for these pectins with the following DE values: 69% (A), 58% (B), 47% (C), 37% (D), and 26% (E).

differential weight fraction versus molar mass curves (**Figure 3**). Therefore, over the deesterification range produced by this PME (69−26% DE) no significant change in the M_w (150000) was observed (**Figure 4**). Whereas PME treatment removed methyl ester groups from pectin, this change to pectin's M_w was not detectable. The preservation of M_w following PME treatment is consistent with the lack of any depolymerizing enzyme activities (*31*) or chemical degradation during our processing of pectins following deesterification. A 16% reduction in the intrinsic viscosity (from 5.55 to 4.64 dL/g) was observed over the course of PME treatment (**Figure 5**). Furthermore, most of the reduction in intrinsic viscosity occurred at a DE of <47%. These changes in IV at constant M_w may indicate a change in the hydrogen bonding pattern that holds pectin aggregates together (*38*, *39*). We also observed that the *z*-average radius of gyration (R_g), the Mark−Houwink exponent (a), the number-average molecular weight (M_n), and the polydispersity index (M_w/M_n) remained relatively constant following PME treatment (32.5 ± 0.07 nm, 0.82 ± 0.02, 62300

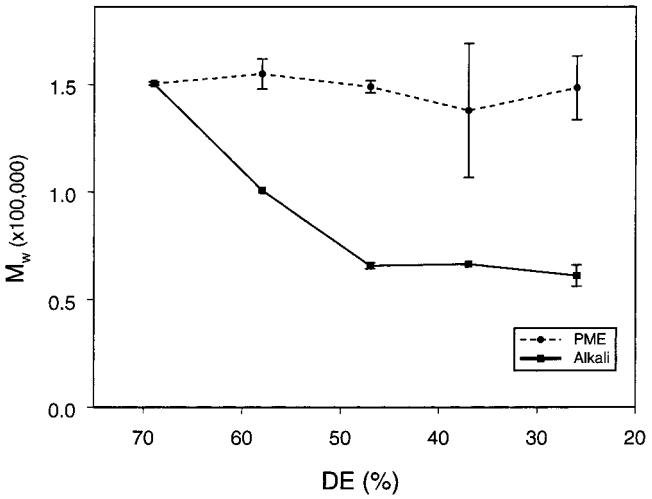

Figure 4. Change in weight-average molecular weight (M_w) of pectin deesterified with orange PME and alkali (pH 10) as determined by HPSEC analysis. The points represent the means (±SD) of the M_w determined from two independent sets of deesterified pectins.

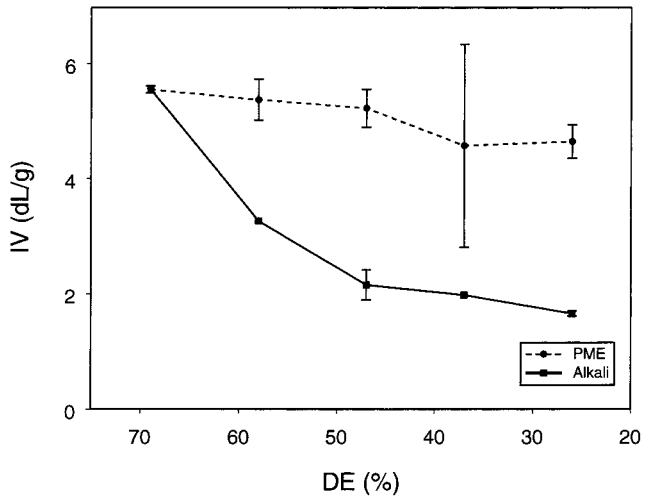

Figure 5. Change in weight-average intrinsic viscosity (IV) of pectin deesterified with orange PME and alkali (pH 10) as determined by HPSEC analysis with three detectors. The points represent the means (±SD) of the weight-average IV values determined from two independent sets of deesterified pectins.

± 3000, and 2.22 ± 0.04, respectively). Possibly, changes in hydrogen-bonding patterns might allow the backbones of solvated pectin aggregates to drain solvent more freely, thereby reducing their IV while M_w, R_g, a, M_n, and polydispersity remained relatively constant.

In contrast, alkali treatment of pectin resulted in both the M_w and IV decreasing by >50% in the first half of the deesterification series (**Figures 4** and **5**). An analysis of variance was performed for each figure comparing the mean values used to generate the PME and alkali curves. In every case during the course of deesterification, the alkali mean was significantly lower than the PME mean ($p < 0.05$). The rapid reduction in M_w and IV following alkali treatment was due to pectin depolymerization by β-elimination (**Figure 2**). Because our objective was to enzymatically deesterify pectin without reducing pectin's M_w, the alkali-deesterified pectins provided a control where we anticipated that the M_w would decrease. Morris et al. (*40*) observed that intrinsic viscosity fluctuated (3.15−4.17 dL/g) for a series of commercial citrus pectins with a DE range of

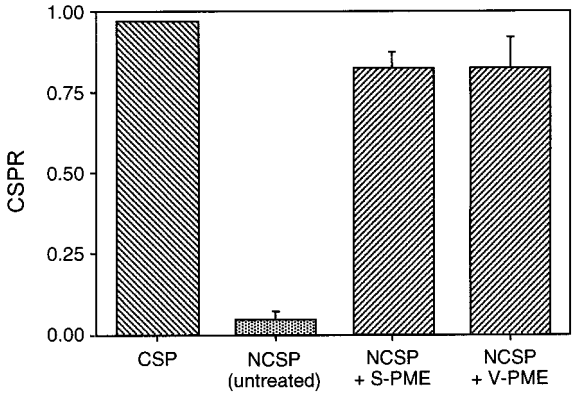

Figure 6. Calcium-sensitive pectin ratio (CSPR) assay of non-calcium-sensitive pectin deesterified by 6% with salt-independent orange PMEs from Sigma (S-PME) and Valencia orange peel (V-PME). The CSPR values for these pectins are compared to those for untreated non-calcium-sensitive pectin (NCSP) and a standard calcium-sensitive pectin (CSP).

78−28%, but constant molecular weight (190000 ± 30000), going through a maximum at DE 38−54%. Although these authors did not address how the commercial pectins were prepared, perhaps the differences in the IV values reported by them (*40*) and us may be due to how the respective pectins were deesterified (random chemical vs blockwise enzymatic mechanisms, respectively).

Calcium sensitivity was rapidly introduced into a HM NCSP that was treated with the salt-independent orange PME. The CSPR dramatically shifted from 4% (NCSP untreated) to 82% (NCSP + PME) following only a 6% reduction in DE (**Figure 6**). Calcium sensitivity requires the presence of blocks of galacturonic acid residues (*15*, *17*), so our data provide the first evidence that this salt-independent orange isozyme acts in a blockwise manner. The dramatic shift in CSPR was demonstrated with both enzyme preparations (salt-independent PME purified from the commercial preparation and that purified directly from fresh orange peel). The majority of the pectin population precipitated in the presence of calcium with a limited reduction in total DE, which suggests that a few critically positioned deesterified blocks were sufficient to introduce calcium sensitivity. However, confirming this distribution of deesterified blocks will require further analysis of treated pectins by high-performance anion-exchange chromatography (*27*).

Coincident with the introduction of calcium sensitivity in the salt-independent orange PME-treated NCSP was the appearance of chemical shifts at 4.75 ppm (**Figure 7A**) and 4.78 ppm (**Figure 7B**) in ^{1}H NMR spectra. These chemical shifts agree with the value for H_5 GGG blocks (4.75 ppm) reported by Neiss et al. (*34*). No chemical shift was observed at 4.75 ppm in the NCSP untreated sample (**Figure 7C**). In the future, we would like to determine the size and distribution of the deesterified blocks produced by salt-independent orange PME using enzymatic fingerprinting (*13*, *41*−*44*) or chromatographic methods (*14*, *27*, *32*). Because the CSPR and NMR data for the CSPs produced by both salt-independent orange PME preparations were essentially identical, these results confirm the conclusions of Savary et al. (*31*) that they were equivalent enzymes.

In support of the CSPR and NMR data demonstrating the blockwise mode of action for the salt-independent orange PME, we also have preliminary evidence that the gel strength of LM pectin (calcium present) treated with this enzyme was weaker than the gel strength of LM pectin produced by random deesterification with alkali (data not shown). Several authors

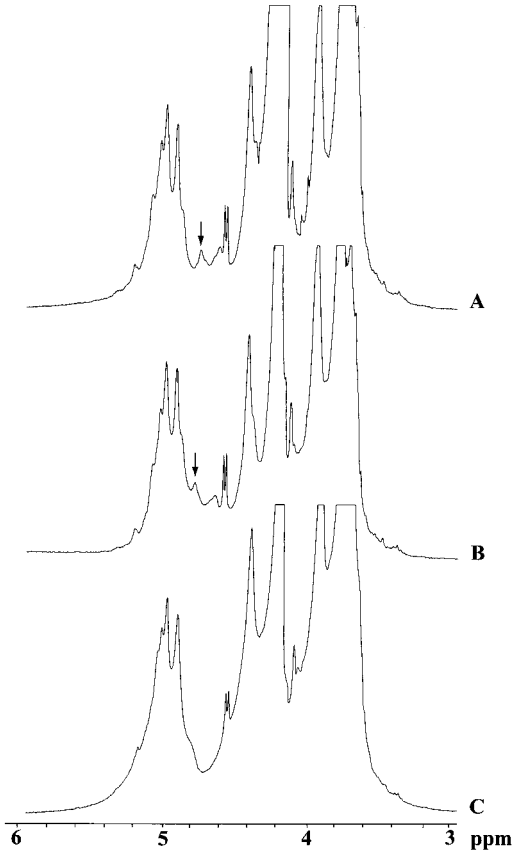

Figure 7. ^{1}H NMR (400 MHz) spectroscopy of pectins deesterified by 6% with salt-independent PMEs from a commercial orange peel preparation (A) and Valencia orange peel (B) compared to the untreated NCSP (C). The GGG triad H_5 chemical shifts are indicated by arrows.

have reported that the calcium gel strength of LM pectins produced by blockwise acting PME was weaker than that observed for randomly deesterified pectins (*5*, *9*, *11*). Ishii et al. (*4*) also found that LM pectin deesterified with a randomly acting PME from *Aspergillus* produced relatively strong calcium gels.

Andersen et al. (*33*) and Grasdalen et al. (*25*) were the first to utilize ^{1}H NMR to directly determine the action pattern of a PME. They used a commercial tomato PME preparation (Sigma Chemical Co.) with a methylated polygalacturonic acid substrate (DE = 92%) and determined the plant PME used a multiple-attack mode of action. The enzyme preparation was used without further purification (*25*, *33*). We demonstrated that this commercial tomato PME preparation contained both salt-independent and salt-dependent PME isozymes as well as PG, which depolymerizes pectin (*30*). Therefore, it remains unclear if the smaller blocks of deesterified pectin produced by the multiple-attack mechanism (*25*, *33*) were due to the salt-independent tomato PME or contaminating pectinases. All evidence indicates that the salt-independent tomato PME is a homologue of our salt-independent PME isozyme from Valencia orange peel (*31*). Neiss et al. (*34*) used 500 MHz ^{1}H NMR and 400 MHz ^{13}C NMR to demonstrate differences in the blockwise distribution of unesterified galacturonic acid residues in lemon pectins produced without the use of PME. Catoire et al. (*26*) used ^{13}C NMR to show that the action pattern of salt-independent α and γ PME isozymes from mung bean hypocotyls shifted from a single-chain mechanism to a multiple-attack mechanism following a pH change from 5.6 (isozymes' pH optimum) to 7.6, whereas a mechanism differing from these two was used to explain the mode of action for the salt-dependent β isozyme at both pH values. Denés et al. (*27*) also reported pH-dependent action patterns for a salt-dependent apple PME isozyme that was single-chain at pH 7 (isozyme pH optimum = 7.5) and multiple-attack at pH 4.5 using high-performance ion-exchange chromatography but could not differentiate between these mechanisms with ^{1}H NMR. Limberg et al. (*13*) determined that a salt-dependent Navelina orange PME isozyme (*20*) deesterified methylated lime pectin (DE = 81%) in a blockwise pattern by enzymatic fingerprinting and an increase in calcium sensitivity but did not confirm this mode of action with NMR. Therefore, our NMR data represents the first NMR evidence for the mode of action of an orange PME.

Whereas the salt-dependent orange PME (*20*) has been used to produce blockwise deesterified pectin (*13*, *14*, *19*, *44*), the M_w and IV of the treated pectins were considerably (nearly 50%) reduced (*13*, *44*). This depolymerization was attributed to β-elimination (*13*). The pH we used (*22*) for pectin deesterification with the salt-independent orange PME isozyme was the same as that used by Limberg et al. (*13*, *44*). The only differences in the two methods was their 10 °C higher temperature of the deesterification reaction (40 vs 30 °C), their heat inactivation of the enzyme (our enzyme was inactivated by ethanol precipitation), and our use of 1000 MWCO dialysis following ethanol precipitation. It is unclear if these processing differences could explain this loss of pectin's molecular weight or if this is a property that distinguishes salt-independent from salt-dependent orange PME isozymes. These questions will be clarified in our further analysis of the salt-independent and salt-dependent orange PME isozymes using common pectin substrate and treatment/processing conditions. Powell et al. (*9*) used an orange PME and Kohn et al. (*10*) used PMEs from alfalfa and tomato to deesterify pectin without observing significant loss of pectin's molecular weight. However, the PMEs used were not characterized so it is unclear which isozyme was used. The loss in M_w and IV, observed in pectins treated with the salt-dependent PME, can be beneficial for the stabilization of proteins in acidic milk-based beverages (*19*), where unesterified galacturonic acid blocks that are too large can cause an undesirable increase in viscosity (*17*). Our demonstration that discrete blocks of deesterified pectin can be introduced into a HM pectin, thereby making it calcium sensitive while maintaining its M_w and causing minimal change to its IV, provides a unique combination of structural and functional properties. These unique properties can be exploited for new food, speciality chemical, and pharmaceutical applications.

ABBREVIATIONS USED

AGA, anhydrogalacturonic acid; CSP, calcium-sensitive pectin; CSPR, calcium-sensitive pectin ratio; DE, degree of esterification; E, esterified galacturonic acid; G, galacturonic acid; HM, high-methoxy; HPSEC, high-performance size exclusion chromatography; IPA, isopropyl alcohol; IV, intrinsic viscosity; LM, low-methoxy; M_w, weight-average molecular weight; MALLS, multiangle laser light scattering; MWCO, molecular weight cutoff; NCSP, non-calcium-sensitive pectin; NMR, nuclear magnetic resonance; PG, polygalacturonase; PME, pectin methylesterase.

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