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Monovalent Salt-Induced Gelation of Enzymatically Deesterified Pectin

SANG-HO YOO,† MARSHALL L. FISHMAN, BRETT J. SAVARY, AND ARLAND T. HOTCHKISS JR.*

Crop Conversion Science and Engineering, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038

Pectin gels were induced by monovalent salts (0.2 M) concurrently with deesterification of high methoxy pectin using a salt-independent orange pectin methylesterase (PME). Constant pH was maintained during deesterification and gelation. If salt or PME was absent, the pectin did not form a gel. The gel strength was influenced by both pH and species of monovalent cation. At pH 5.0, the pectin gel induced by KCl was significantly stronger than the NaCl-induced gel. In contrast, a much stronger gel was produced in the presence of NaCl as compared to KCl at pH 7.0. LiCl did not induce pectin gelation at either pH. Molecular weights of pectins increased from 1.38×10^5 to 2.26×10^5 during NaCl-induced gelation at pH 7. One proposal to explain these pectin molecular weight changes is a hypothetical PME transacylation mechanism. However, these pectin molecular weight changes can also be explained by metastable aggregation of the enzymatically deesterified low methoxy pectin. We postulate that gelation was induced by a slow deesterification of pectin under conditions that would normally salt out (precipitate) low methoxy pectin in the absence of PME.

KEYWORDS: Pectin methylesterase; pectin; monovalent salts; gel strength; molecular weight; intrinsic viscosity

INTRODUCTION

Commercial pectins are widely utilized in food, personal care, and pharmaceutical applications (*1*−*3*). Pectins are heteropolysaccharides of various neutral and acidic sugars and generally act as anionic polyelectrolytes. Commercial pectins extracted from the plant cell walls of citrus peel and apple pomice consist of at least 65% anhydrogalacturonic acid content and have a high degree of methyl esterification (65−80%) (*1*). LM commercial pectins are produced by acid or base hydrolysis of the methyl esters from galacturonic acid C_6 carboxyl groups such that the DE is less than 50% (*1*). The HM and LM pectins undergo two distinctly different gelation mechanisms, which result in very different physical properties (*4*). HM pectins require large amounts of soluble solids and low pH for gel formation, whereas LM pectins form gels with or without solids in the presence of divalent cations such as calcium (*4*, *5*). LM pectin gelation is less pH sensitive than HM pectin gelation. Alkali deesterification of pectin results in degradation of the homogalacturonan backbone by β-elimination, which leads to poor gel quality. In contrast, a salt-independent PME from orange deesterified pectin without altering its molecular weight, thereby improving the quality of the resulting LM pectin gels (*6*). Blockwise deesterification with the salt-independent orange PME produced HM pectins with high calcium sensitivity, which is known to provide stabilization of casein in acidic dairy beverages (*6*−*8*).

Increases in the molecular size of pectin associated with PME were described in several papers. During juice clarification, particle size increased mainly due to the association of calcium sensitive pectins with themselves and with cloud particles (*9*, *10*). It has been proposed that PME interacts directly with cloud particles in reconstituted orange juice (*10*). Increase in the particle size of pectin associated with a pea sprout PME was shown using a laser (90°) particle size analyzer (*11*). To explain the size increase in pectin particle size, the authors proposed that PME had transacylating activity that catalyzed the esterification between GA carboxyl groups and hydroxyl groups on other pectin molecules immediately following their deesterification (*12*). An increase in citrus pectin molecular weight was also observed following treatment with jelly fig achene PME (*13*). In contrast, MacKinnon (*14*) reported that there was no increase in nonmethyl ester linkages in potato cell walls under conditions where PME effectively deesterified pectin. Additionally, no evidence was observed for transglycosylation between radiolabeled oligogalacturonides (DP 2−12) and nonradioactive pectic polysaccharides in the presence of various plant cell wall proteins (*15*). Schmelter et al. (*16*) reported that pectin gelation occurred using a heat-inactivated fungal PME in the presence of NaCl. Because no PME activity was involved in their system, it was suggested that physical interactions such as hydrogen

* To whom correspondence should be addressed. Tel: (215)233-6448. Fax: (233)233-6406. E-mail: ahotchkiss@arserrc.gov.
† Present address: Department of Food Science and Technology, Sejong University, 98 Gunja-Dong, Gwangjin-Gu, Seoul 143-747, Korea.

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bonding and hydrophobic interaction were the major forces involved in gelation. Currently, it is not clear whether covalent or noncovalent interaction is critical for the aggregation of pectin chains during increase in particle size, molecular weight, or gelation. Thus, the mechanism of pectin aggregation associated with PME needs to be addressed systematically using various analytical approaches. Here, we describe a pectin gelation system that utilizes a salt-independent orange PME in the presence of monovalent salts. The chemical and molecular properties of pectin in the resulting gel were analyzed, and the possible explanations of salt-induced gelation were discussed.

MATERIALS AND METHODS

PME and Pectin. The major PME (EC 3.1.1.11), a salt-independent isozyme, from Valencia orange peel was purchased from Sigma Chemical Co. (St. Louis, MO; P5400). The enzyme preparation was free of polygalacturonase and other depolymerizing activities (*17*). Commercial pectin (Sigma Chemical Co.; P9135) from citrus peel was used for this study. Other reagent grade chemicals were purchased from Sigma Chemical Co. and used without further treatments. Pectin solutions were prepared by dissolving 2% (w/w) of the commercial pectin in salt-containing buffered solutions overnight at 4 °C with gentle stirring.

PME Activity and Apparent DE Change by Autotitration. PME activity was determined at pH 7.0 by titration assay using a Radiometer pH stat as described previously (*18*). One unit of activity was equivalent to 1 μmol of sodium hydroxide consumed per min. Methyl ester hydrolysis of PME deesterification was continuously monitored using the autotitrator system. A pectin solution (15 mL; 1.0%, w/v) was titrated using 0.25 M NaOH. The amount of NaOH consumed was converted to mmol of hydrolyzed methoxyl groups, which was used to estimate the apparent DE value. The PME dosages at pH 5.0 and 7.0 were 0.05 and 0.025 units/mg pectin, respectively. Each autotitration PME assay was repeated three times.

DE. GA content was determined with a colorimetric method described by Filisetti-Cozzi and Carpita (*19*), with some modifications. To 0.4 mL of a pectin sample (100 μg/mL), 40 μL of 4 M sulfamate solution and then 2.5 mL of 75 mM sodium tetraborate in concentrated sulfuric acid were added and mixed vigorously. The mixture was cooled to room temperature in an ice bath before it was heated in a boiling water bath for 15 min. The sample was immediately cooled in an ice bath for 1.5 min to bring it to room temperature. Finally, 80 μL of 0.15% 3-phenylphenol (w/v) in 0.5% NaOH (w/v) was added and the sample was well mixed prior to reading its absorption at 525 nm within 3−5 min. The GA concentration was divided by the initial sample concentration to give the GA content (%) of the pectin. Methanol content was determined using the high-performance liquid chromatography (HPLC) procedure described by Voragen et al. (*20*). About 30 mg of a pectin sample was mixed in 1 mL of 1:1 water−2-propanol solution containing 0.4 M NaOH for 2 h at room temperature. The sample was clarified using a microcentrifuge at 10 000*g* for 5 min, followed by filtration (Millipore Nylon 0.45 μm, 4 mm; Bedford, MA). Then, the sample (20 μL) was injected onto an Aminex HPX-87H ion exchange HPLC column (BioRad, Richmond, CA) and a H^+ guard column, heated at 30 °C. The mobile phase was 5 mM sulfuric acid with a flow rate of 0.6 mL/min. An ERMA-ERC 7510 refractive index detector (40 °C) was used for analysis of the column effluent. The DE (%) was calculated from the molar ratio of methanol to GA.

Pectin Gel Strength. Gels were prepared by mixing 0.3 g of the pectin in 15 mL of buffer solution plus the PME (0.1 units/mg pectin). Sodium citrate buffer (0.1 M) was used at pH 5.0 and sodium phosphate buffer (0.1 M) at pH 7.0. The beaker containing the reaction mixture was stirred for 2 min. The homogeneous mixture was then poured into a Petri dish (10 mm × 35 mm) that had been wrapped around the top with tape in order to allow the gelling solution to be poured to above the rim of the Petri dish. A lid was placed on the top of the Petri dish, and then, it was placed in a 30 °C incubating chamber. The mixture was incubated for 24 h. The tape was then removed, and the part of the gel above the rim was cut off using a razor blade to obtain a flat gel surface. Gel strength was determined using a Stable Micro Systems TA-XT2 Texture Analyzer (Robbinsville, NJ). Probe speed was 0.5 mm/s and the 0.5 in. diameter Delrin probe was allowed to penetrate to a depth of 5 mm. The maximum point in the force−penetration curve was taken as the gel strength at break. Three replicates were run for each sample.

Syneresis Test. The water loss after the storage at 4 °C for 72 h was measured for this analysis. The amount of water exuded from the gel matrix was determined after centrifugation at 3000*g* for 30 min. Degree of syneresis was expressed as the weight percentage of the water fraction expelled from the gel matrix.

Pectin Molecular Properties. To determine the molecular properties of PME-treated pectin samples during gelation, we stopped the PME reaction (0.2 M NaCl; 0.0125 units/mg pectin; pH 7.0) at 0.5, 1.0, 2.0, 4.0, and 6.0 h and inactivated PME in a boiling water bath for 2 min. The heated pectin gel mixtures were dialyzed against water for 72 h, resulting in complete dissolution of the gel matrix. These pectin solutions were lyophilized and dissolved in 50 mM $NaNO_3$ for HPSEC analysis. M_w, $[\eta]$, and the Rg_z for pectin samples were determined using 50 mM $NaNO_3$ as the eluent (0.7 mL/min) for HPSEC combined with MALLS (Wyatt Technology Corp., Santa Barbara, CA), refractive index (Shodex, Tokyo, Japan), and differential viscometer detectors (Viscotek Corp., Houston, TX) (*7*).

RESULTS AND DISCUSSION

Effects of Monovalent Salts and pH on PME Activity. During continuous monitoring of PME deesterification by autotitration at pH 7.0, we observed that NaOH consumption rapidly increased during the first 1 h and then reached a plateau (**Figure 1A**). The reaction rates of the deesterifications were almost identical regardless of which 0.2 M salt solution was used (**Figure 1A**). On the basis of these data, it appeared that different monovalent salts (i.e., Li, Na, and K) did not affect the PME activity at pH 7.0. On the other hand, at pH 5.0, the rate of PME deesterification was 14% higher with NaCl than with the other two salts (**Figure 1B**). Even though the activity of the salt-independent orange PME may not be affected by salt at neutral pH (near pH optimum), this enzyme's activity drops significantly without salt at acidic pH (*18*). Lineweaver and Ballou (*21*) reported that at pH 6.0 the relative activity of alfalfa−PME with NaCl is greater than that with KCl, which suggested that various monovalent salts may affect PME activity differentially and also that final apparent DE may change with pH. We observed higher rates (14−33%) of NaOH consumption at pH 7.0 as compared to pH 5.0 when approaching the limit of deesterification. When more PME was added (0.05 and 0.025 units/mg pectin for pH 5.0 and 7.0, respectively) at 90 min, there was no further increase in the NaOH consumption rate.

Effects of Monovalent Salts and pH on Pectin Gelation. Divalent cation cross-linked LM pectin gels can be produced at pectin concentrations ranging from 0.5 to 1% (w/w) and Ca^{2+} concentrations ranging from about 5 to 57.7 mM (*22*). Axelos (*23*) also reported that the addition of NaCl allows LM pectin to gel with lower concentrations of Ca^{2+} than when it was absent. In the absence of Ca^{2+}, here, we produced a homogeneous pectin gelation system consisting of 2.0% (w/v) pectin, PME (0.1 units/mg pectin), and monovalent cation salts (0.2 M) in a 0.1 M buffer for pH control. If any one of our gelation system components was removed, then gelation did not occur. Furthermore, heat-inactivated salt-independent orange PME did not induce pectin gelation in our system. The final DEs of pectin gels produced with monovalent salts at different pH values ranged from 9 to 17 (**Table 1**). In the presence of LiCl, lower DE pectin gels were produced at both pH values. Pectin gels produced at pH 7.0 had lower DEs than did the gels produced with the same monovalent salt at pH 5.0, which was in

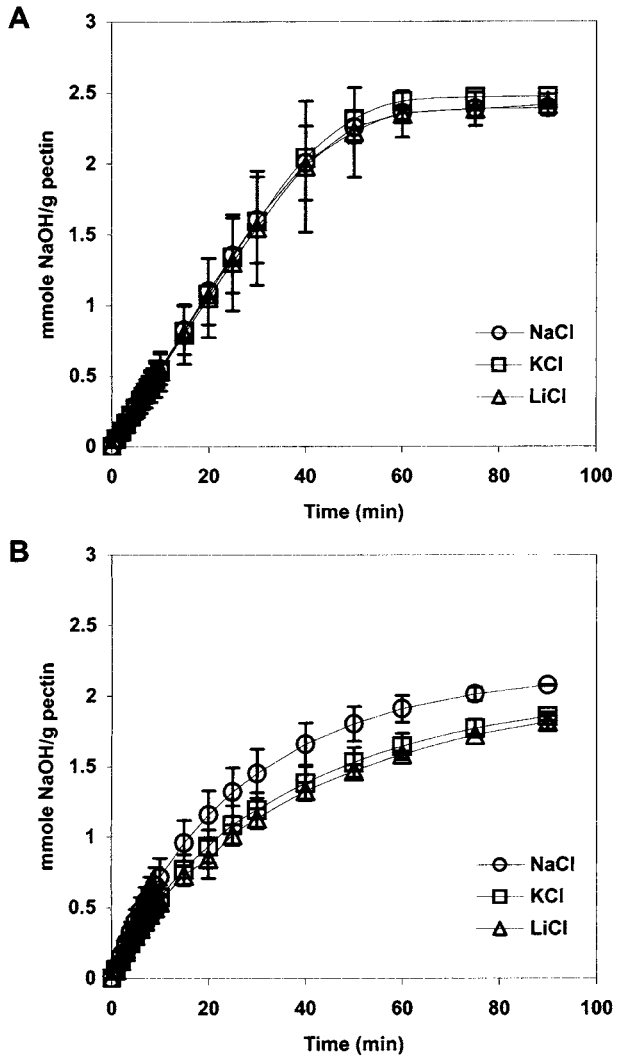

Figure 1. Deesterification of commercial pectin from orange peel. The time course analyses of salt-independent PME deesterification were achieved at pH 7.0 (**A**) and pH 5.0 (**B**); (**A**) 0.025 units/mg pectin and (**B**) 0.05 units/mg pectin.

Table 1. DE of Pectins from PME-Mediated Salt-Induced Gels[a]

salt	pH 5.0	pH 7.0
LiCl	12.6 (0.6)	7.3 (0.2)
NaCl	16.0 (0.5)	12.9 (0.6)
KCl	17.2 (0.1)	9.4 (0.4)

[a] GA content was measured by a colorimetric assay using 3-phenylphenol, and the amount of methanol was quantified using a HPLC method. The DE (%) was calculated from the molar ratio of methanol to GA. The average values were determined from two pectin gels prepared independently. Values in parentheses are the standard deviations.

agreement with the final DE predicted by the methyl ester hydrolysis curves (**Figure 1**).

At pH 5.0, the KCl-induced gel strength at break (323.8 ± 3.2 g) was much larger than the NaCl-induced gel (97.9 ± 18.6 g), whereas LiCl did not develop any apparent gel network as indicated by its negligible gel strength (**Figure 2**). This trend of increasing salt-induced gel strength directly correlated with the ionic radius of the cation used. It has been reported that anionic biopolymers such as carrageenan (*24*), dextran sulfate, and alginate (*25*) formed stronger gels with salts in the order of $K^+ > Na^+ > Li^+$. Furthermore, Watase and Nishinari (*24*)

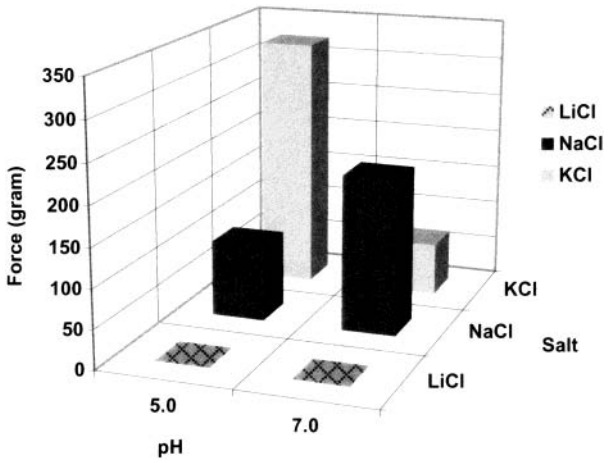

Figure 2. Gel strength of monovalent salt-induced pectin gels. The pectin gels were obtained with the PME deesterification at pH 5.0 and 7.0 at 30 °C for 24 h.

reported that the elastic modulus of κ-carrageenan gels increased remarkably by adding alkali metal ions, following the order $Cs^+ > K^+ \geq Na^+ > Li^+$. They suggested that the electrostatic repulsion of sulfate groups in κ-carrageenan gels was prevented by the shielding action of the alkali metal ions, which stabilized the double helical structure of κ-carrageenan gels. An ion with a smaller ionic radius such as Li^+ is highly hydrated and acts as a structure ordering ion, whereas a larger ion such as K^+ is weakly hydrated making it more likely to directly interact with free carboxyl groups thereby shielding them (*26*, *27*). Hales et al. (*25*) found that Li^+ could solvate alginate carboxyl groups better than Na^+ or K^+. Furthermore, they found that the enhanced solvation of alginate by Li^+, as compared to that of Na^+ or K^+, increased the exothermic change in enthalpy due to Li^+'s smaller ionic radius. Hales et al. (*25*) suggested that these findings were the basis for Li^+ alginates being more stable in solution than K^+ or Na^+ alginates. Fishman et al. (*28*) showed that in 0.2 M NaCl or KCl, LM pectin formed a gel and/or precipitated even at 0.25% (w/w) whereas in LiCl no gelation or precipitation occurred. They also found that commercial pectins with a range of DEs had higher M_w values when dissolved in 0.05 M $NaNO_3$ than the same pectins dissolved in 0.15 M LiAc/HAc buffer (pH 4.8). These results provide further evidence for the greater solvating ability of Li^+ as compared to Na^+.

In contrast to the gelation at pH 5.0, pectin gelled with NaCl (200.0 ± 22.7 g) gave a substantially stronger gel than pectin gelled with KCl (67.5 ± 0.7 g) at pH 7.0. Other factors must be involved in salt-induced pectin gelation at neutral pH in addition to the increase in ionic radius, charge shielding, cation hydration, and carboxyl group solvation. The pH may differentially affect pectin gel formation and the gel strength in the presence of the different cation salts. For a given DE, there is an optimal pH for pectin gel strength (*29*). Additionally, there may be a change in pectin intramolecular conformation between pH 5 and pH 7 (*30*). Changes in pectin conformation could possibly affect cation binding by altering the optimal ionic radius for cation binding and shielding. The anionic buffer (citrate or phosphate) used at the different pH values also may have influenced pectin gelation.

Monovalent cations are known to be activators of PME, even though they are not as effective as divalent or trivalent cations on a molar basis (*31*, *32*). Plant PMEs are generally much more sensitive to their ionic environment as compared to microbial PMEs (*33*). The mechanism of PME activation is not well-

Table 2. Syneresis of Salt-Induced Pectin Gels[a]

salt	pH 5.0 (%)	pH 7.0 (%)
LiCl	N/A[b]	N/A
NaCl	40.0 (1.9)	36.8 (0.6)
KCl	19.0 (1.1)	19.5 (2.1)

[a] The weight fraction of water expelled from the gel matrix was determined after the storage at 4 °C for 72 h. The average values were determined from two pectin gels prepared independently. [b] There was no gelation with LiCl at either pH. Values in parentheses are the standard deviations.

understood, but it is thought that the cations interact primarily with pectin rather than with the PME and may dissociate positively charged PME from the inactive PME−pectin complex (*34*). We found that there was no pectin gelation with LiCl at pH 7.0, even though the PME in this salt solution actively deesterified methoxyl groups. This result suggests that while the highly hydrated Li^+ cannot induce pectin gel formation at 0.2 M, it may be very active in promoting the dissociation between PME and its pectin substrate.

KCl-induced gels were much more stable at storage temperature (4 °C) over time (3 days) than NaCl-induced gels, regardless of the pH in which the gels were prepared (**Table 2**). During the gelation, the NaCl-induced gel exuded water even before storage at 4 °C, but syneresis was not apparent from the KCl-induced gel. After storage for 3 days at 4 °C, NaCl gels showed about twice as much syneresis as did KCl gels at both pH values. There was no significant difference in the degree of syneresis between the same salt-induced gels at either pH.

Of practical interest, commercial orange juices contain up to 60 mM levels of K^+ (35). Because limited salt-induced pectin gelation occurs at this concentration, K^+, in addition to Ca^{2+}, can contribute to juice cloud particle aggregation in orange juices. By controlling the level of K^+ in fruit juices, it may be possible to prevent juice cloud loss and to make Ca^{2+} more bioavailable in Ca^{2+}-fortified juices. To K^+ fortify orange juice, it will be critical to remove PME activity to avoid salt-induced pectin gelation and juice cloud loss.

The conditions used in our salt-induced gelation system for enzymatically deesterified pectin were selected to reproduce those used by Jiang et al. (*13*) and Schmelter et al. (*16*) as evidence for and against PME transacylation activity, respectively. We have elucidated for the first time the roles of different monovalent cations in this type of pectin gelation system. The salt-independent PME that we used was unique as compared to the PMEs used by Jiang et al. (*13*) and Schmelter et al. (*16*) in that it deesterified pectin without significantly altering its M_w and produced only a 16% loss in the pectin's [η] (*6*).

Effect of Deesterification on the Molecular Properties of Pectin in Gel Network. In **Table 3**, we have summarized selected molecular properties of pectins that were prepared by PME deesterification and NaCl-induced gelation at pH 7.0 followed by dialysis against water for resolubilization. As a basis for comparison, we have included in **Table 3** the untreated HM pectin (DE 71.2) dissolved in 0.2 M NaCl and dialyzed against water. M_w and [η_w] values increased as DE decreased, whereas polydispersity decreased (i.e., M_w/M_n and M_z/M_n). The changes in M_w, [η_w], M_w/M_n, and M_z/M_n of pectin solubilized from gels clearly indicate an increase in the molecular weight of pectin. The differential molecular weight distributions shown in **Figure 3** also revealed that the entire distribution shifted to higher molecular weight with decreasing DE. Incomplete pectin solvation resulting from the pectin−pectin interactions could lead to metastable pectin aggregation as shown previously (*36*, *37*), which could explain the increase in molecular weight of pectin following deesterification and NaCl-induced gelation.

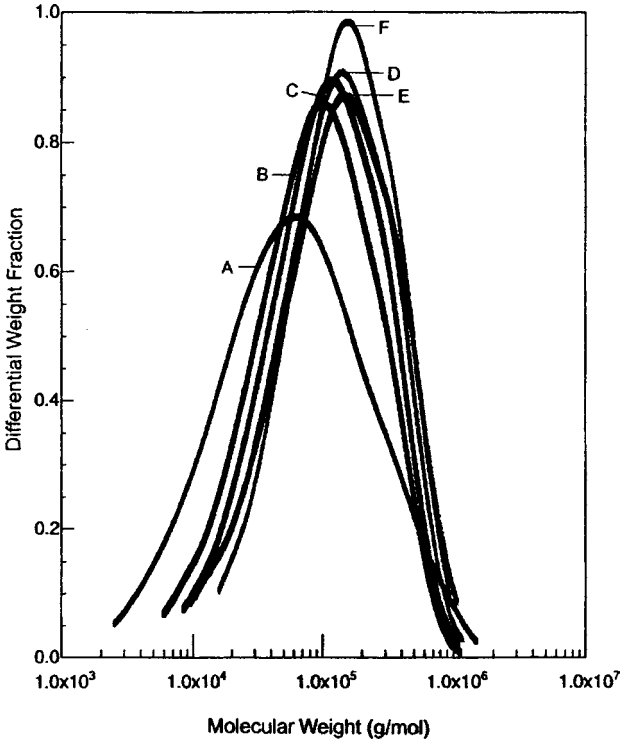

Figure 3. Change in molecular weight of pectin during the PME-driven salt-induced gelation reaction at pH 7.0. Overlaid differential weight fraction vs molecular weight curves are shown for the pectins treated with the PME with the following DE values: 71.2 (A), 39.9 (B), 23.8 (C), 16.1 (D), 14.1 (E), and 12.9% (F).

Figure 4 contains Mark−Houwink plots for pectins solubilized from gels with three different DEs. From the slopes of the curves, one obtains the Mark−Houwink (M−H) exponent, **a**, which is correlated with the ratio of the molecular volume to the molecular weight for the molecules in the distribution. Furthermore, **a** is semiquantitatively correlated with molecular shape. The nonlinearity of the curves indicates that the molecules

Table 3. Effect of Deesterification on Molecular Properties of Pectin[a]

reaction time (h)	DE[b]	% recovery	M_w ($\times 10^{-3}$)	M_w/M_n	M_z/M_n	Rg_z (nm)	[η_w] (dL/g)	a[c]
0	71.2	110	138 (2)[d]	2.39 (0.03)	8.10 (0.35)	38.6 (0.6)	2.49 (0.02)	0.79 (0.01)
0.5	39.9	86.6	135 (1)	1.92 (0.02)	4.15 (0.13)	37.3 (0.3)	3.01 (0.02)	0.82 (0.02)
1.0	23.8	95.4	164 (2)	1.83 (0.04)	3.67 (0.19)	39.5 (0.8)	3.46 (0.03)	0.81 (0.01)
2.0	16.1	81.4	197 (5)	1.74 (0.01)	3.44 (0.03)	40.5 (0.4)	3.48 (0.03)	0.82 (0.01)
4.0	14.1	97.3	216 (5)	1.78 (0.02)	3.72 (0.08)	39.7 (0.4)	3.60 (0.01)	0.78 (0.01)
6.0	12.9	98.1	226 (2)	1.80 (0.01)	4.10 (0.07)	40.7 (0.5)	3.30 (0.02)	0.80 (0.01)

[a] The pectin (DE 71.2) was enzymatically deesterified using salt-independent orange PME in 0.1 M sodium phosphate buffer (pH 7.0) in the presence of 0.2 M NaCl. [b] Degree of methylesterification. [c] Mark–Houwink exponent. [d] Standard deviation of triplicate analysis.

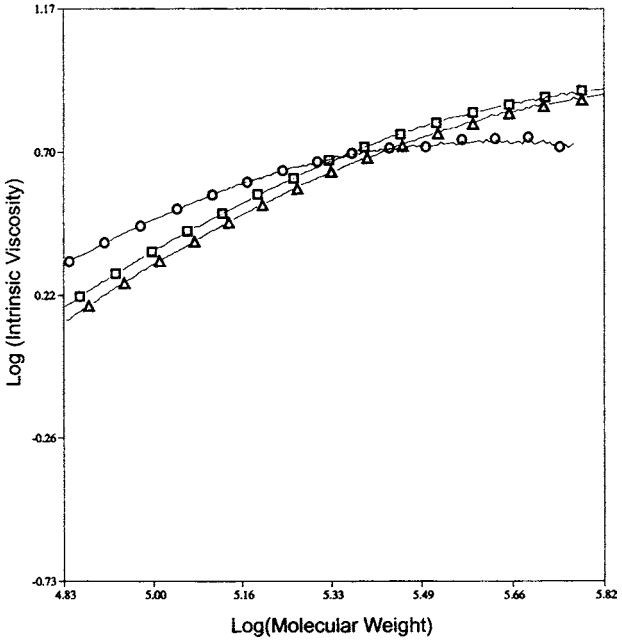

Figure 4. Overlaid Mark–Houwink curves for pectin samples with DE values of 71.2 (○), 14.1 (—), and 12.9% (- - -).

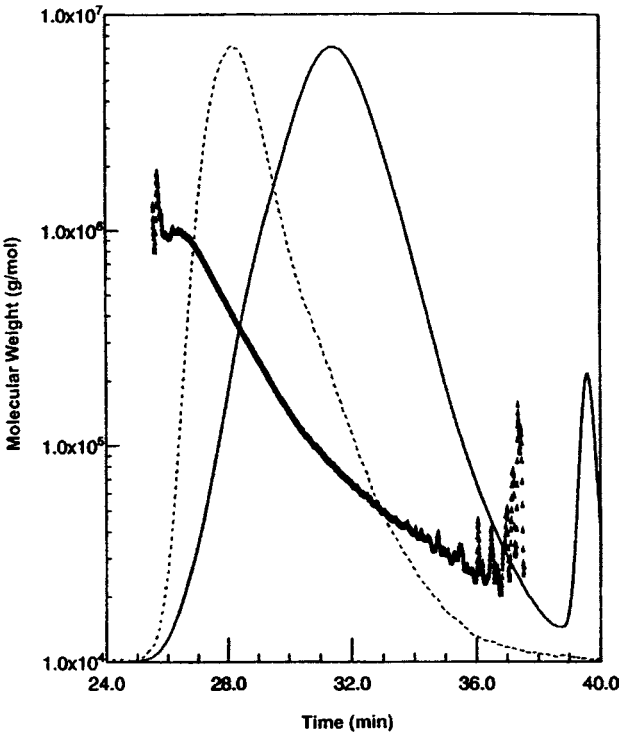

Figure 5. Molecular weight calibration curve (—) superimposed on RI (—) and 90° LS (- - -) signal profiles for DE 71.2 pectin.

in the distribution have more than one shape. Generally, **a** decreases as the shape of the macromolecule becomes more compact. The M−H exponents for pectins in **Table 3** were obtained from the "best" linear least squares lines through the curvilinear data. These values were relatively constant during deesterification but only indicated an average value of compactness. Comparison of the curves in **Figure 4** revealed that deesterified pectins at high ends of the distributions had larger values of $[\eta]$ than the untreated DE 71.2 pectin, whereas deesterified pectins at the low ends of the distribution had lower values of $[\eta]$ than the DM 71.2 pectin. **Figure 5** contains the

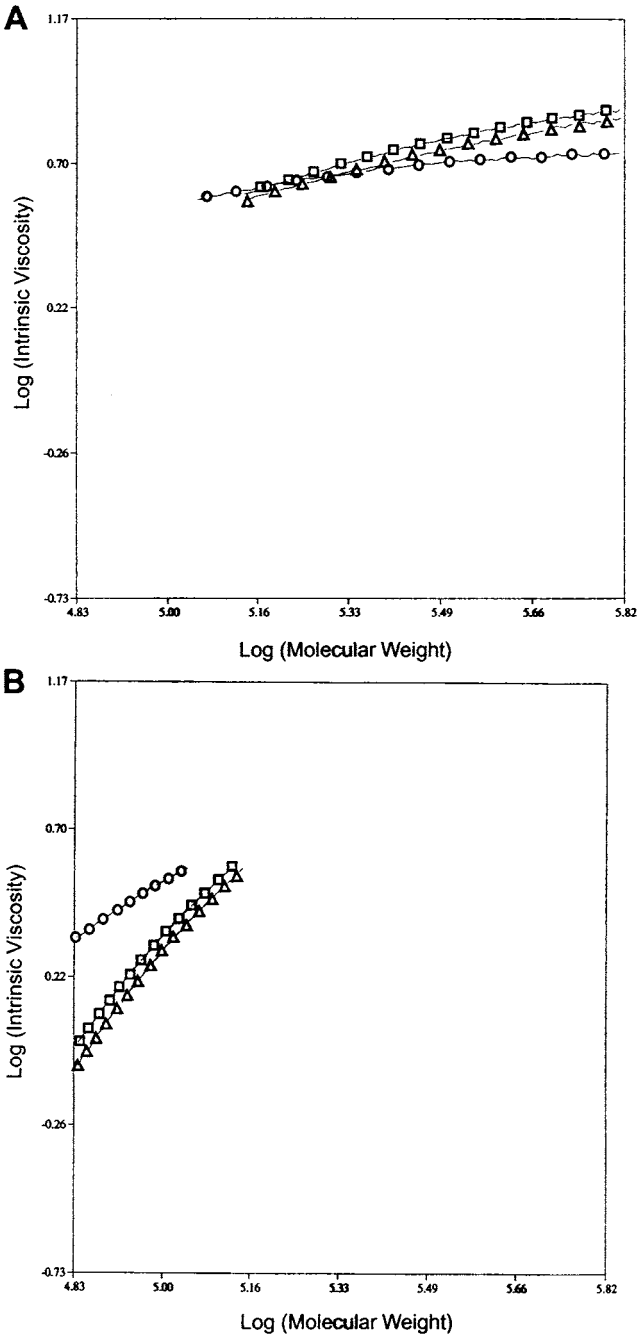

Figure 6. Overlaid partial Mark–Houwink curves were produced for fraction I (**A**) and fraction II (**B**) of the pectin samples with DE values of 71.2 (○), 14.1 (□), and 12.9% (◇).

molecular weight calibration curve superimposed upon differential refractive index and the 90° light scattering chromatograms for untreated DE 71.2 pectin. At 30 min, there was a marked change in slope of the calibration curve. Calibration curves for the deesterified pectins had a similar change in slope. Thus, we integrated the chromatogram into two parts, a high molecular weight fraction (I) and a low molecular weight fraction (II). A linear M−H plot was approximated for each fraction. The boundary between the parts was chosen at the elution volume where there was a marked change in the calibration curve slope following the procedure described previously (*28*). **Figure 6A**,**B** is the M−H plots from which the M−H exponents were extracted after integration by parts. Molecular properties were calculated for these fractions from the appropriate areas of their chromatograms. The data from those calculations are

Table 4. Effect of Deesterification on Molecular Properties of Pectin Fractions[a]

DE[b]	fraction	% fraction	M_w ($\times 10^{-3}$)	M_w/M_n	M_z/M_n	Rg_z (nm)	$[\eta_w]$ (dL/g)	**a**[c]
71.2	I	36.2	308 (5)[d]	1.61 (0.01)	2.83 (0.09)	40.0 (0.6)	4.74 (0.1)	0.19 (0.01)
14.1	I	49.7	334 (8)	1.40 (0.01)	2.03 (0.05)	39.6 (0.4)	5.94 (0.04)	0.38 (0.03)
12.9	I	47.1	359 (3)	1.24 (0.01)	2.21 (0.02)	40.4 (0.2)	5.65 (0.1)	0.35 (0.01)
71.2	II	63.8	54 (2)	1.11 (0.02)	1.22 (0.02)	21.4 (1.1)	2.03 (0.01)	1.34 (0.03)
14.1	II	50.3	87 (3)	1.03 (0.01)	1.06 (0.01)	27.3 (1.1)	2.13 (0.08)	1.89 (0.09)
12.9	II	52.9	90 (1)	1.03 (0.01)	1.06 (0.01)	29.6 (0.7)	1.97 (0.03)	2.02 (0.07)

[a] HPSEC-RI profile of pectin sample was divided into two fractions at an elution volume where there was a marked change in slope of molecular weight calibration curve (see **Figure 5** and the text). [b] Degree of methylesterification of combined fractions (I + II) of individual pectin sample. [c] Mark–Houwink exponent. [d] Standard deviation of triplicate analysis.

found in **Table 4** and are for the same fractions as found in **Figure 6**. The data in **Table 4** revealed that there was an increase in the amount of fraction I at the expense of fraction II after deesterification. Values of M_w and $[\eta_w]$ increased, whereas values of polydispersity (i.e., M_w/M_n and M_z/M_n) decreased for fraction I after deesterification. In the case of fraction II, values of M_w and Rg_z increased whereas polydispersity decreased. These results indicated that both fractions were undergoing interactions. Furthermore, values of **a** for both fractions increased after deesterification, which indicated a decrease in compactness. Nevertheless, as reported previously for pectin, the high molecular weight fraction of the distribution was significantly more compact than the low molecular weight fraction of the distribution (*28*, *38*). In this study, the high molecular weight fractions approached the behavior of hard spheres for both HM and LM pectins. As indicated by our earlier work on pectins imaged from dilute solution by electron microscopy (*39*, *40*), fraction II of the HM pectin (DE = 71.2%) could be a mixture of rods, segmented rods, and kinked rods, whereas fraction II of the LM pectins (DE = 14.1 and 12.9%) appeared to approach purely rod behavior.

Possible Role for PME in Gelation. Toft et al. (*41*) reported thermally reversible gels with mixtures of alginates and HM pectin by slow acidification through the addition of D-glucono-δ-lactone. These gels formed in the absence of sugar, below pH 3.8 and in the cold. The gelation was termed synergistic in that gels formed under conditions in which neither pectin nor alginate would gel alone. They suggested that partially or fully protonated blocks of acid groups in alginate interact with esterified pectin chains to form junctions in the gel network. In our study, slow conversion of HM pectin to LM pectin with blocks of deesterified carboxyl groups in the presence of a relatively high concentration (0.2 M) of NaCl may suppress counterion dissociation enough to allow sufficient chain−chain interactions among pectins for gelation to occur. Fishman et al. (*37*) studied the binding of sodium ions to the carboxyl group of galacturonate, and they found that LM pectin had a larger fraction of bound counterions than did HM pectin. Thus, in the presence of 0.2 M NaCl, in situ deesterification of pectin at pH 5−7 by PME would decrease pectin solubility slowly under conditions of suppressed ion dissociation.

The ultrastructure of a large collection of aggregated HM pectin molecules in a dilute aqueous solution has been shown to be circular microgel networks or larger gel networks of indeterminate shape possibly formed by the coalescence of several circular microgel networks (*39*, *40*). These networks dissociate in the presence of low levels ($\leq$50 mM) of NaCl. At 5 mM NaCl, the microgel network dissociates into branched structures. At concentrations of 50 mM NaCl, the branched structures are dissociated into rods, segmented rods, and kinked rods. When PME is present, pectin gelation is induced by monovalent cation concentrations of 50 mM and higher (data not shown). Therefore, HM pectin microgel networks in an aqueous environment result from the proper combination of forces, some of which promote gel network formation, some of which promote precipitation, and others that promote solubilization.

These forces that stabilize different forms of pectin aggregation are complex. Hydrogen bonds between hydroxyl, carboxyl, and reducing end groups promote network structure because they are responsible for the trifunctional cross-linking of pectin rods into a HM pectin microgel network. Repulsive forces between negatively charged pendent carboxylate ions prevent excessive intermolecular interactions between galacturonate residues in the pectin backbone, which could lead to precipitation. Carboxylate ions cannot participate in hydrogen bonding while nonionized carboxyl groups can. Thus, solution pH is an important factor in determining whether HM pectin gels form. At pH 5−7, most galacturonate residues are in the carboxylate form. Therefore, at this pH, the opposing forces of hydrogen bonds and carboxylate ion repulsion stabilize the HM pectin gel network. Equally important is the concentration of added monovalent cations. As indicated above, low concentrations break hydrogen bonds; yet, at higher concentrations, they shield the repulsion between negatively charged carboxylate groups. In addition, added monovalent cations promote cation binding, which in effect neutralizes the charge on carboxylate ions by forming ion pairs. At some point, increasing the concentration of added monovalent ions will cause sufficient loss of entropy and charge through ion pairing to induce precipitation of pectin chains. This last process has been called the "salting out" effect. Another important factor is the degree of pectin methyl esterification. In a polar environment, ester groups will favor intermolecular aggregation and eventually precipitation due to hydrophobic interactions. Nevertheless, hydrophobic driven interactions on a molar basis are weaker and more easily reversed than interactions driven by hydrogen bonding. At pH values of 5−7 and ionic strengths of 0.2−0.3 M due to added salt and buffer, HM pectins are soluble; yet, LM pectins tend to precipitate due to salting out. If HM pectins are slowly deesterified by PME under these conditions, they will become less soluble as deesterification progresses. When those forces that aid pectin gel network formation, precipitation, and solubilization are in a proper balance, a metastable intermediate state, namely, the gel state, will form. Thus, the gel state results from a failed precipitation due to the influence of other forces that favor gel network formation and solubilization.

CONCLUSIONS

The changes in the molecular properties of pectins dissolved from gels could be explained by two possible mechanisms. First,

transacylation of pectin by PME may create one or more covalent ester bond(s) between pectin chains. Second, we have proposed an explanation for gel formation in the presence of PME and 0.2M NaCl/KCl as a slow salting out process that ceased at the gel state prior to precipitation. This was caused by the slow PME deesterification of HM pectin under conditions of relatively high salt concentration. When we dialyzed pectin gel samples against water, the gels completely dissolved, reflecting that salt removal from the gel network reduced attractive (cation-shielded) noncovalent interactions between pectin chains. Gels formed by covalent cross-links would not be expected to be reversible following dialysis. However, because the molecular weight of pectins deesterified by PME in the presence of 0.2 M NaCl/KCl did increase, at least some of the salt-induced aggregation state of pectin is retained following dialysis. While we do not rule out the possibility of a PME transacylation reaction, direct chemical or physical evidence is required to demonstrate covalent bond formation resulting from this reaction in pectin gelation. So far, direct chemical or physical evidence supporting pectin transacylation by PME has not been reported.

ABBREVIATIONS USED

DE, degree of esterification; GA, galacturonic acid; HPSEC, high-performance size exclusion chromatography; HM, high methoxy; LM, low methoxy; M_w, weight average molecular weight; [η], intrinsic viscosity; Rg_z, z average radius of gyration; MALLS, multiangle laser light scattering; PME, pectin methylesterase.

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