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Rheological Investigation of Pectin Deesterified using Salt-independent Pectin Methylesterase from Citrus

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During deesterification, the ester groups on the pectin from citrus peel by-products can be removed in a random or block-wise (sequential) manner. Pectins containing these blocks of unesterified groups on the chain are highly reactive to divalent cations such as calcium ion. In solution, in the presence of calcium ions, pectins demethylated by plant pectin methylesterases (PME) can exhibit unique rheological properties. One such property is that they can produce pseudoplastic solutions that are needed for suspension aids for food and non-food applications. A non-calcium sensitive pectin with a 94% degree of methylation was demethylated to a value of either 55% degree of methylation (DM) at pH 4.5 or 62% DM at pH 7.5 using monocomponent citrus salt-independent PME. Rheology measurements on these pectins indicated that to reach a maximum value storage modulus G´, a cure time of 20 hours was needed. At an angular frequency of 0.01 rad·s^{-1} the G´, G˝ (loss modulus), and tan (δ) (ratio of loss to storage modulus) values were 9.69 Pa, 0.247 Pa, and 0.0255 Pa, respectively, for the pH 4.5 sample. This indicates that the modulus is largely comprised of an elastic component that is important for suspension applications. A maximum value of storage modulus was observed at mole ratios (calcium ion to free unesterified carboxyl groups) of 1.0 and 1.2, where the G´ values were 34.5 and 52.1 Pa, respectively. The decrease in G´ to 0.14 Pa observed after shearing for 15 seconds signifies the need for improvement of modulus recovery after shear.

The problem encountered with mixtures containing insoluble components, such as pulps, essential oils, and the like, is the tendency of the insoluble components to separate, e.g., via sedimentation or creaming. In order to maintain insoluble components in suspension, gums such as xanthan have been added to fruit drinks to raise the viscosity or alter the rheology of the drink. Xanthan, when added to aqueous solutions, even at low concentrations, exhibits very strong pseudoplasticity (Holme et al., 1988; Lapsain et al., 1995). In the work described herein, solutions, containing enzyme modified pectins, have been investigated for pseudoplasticity.

A major component of citrus peel is pectin and one approach toward utilization of citrus by-products is to modify the pectin component prior to use to provide pseudoplastic properties. Pectin is a complex polysaccharide composed of at least five different sugar moieties where 80% to 90% of its dry weight is anhydrogalacturonic acid (AGA). The majority of the AGA is found in homogalacturonan (HG) regions of pectin as unbranched polymers of AGA in which a variable proportion of the AGA residues contain a methyl ester at their C6 position (Ridley et al., 2001; Vincken et al., 2003). Pectin's functional properties and reactivity toward calcium and other cations are largely dependent on the amount of methylated galacturonic acid subunits and their distribution pattern within the HG stretches (Powell et al., 1982; Willats et al., 2001). Two general patterns of methyl ester distribution are recognized, i.e., random or ordered (Willats et al., 2001). For enzymatic demethylation of pectin, three different modes of action have been hypothesized (Denes et al., 2000), two of which lead to ordered (blockwise) removal of esters. Previous studies have demonstrated that plant PMEs can demethylate pectin in an apparent ordered process, described as processive hydrolysis of the methyl esters. Catalysis by plant PMEs creates demethylated blocks (Daas et al., 1999; Denes et al., 2000; Hotchkiss et al., 2002; Limberg et al., 2000a, 2000b; Savary et al., 2002) that are reactive to calcium ion. The presence or absence of these blocks, and how these blocks are produced, is important since it affects the reactivity of pectin with cations such as calcium.

PME action patterns and the presence of demethylated blocks have been shown to have significant effects on the rheological properties of pectin such as pseudoplasticity after binding calcium ion (Luzio, 2003; Luzio et al., 2007; Powell et al., 1982; Schmelter et al., 2002; Willats et al., 2001). Pseudoplasticity is a rheological behavior most desired for stabilization of particles in aqueous solution. Aqueous solutions are characterized as being pseudoplastic if they can display decreasing viscosity with increasing shear rate and are non-Newtonian fluids such as a Bingham plastic (Steffe, 1996). An important characteristic of a Bingham plastic is the presence of yield stress that requires a finite stress to achieve flow, which acts as an aid to suspend particles. Yield stress measurements for pectins with a blockwise distribution of unmethylated GalAs indicate that interchain associations in the presence of calcium ion are stronger than those involving pectins with random distributions for degree of methylation (DM) values greater than 45% (Powell et al., 1982). A recent study indicates that the average block number is relatively small (>2.0) when storage modulus first appears (Luzio et al., 2007), but the calcium levels required for optimum gelation and shearing effects after gelation were not investigated.

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Two of the pectins from that study, deesterified by a salt-independent citrus PME at pH 4.5 (DM = 55%) and pH 7.5 (DM = 62%), were examined further for storage and loss moduli, G´ and G˝, before and after shearing the sample. In addition, the optimum amount of calcium needed to maximize the storage modulus and minimize the loss modulus was also determined. These are important physical properties measured for gels made by cross-linking pectin with calcium ions for optimizing suspension properties. These particular samples were chosen for further investigation since they had relatively high observed yield point and storage modulus values.

The storage modulus G´, corresponding to the stress (applied force per unit area of the gel sample) in phase with the strain (ratio of the displacement caused by the stress relative to the thickness of the gel sample), is a measure of the energy that can be stored in the material; for a gel, it is directly related to the cross-link density of the network and is the elastic component of a gel. This elastic component is important for suspension since this cross-linked structure inhibits particle settling under conditions of low shear. The loss modulus G˝, corresponding to the 90° out-of-phase stress amplitude, is a measure of the energy dissipated as heat (Axelos et al., 1991) and is an apparent viscous component present in a gel. These values are typically obtained by doing an oscillatory frequency scan at low levels of stress or strain to minimize disruption of the gel. By scanning the frequency, the mechanical response of the material can be probed and the gel characterized without irreversibly altering the cross-links. Strong gels typically have G´ values that are an order of magnitude or greater than G˝, whereas with weak gels the difference between modulus values is much smaller.

The purpose of the work described herein is to study the effects of altering the pectin-to-calcium ion ratio and to examine the recovery of modulus before and after shearing to optimize yield stress behavior for suspension applications.

Material and Methods

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Pectin demethylation, demethylation analysis, and block determination. Pectin samples and methods for pectin demethylation and demethylation analysis were described previously (Cameron et al., 2007) for the pH 4.5, 55% DM pectin and the pH 7.5, 62% DM pectin.

Rheological Measurements

Oscillatory experiments before shearing. The viscoelasticity of the gel was characterized by oscillatory measurements using a stress-controlled AR 2000 Rheometer (TA Analytical, Wilmington, DE) equipped with 60-mm acrylic parallel plate geometry. The rheological measurements were conducted in three replicates. Pectin solutions at 0.2% concentration were prepared and stirred for 24 h at room temperature. The pH was adjusted to 7.0 and 0.02 g of CaH_2PO_4 was added and mixed overnight, with no gel formation observed. The pH was then adjusted to 5.9 by addition of an aliquot of freshly prepared 1.5% gluconolactone solution, which results in a uniform time release of calcium ion into solution and is similar to a previously described method of calcium-pectin gel formation (Powell et al., 1982). After brief mixing for 15 s, a 4-mL sample was placed under the parallel plates. After gap adjustment, 1000 µm, a manufacturer-designed cover was used over the geometry to prevent evaporation, which utilizes a water seal built into the geometry design. The time sweep was performed for 20 h at a strain of 0.02 and a frequency of 0.2 Hz.

The frequency scan was performed immediately after the time sweep without disturbing the sample. The measurements were performed at 20.0 °C at a frequency range of 0.005 to 600 rad·s^{-1} in log mode to measure G´ and G˝ and tan (δ) = G˝/G´.

Oscillatory experiments after shearing. Immediately following the oscillatory experiments where no shear was applied, the samples were subjected to a rotational shear rate of 20 s^{-1} for 15 s and then a time sweep was performed for 2 h at a strain of 0.02 and a frequency of 0.2 Hz. Another frequency scan was performed immediately after the time sweep without disturbing the sample. The measurements were performed at 20.0 °C at a frequency of range of 0.005 to 600 rad·s^{-1} in log mode. Standard deviations were determined from values obtained in duplicate runs.

Result and Discussion

In earlier studies (Luzio, 2007), pectin samples were exposed to shearing while loading onto the instrument. This procedure has limited value to study the effects before and after shearing, which is important for suspension applications where intermittent shearing is applied. The earlier procedure could not be used under this conditions, since the loading procedure irreversibly disrupted the gel properties under study prior to taking shearing measurements. Shearing can irreversibly alter a gel in such a manner that a portion of the storage modulus is not recovered, reducing the capacity of the suspension aid. Consequently, it is important to quantify this possible capacity loss.

A new sampling procedure was adopted to examine the effects of added calcium ion and shearing on the rheological properties of this pectin. This was accomplished by gelling a sample in-situ by lowering the pH with gluconolactone to induce calcium release after placing the pectin mixture under the instrument geometry. In this manner, one can examine an undisturbed gel sample. Gelling is affected by the duration of time it takes to release the calcium (among other factors), so modulus values were monitored for a minimum of 20 h after placing the sample on the instrument.

As shown in Figure 1, the maximum value of G´ of 9.92 (±3.14) Pa and the value of G˝ reached 0.280 (±0.013) Pa after the 20-h gel time for the pH 4.5 deesterified sample at a concentration of 0.2% with calcium hydrogen phosphate concentration of 0.06%. Standard deviations were calculated from values for duplicate runs and are given in parenthesis. The tan (δ) = G˝/G´ reached a minimum value of 0.028 (±0.018) after 20 h. A minimum value of tan delta indicates a maximum value for gel strength. A low value for tan (δ) indicates that the major contribution to the modulus is provided by the storage modulus, which is an elastic component needed for particle suspension. All experiments performed showed a similar cure time of 20 h to reach a minimum tan (δ) (data not shown) for both the pH 4.5 and pH 7.5 deesterified pectins. Thus all samples were allowed to cure for 20 h before running a frequency sweep experiment.

As noted previously, by scanning the frequency the mechanical response of the gel can be explored and characterized. The data for the frequency scan for a pH 4.5 deesterified pectin are shown in Figure 2. For this sample the linear viscoelastic range (LVR) was from an angular frequency of 0.005 to approximately 60 rad·s^{-1} for G´, whereas the LVR for G˝ was from 0.005 to 1

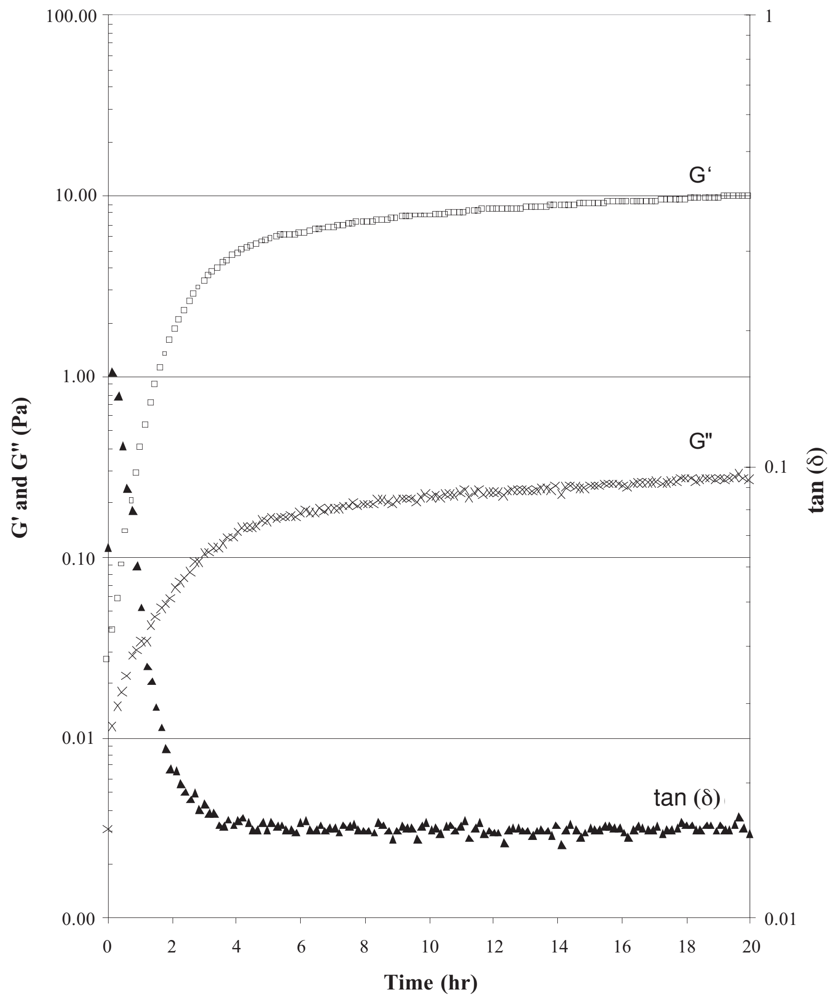

Fig. 1. Time sweep at 0.2% pectin deesterified at pH 4.5 to a DM of 55% at a strain of 0.02 and frequency of 0.2 Hz: (❑) storage modulus G´; (✕) loss modulus G˝; (▲) tan (δ).

rad·s–1 with a strain of 0.02 (strain = displacement/gap and thus is a unit-less number). Similar data were observed for the pH 7.5 sample (data not shown). It is important to measure the modulus values in the LVR where the testing procedure is not adversely affecting the gel cross-links being measured, while sufficient strain is being induced to provide a quantifiable measurement of gel bond strength. In the LVR, one is typically testing the strength of the calcium cross-links without breaking the cross-links and inducing an irreversible alteration to the gel structure. In the experiment, the frequency is gradually increased from a low value to a higher value until the measurement reaches a sufficiently high frequency where gel bond cross-links can be temporarily disrupted and the sample takes on characteristics more similar to a liquid than a solid. A low angular frequency in the LVR of 0.1 rad·s–1 was chosen as the frequency at which the modulus values were taken for comparison. At this angular frequency the G´, G˝, and tan (δ) values were 9.69 (±3.54) Pa, 0.247 (±0.028), and 0.0255 (±0.0131), respectively, as shown in Figure 2. As expected, this is similar to the values obtained in the time sweep experiments, which are performed using similar oscillation and strain values. These data indicate that the complex modulus is mostly comprised of an elastic component, G´, which again is an important component in this system for suspension. The lack of change in modulus with change in angular frequency, until a frequency value of greater than 100 rad·s–1 is reached, is consistent with previous findings that this gel is a pseudoplastic material (Luzio, 2003).

Having determined the required gelation time as 20 h, and selecting oscillation settings of 0.1 rad·s–1 for measuring the gel strength in the LVR, then the effects of added calcium ion on the storage modulus values and gel strength could be studied. These data are given in Figure 3 for the pH 7.5 deesterified sample, where the pectin concentration remained constant, but the calcium phosphate concentration was increased stepwise in a range of 0.01% up to 0.1%. The data are plotted as the storage modulus vs. the molar ratio of calcium ion to AGA (as free acid). The free acid value only takes into account the moles of AGA carboxyl groups in the pectin molecule that are not esterified, since they are the only functional groups present that can form an ionic bond with calcium ion. At low molar ratio values <0.2, the storage modulus values were <2.5 Pa. A maximum value of storage modulus was observed at mole ratios of 1.0 and 1.2 where the G values were 34.5 and 52.1 Pa, respectively. This would correspond to one calcium ion per free acid site regardless of whether that represents a random demethylation site or a block of demethylated AGA units. At higher mole ratios the G´ values decreased to where a mole ratio of 1.8 G´ was 3.4 Pa. At low mole ratios, G´ is low since there are insufficient calcium ions

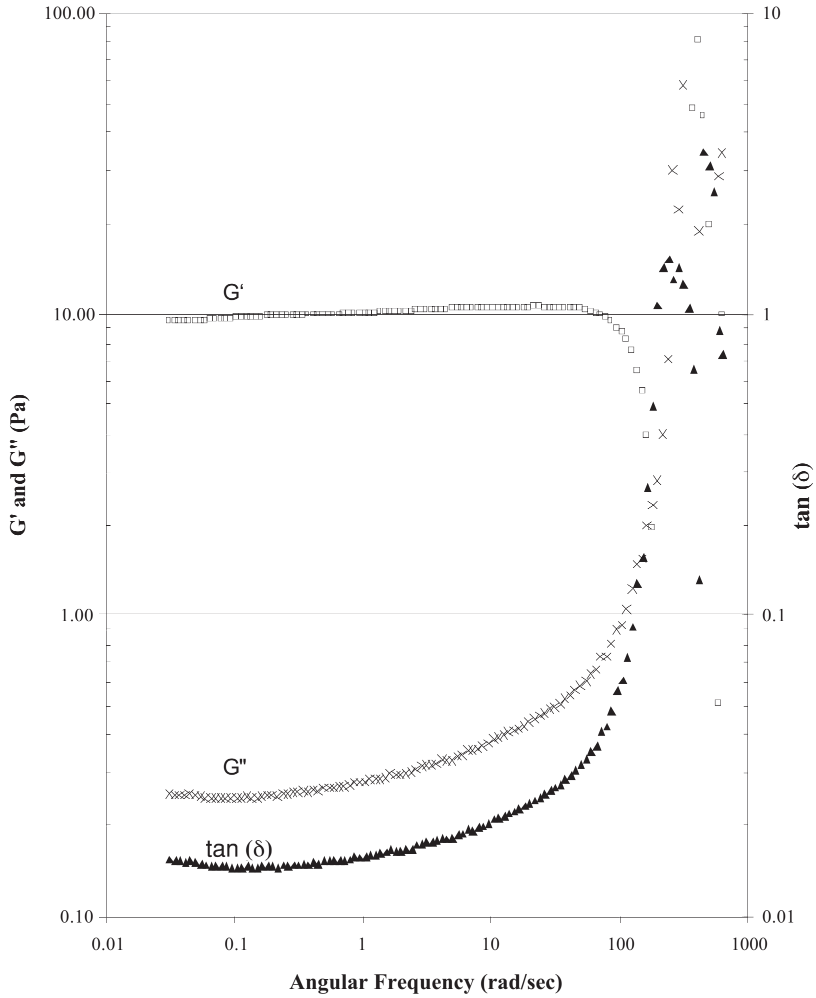

Fig. 2. Frequency sweep at 0.2% pectin for pectin deesterified at pH 4.5 to a DM of 55% at a strain of 0.02: (❑) storage modulus G´; (✕) loss modulus G˝; (▲) tan (δ).

present to form all of the cross-links and functional bonds are not fully formed. At high mole ratio values, the lowering of the G´ may be due to gel contraction and aggregation of the pectin molecules caused by the collapse of the gel in the presence of excess calcium salt ions. These data demonstrate the importance of controlling the calcium ion in order to reach maximum storage modulus values required for suspension applications.

Shearing effects were studied at 0.3% pectin with 0.09% calcium hydrogen phosphate, which is equivalent to a 1:1 mole ratio for calcium ion vs. AGA as free acid. Data are given in Figure 4 for storage and loss modulus before and after shearing the gel. Rotational shear rate was held 15 s at a rotational rate of 20 s^{-1}.

A higher concentration of pectin was used to provide for higher modulus values after shear. As previously shown for shear at an angular frequency of 0.1 rad·s^{-1}, G´ was 48.1 Pa and after shear this decreased to 0.13 Pa for a decrease in storage modulus of 99.7%. There is a frequency dependence for the storage modulus above 0.1 rad·s^{-1}, which indicates that this system behaves more

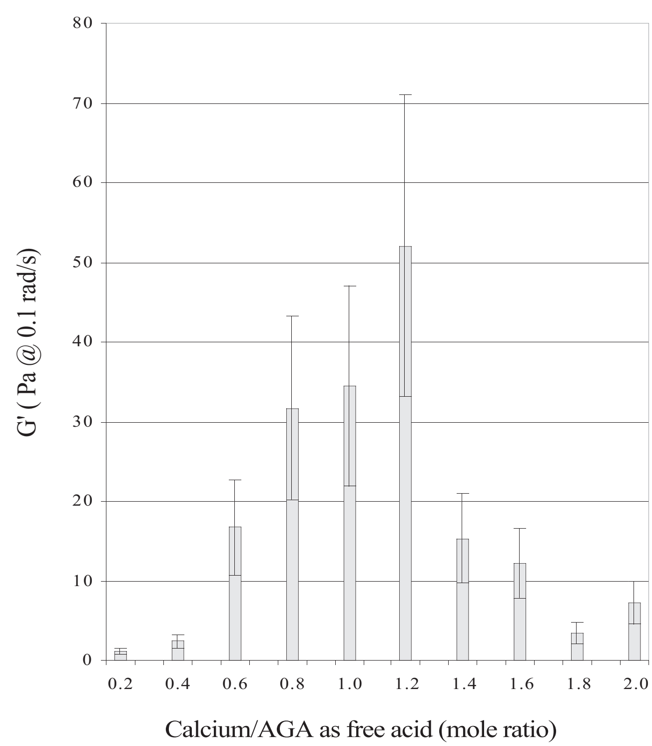

Fig 3 (**right**). Storage modulus G´ measured at a strain of 0.02 and frequency of 0.2 Hz vs. calcium/AGA as free acid mole ratio for at 0.2% pectin deesterified at pH 7.5 to a DM of 62% at a strain of 0.02. Pectin concentration held constant at 0.2% and calcium ion concentration adjusted to achieve particular mole ratio. pH adjusted to 5.9 ± 0.2 using gluconolactone. Error bars represent standard deviation.

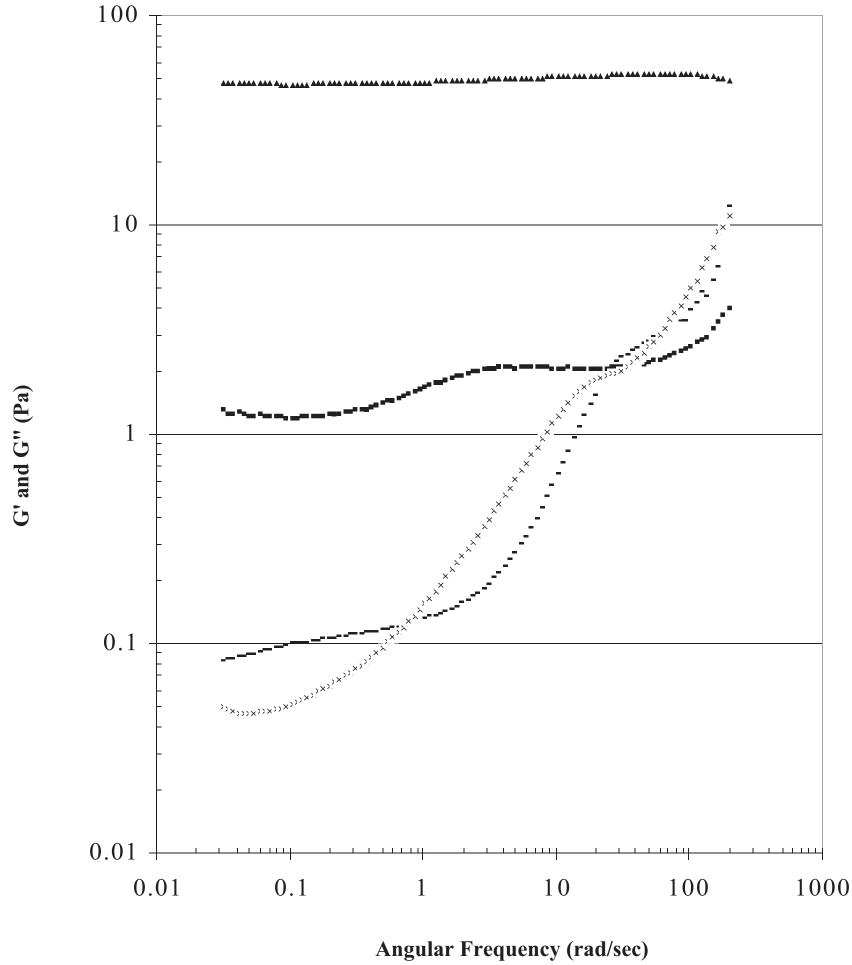

Fig. 4. Frequency sweep at 0.3% pectin for pectin deesterified at pH 4.5 to a DM of 55% at a strain of 0.02: (– – –) storage modulus G´ before shear; (■) loss modulus G˝ before shear; (✕) storage modulus G´ after shear; (◆) loss modulus G˝ after shear.

like a liquid than a gel. There was also a decrease in loss modulus G˝ from 1.66 Pa before shearing to 0.15 Pa after shearing for a decrease of 90%. Loss in yield stress behavior will also occur in this mixture since decrease in modulus is observed. This experiment illustrates the importance of adopting the gel-in-place procedure, used herein, since accurate measurements can now be obtained to illustrate the effects of shear on the gel sample.

Conclusions

Use of citrus pectin methylesterase to deesterify pectin resulted in a polysaccharide that, in solution with calcium ion, exhibited pseudoplastic properties. These solutions behave as if they are weak, shear thinning gels that could be useful for suspension applications such as suspension of pulp in fruit juices. The optimum level for calcium ion concentration to reach a maximum in storage modulus appears to be nearly equivalent to the molar concentration value for the free carboxyl groups present on the pectin molecule and the minimum pectin concentration required is approximately 0.5%. Improvements in yield stress will require finding conditions by which greater retention of modulus is observed after shearing.

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