Characterization of Pectins by Size Exclusion Chromatography in Conjunction with Viscosity Detection

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High-performance size exclusion chromatography (HPSEC) with detection by refractive index and viscosity was employed to characterize citrus pectins with varying degrees of methylesterification (DM). Studies in water revealed that high-methoxy pectins undergo a sequential size expansion with decreasing concentration. Nevertheless, both intrinsic viscosity and radius of gyration as a function of concentration gave typical hyperbolic behavior. In contrast, decreasing the ionic strength of the solvent induces a size expansion in all pectin molecules. Frontal-type experiments on nonfractionating HPSEC columns with 0.05 M NaCl as the solvent revealed virtually no dependence of reduced viscosity on pectin concentration. A plot of log (intrinsic viscosity) against log (radius of gyration) gave a linear plot for pectic substances with DM between 0 and 73.9. It was shown that such a plot can give shape information.

Since pectins are a class of complex polysaccharides whose main components are α-D-galactopyranosyluronate residues (BeMiller, 1986), it is important to determine which structural features of pectins are important in determining their functional properties in foods. One feature that must be considered is the molecular weight distribution. A rapid method for determining molecular weight distributions in addition to other structural features is high-performance size exclusion chromatography (HPSEC), in conjunction with viscosity measurements. Anger and Berth (1985) fractionated sunflower pectin on a gel filtration column and separately determined the intrinsic viscosity of the individual fractions they collected. Deckers et al. (1986) employed HPSEC and capillary viscosity measurements on whole samples to characterize apple pectins by universal calibration. Here, we employ HPSEC, a viscosity detector, and a refractive index detector in series to measure radii of gyration, molecular weights, and intrinsic viscosities of citrus pectins with different degree of methylesterification (DM). This system, in addition to being more rapid and more readily automated than previous systems, has the advantage of yielding all the information on a single sample under identical conditions. Thus, ambiguous results arising from changes in the size of pectin aggregates due to differences in conditions of measurement, sample concentration, and method of preparation (Fishman et al., 1984, 1985, 1986a,b) are likely to be eliminated or significantly reduced by the method employed here.

EXPERIMENTAL SECTION

Materials. Dextran and pullulans standard have been described previously (Fishman et al., 1987). Characterization and preparation of sodium polygalacturonate and citrus pectins with DM 35, 37, 57, 58–60, 72, and 73 were described earlier (Fishman et al., 1984, 1986a). Two samples of commercial lime pectin were gifts from Grindsted Products, Inc. (Industrial Airport, KS). One sample had a DM of 73.9 and a uronate content of 81.6% of total sugars by weight whereas the other had a DM of 65.6 and a uronate content of 93.4% of total sugars by weight as determined by the manufacturer. The lime pectins were prepared in the same manner as the citrus pectins.

Chromatographic Analysis. Macromolecular solutions were passed through a 0.4-μm nucleopore filter and equilibrated overnight at 35 °C in capped bottles prior to chromatography. Sample concentrations were adjusted to give a minimum signal

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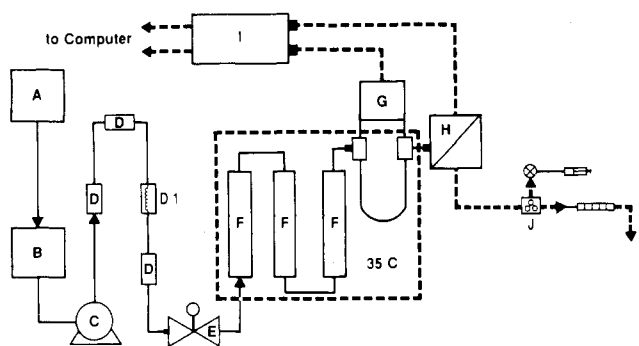
to noise of 10:1 from the viscosity detector but usually was in the range of 20 or 30:1. The injected sample volume was 1.54 mL for experiments with nonfractioning columns and 100 μL for experiments with fractionating columns. The schematic of the chromatographic system is depicted in Figure 1. The mobile phase was either distilled water passed through an ion-exchange system or the same water, which was 0.05 M in NaCl. Solvent was degassed prior to connecting to the system and inline with a Model ERC 3120 degasser (Erma Optical Co., Tokyo). Two pumps were employed during the course of the research. A Waters Associates (Millford, MA) Model M6000A was fitted with three M45 pulse dampeners. Two of the pulse dampeners were mounted on a plate and separated by 15 ft of coiled capillary tubing (i.d. 0.01 in.). Also employed was a Beckman Model 334 solvent delivery system (Beckman Instruments, Palo Alto, CA). With the latter pumping system, one of the M45 pulse dampeners was replaced with a Beckman pulse filter. In addition, periodic pump noise was removed with an empirical, digital filtering program for all pectin samples with DM 37 or less dissolved in 0.05 M NaCl. Sample injection was with a Beckman Model 210 valve. Nonfractionating experiments were performed with two columns in series, Synchropak GPC 100 (250 × 4.6 mm) (Synchrom, Inc., Linden, IN). Three columns in series, a μ-Bondagel E-High, E-1000, and a Synchropak GPC-100, were employed in fractionating experiments. Column dimensions were 300 × 3.9 mm for the μ-Bondagel columns and 250 × 4.6 mm for the GPC-100. The homemade viscosity detector and the inclusion of pulse dampening were similar to that described by Malihi et al. (1984). The nominal value of the capillary tubing i.d. was 0.007 in. Nevertheless, an i.d. of 0.0124 in was obtained from the pressure drop of water (2.83 lb/in² at 23.8 °C and 0.5 mL/min) as calculated from Poiseuille's law. Differential refractive index (DRI) was measured with a Model ERCC 7810 refractive index monitor (ERMA Optical). Chromatography columns and the capillary tubing of the transducer were thermostated in a temperature-controlled water bath at 35 ± 0.003 °C, and the cell of the DRI monitor was thermostated also at 35 °C. Measurement of flow rate, data acquisition, and data reduction have been described previously (Fishman et al., 1987). All samples were chromatographed at 0.5 mL/min.

THEORY

According to the Hagen–Poiseuelle law (Lecacheux and Lesec, 1982), the longitudinal pressure drop, ΔP, produced by constant laminar flow through capillary tubing, is proportional to the kinematic viscosity, η, of the moving fluid:

$$\eta = k\Delta P \tag{1}$$

The specific viscosity, η_{sp}, of a macromolecular solution is defined by eq 2. The subscript s refers to the macro-

$$\eta_{sp} = (\eta_s - \eta_o)/\eta_o \tag{2}$$

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Figure 1. Schematic of chromatography system: A, reservoir; B, degasser; C, pump; D, pulse dampener; D1, coil; E, injection valve; F, chromatography column; G, viscometer; H, differential refractometer; J, flow meter; I, recorder.

molecular solution, whereas the subscript o refers to the solvent. Substitution of eq 1 into 2 enables one to obtain the specific viscosity of a macromolecular solution from eq 3 by measuring the pressure drop of solvent and solu-

$$\eta_{sp} = (\Delta P_s - \Delta P_o)/\Delta P_o \tag{3}$$

tion across the tubing length. According to the Huggins equation (Tanford, 1961), in dilute solution η_{sp} is proportional to the concentration of macromolecules in solution, c, and the proportionality constant is called the intrinsic viscosity, $[\eta]$:

$$\eta_{sp} \approx [\eta]c \tag{4}$$

The reduced viscosity, η_{red}, is defined by eq 5 and in dilute solution is equal to $[\eta]$. The intrinsic viscosity is related

$$\eta_{red} = \eta_{sp}/c \approx [\eta] \tag{5}$$

to the root-mean-square radius of gyration, R_g, and the molecular weight, M (Tanford, 1961), by means of eq 6.

$$[\eta] = A(R_g)^3/M \tag{6}$$

Here, A is constant for a series of macromolecules with similar shape, whose molecular weight increments by an increase in a linear dimension. It has been found that R_g is related to the molecular weight through eq 7 (Yau et al.,

$$M = B(R_g)^{1/x} \tag{7}$$

1979). The exponential x is related to the shape of the macromolecule in solution. Substituting eq 7 into eq 6 yields eq 8. According to eq 8, a plot of log $[\eta]$ against

$$[\eta] = C(R_g)^{3-(1/x)} \tag{8}$$

log R_g will yield a straight line whose slope will equal 3 – $(1/x)$. Since R_g can be measured from SEC (Fishman et al., 1987), SEC in conjunction with viscosity detection will yield shape information.

Calculations of Intrinsic Viscosity from Online Experiments. The intrinsic viscosity of a solute molecule as a function of elution volume from a chromatographic column is given by eq 9 and 10 (Malihi et al., 1984). Here, concentration of the eluting solute $C(v)$ is monitored by refractive index, and $\Delta P(v)$ is measured by a pressure drop viscometer

$$[\eta](v) = [1/C(v)][\Delta P_s(v) - \Delta P_o]/\Delta P_o \tag{9}$$

$$C(v) = [W\Delta RI(v)]/\int \Delta RI(v) \, dv \tag{10}$$

where $\Delta RI(v)$ is the refractive index response of solute as a function of elution volume. W is the weight of injected macromolecule. The integral is the area of the refractive
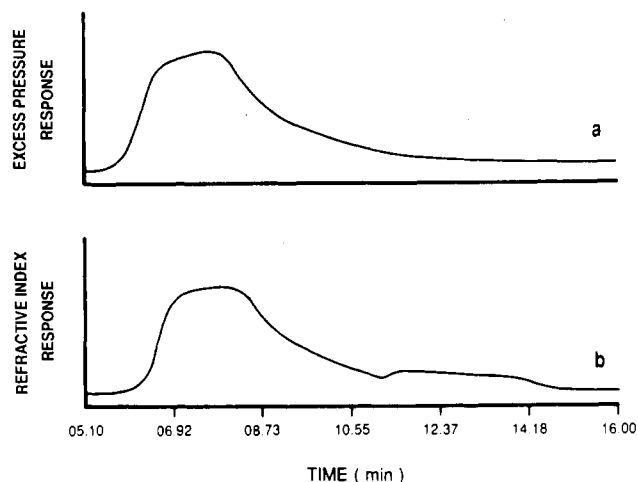

Figure 2. Chromatograms from plateau experiment on non-fractionating columns: (a) viscometric detector; (b) differential refractometer detector. Conditions: DM 72–73 pectin, protonated form; mobile phase, 0.05 M NaCl; sample concentration, 1.12 mg/mL; injection volume, 1.54 mL.
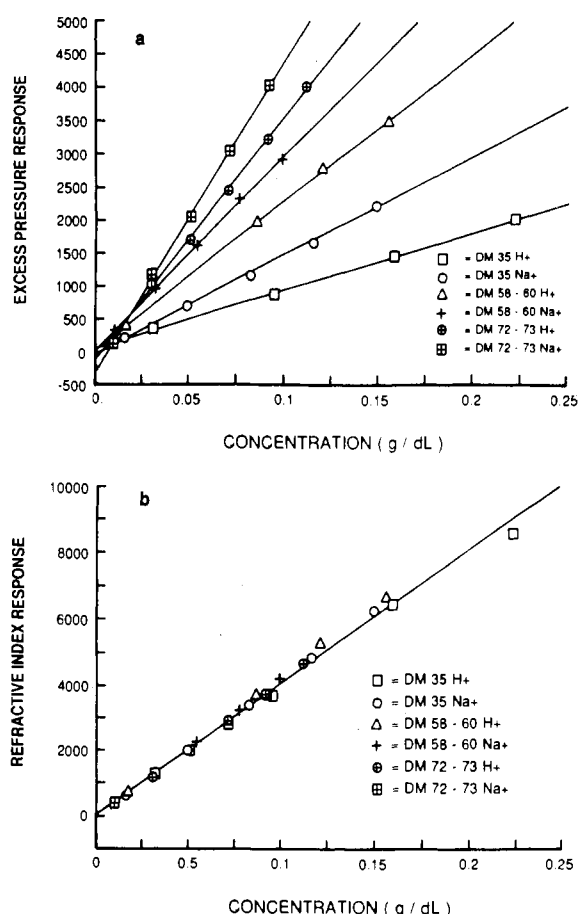

Figure 3. Detector linearity: (a) dependence of excess pressure response on pectin concentration; (b) dependence of refractive index response on pectin concentraction. Conditions: mobile phase, 0.05 M NaCl; injection volume, 1.54 mL.

index peak as determined from analog to digital conversion of the ΔRI signal. For the case of a sample that elutes in the void volume of an SEC column, $C(v)$ can be taken as the average concentration of the entire sample. Furthermore, if eq 3 and 4 are combined and rearranged, eq 11 is obtained. Since

$$\Delta P_s - \Delta P_o = (\text{const})C(v) \tag{11}$$

$$\Delta RI = (const') C(v) \qquad (12)$$

then

$$\Delta RI = (const'')(\Delta P_s - \Delta P_o) \qquad (13)$$

Substituting eq 13 into eq 10 and eq 10 into eq 9 gives eq 14. The integral in the numerator of eq 14 is the area of

$$[\eta] = \int (\Delta P_s - \Delta P_o) \, dv / W \Delta P_o \qquad (14)$$

the viscosity detector peak when the detector output potential is set at zero with mobile phase flowing. The term $\Delta P_s - \Delta P_o$ is proportional to the excess viscosity arising from dissolved polymer. The pressure drop of the solvent, ΔP_o, is obtained by zeroing the output of the detector with the pump off and then measuring the detector output under mobile phase flow.

In dilute solution, it can be assumed that the specific viscosities of individual molecules are additive (Flory, 1953), a statement embodied in eq 15.

$$\eta_{sp} = \sum \eta_{sp}(v) \qquad (15)$$

A consequence of eq 15 is that eq 14 applies regardless of whether the macromolecular solution eluted in the void volume of a column or was fractionated by the column; i.e., in either case the area under the viscosity detector trace gives the intrinsic viscosity of the entire sample.

An elution band with a plateau can be produced by adopting the procedure of Barford et al. (1978): A small sample (e.g. 100 μL) is injected onto the column and the retention volume of the eluted peak width measured (ca. 5σ for a Gaussian peak). A sample volume is injected onto the column, which is 6.25σ. For any point on the plateau, $C(v) = C(i)$ and $\Delta P(v) = \Delta P(i)$. Substituting these quantities into eq 9 gives eq 16. Thus, by measuring the

$$[\eta] = [1/C(i)][\Delta P(i) - \Delta P_o]/\Delta P_o \qquad (16)$$

pressure drop along the plateau region of the chromatogram, $\Delta P(i)$, and from knowledge of the sample concentration prior to injection, $C(i)$ the intrinsic viscosity of the macromolecule is obtained.

If the macromolecule elutes in the void volume of the column set, the experiment is a form of flow injection analysis applied to viscometry, whereas if the macromolecule undergoes fractionation in the column set, additional information may be obtained concerning the viscosity of each molecular weight species through frontal analysis of the buildup portion of the chromatogram.

RESULTS AND DISCUSSION

Plateau Experiments. Parts a and b of Figure 2 contain typical traces from the viscosity and refractive index detectors, respectively. Pectin dissolved in 0.05 M NaCl was pumped through two nonfractionating GPC-100 columns in series. In Figure 3a, excess pressure drop at the plateau is plotted against concentration for several pectins. These plots demonstrate the linearity of the detector over the concentration range studied. Theoretically, these lines should pass through zero. All lines but that of the sodium salt of DM 72–73 pectin had intercepts close to zero. The nonzero intercepts were assumed to arise from systematic errors. Thus, the lines were adjusted upward or downward, point by point, by the nonzero value of their respective intercepts. In Figure 3b, differential refractive index at the plateau is plotted against concentration. Interestingly, all points regardless of DM fall on the same straight line, and the line passes through the origin. Surprisingly, Huggins plots (see Figure 4) have slopes close to zero. Thus, eq 5 appears to approximate the dependence of specific viscosity on concentration for pectin over the range studied. These results confirm the assumption
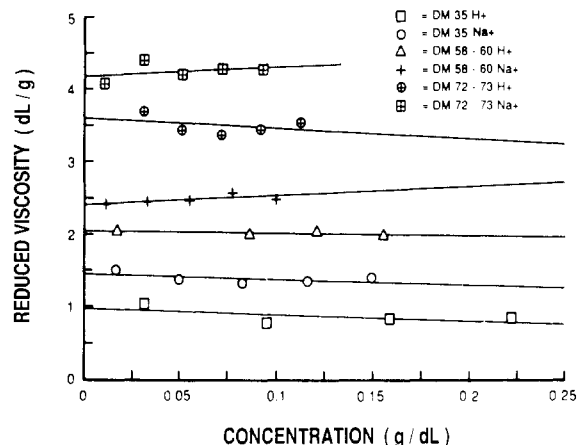

Figure 4. Huggins plot for pectins in 0.05 M NaCl.

Table I. Viscosity[a] of Dextrans from Fractionating Columns

sample	concn, mg/mL	eq 14	lit. value[b]
T500	17.45	0.564	0.53
T250	16.21	0.418	0.44
T110	16.58	0.328	0.32
T70	19.45	0.262	0.26
T40	23.00	0.203	0.210
T20	22.51	0.141	0.148
T10	22.48	0.085	0.090

[a] Deciliters per gram. [b] Obtained from supplier.

that the reduced viscosity can be equated with intrinsic viscosity for pectins, in 0.05 M NaCl and at the concentrations employed here.

Fractionating Experiments. Intrinsic viscosities of dextran standards were calculated from areas under the excess pressure drop chromatogram to check the validity of eq 14 and 15, for the fractionating case. The results of these measurements are in Table I.

The ability of dissolved pectin to undergo size contraction with increases in ionic strength of the solvent is well documented (Michel et al., 1984). Decreasing the concentration of pectin in pure water significantly decreases the ionic strength of the solvent by simultaneously decreasing the concentration of counterions. Parts a and b of Figure 5 contain chromatograms of DM 72, 73 pectin in the sodium form run at several concentrations. Reduced viscosity from the areas under the curves in Figure 5a are plotted against concentration in Figure 6. Also included in that figure are data points for DM 73.9 lime pectin in the protonated form (chromatograms are not shown). The nonlinear increase in size with decreased concentration is similar to that observed previously when the reduced viscosity as a function of pectin concentration was determined by conventional capillary viscometry (Pals and Hermans, 1952). Furthermore, as found previously, the data are linearized by constructing a Fuoss plot (see upper straight lines in Figure 6) (Michel et al., 1984). The intercept of the Fuoss plot gave values of 82 ± 19 and 147 ± 13 dL/g, for the intrinsic viscosity of DM 72, 73 Na and DM 73.9 H, respectively. Moreover, combining HPSEC with viscometric detection has revealed new information about the so-called "polyelectrolyte effect" in the case of pectin. Apparently, the increase in size with decreasing pectin concentration is a sequential process. As the concentration of pectin increased, smaller sized species appeared in addition to the larger sized species persisting from lower concentrations.

Increasing the ionic strength of the solvent by adding 0.05 M NaCl also reduced the size of pectin, but the se-
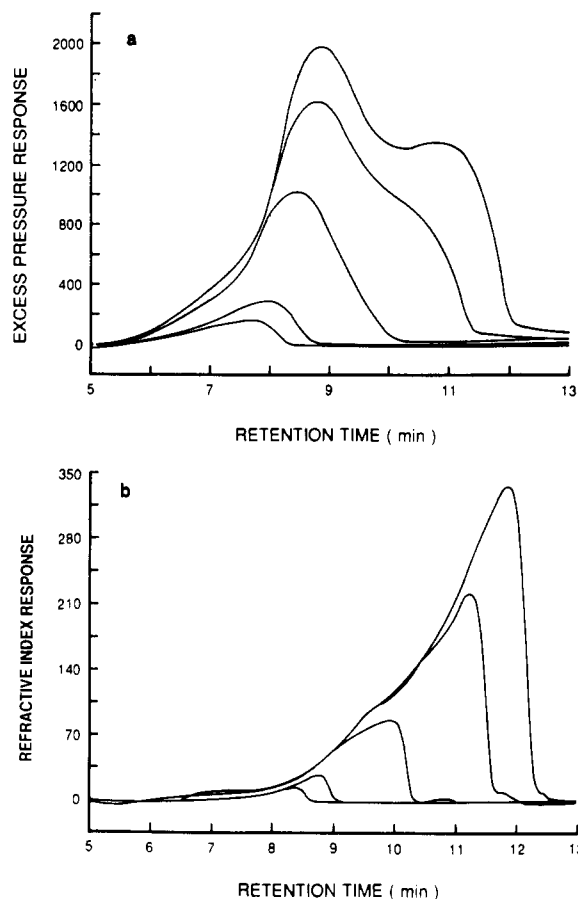
Figure 5. Chromatograms of sodium pectinate: (a) viscometric detector; (b) refractive index detector. The concentration is proportional to the area under the curve. Conditions: DM 72–73 on fractionating columns; mobile phase, water; injection volume, 100 μL; concentrations, 0.35, 0.25, 0.15, 0.05, 0.01 g/dL.
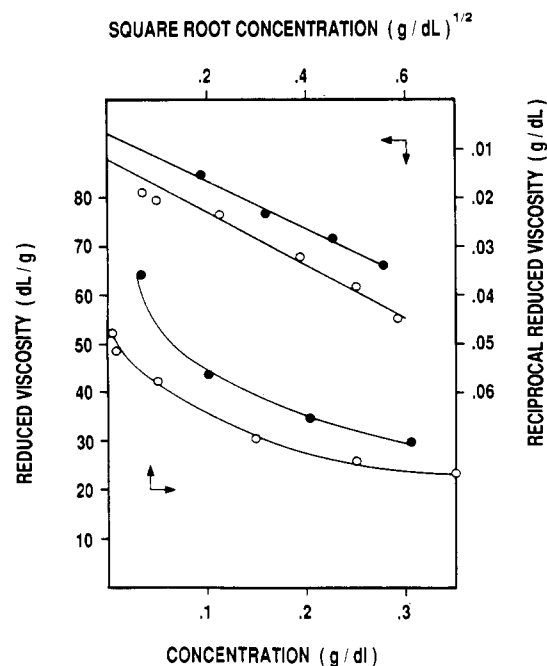

Figure 6. Concentration dependence of reduced viscosity from area under excess pressure response chromatogram, according to eq 14 in the text: open circles, sodium pectinate, DM 72–73; solid circles, protonated lime pectin, DM 73.9. Linear curves plotted according to equation of Fuoss (Michel et al., 1984).

quential change in the pectin size distribution has disappeared. Chromatograms of DM 72, 73 H in water and 0.05
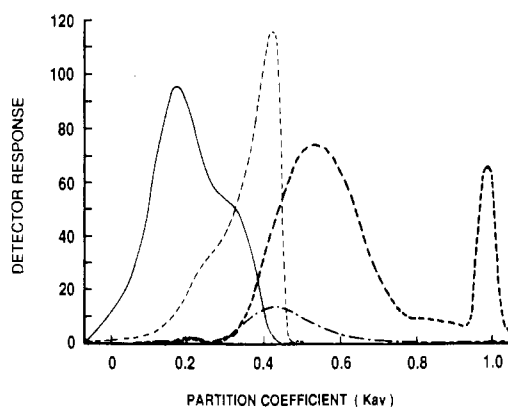

Figure 7. Effect of NaCl on pectin chromatographed on fractionating columns: solid line, excess pressure trace in water; thin dashed line, refractive index trace in water; dotted–dashed line, excess pressure trace in 0.05 M NaCl; thick dashed line, refractive index trace in 0.05 M NaCl. Conditions: sample, protonated pectin, DM 72–73; injection volume, 100 μL; concentration 2.5 mg/mL.
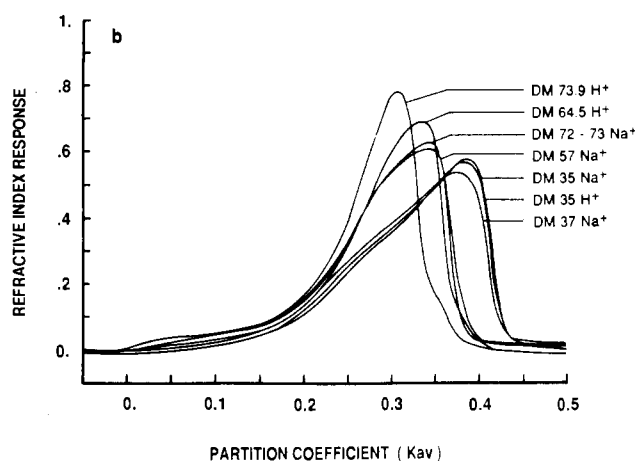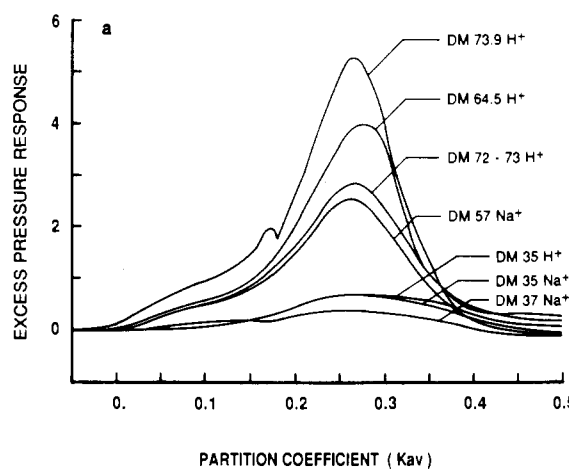

Figure 8. Pectins in water chromatographed on fractionating columns: (a) viscometric detector; (b) refractive index detector. Conditions: injection volume, 100 μL; sample concentrations, DM 35 (Na) 0.46 mg/mL, DM 35 (H) 0.42 mg/mL, DM 37 (Na) 0.36 mg/mL, DM 57 (Na) 0.38 mg/mL, DM 72–73 (H) 0.39 mg/mL, DM 64.5 (H) 0.36 mg/mL, DM 73.9 (H) 0.34 mg/mL.

M NaCl are in Figure 7. The concentration of pectin in water was 0.389 mg/mL whereas in salt it was 2.52 mg/mL. The attenuation of the differential refractive index response was increased in the case of the chromatogram with salt as the solvent, so that it could be compared with the chromatogram run in water. The excess pressure traces for both solvents are run at the same attenuation. Parts

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Table II. Pectin Viscosity from Fractionating Columns

| | water | | | | 0.05 M NaCl | | | |
| | Na⁺ | | H⁺ | | Na⁺ | | H⁺ | |
DM[a]	concn[b]	η_{sp}/c	concn	η_{sp}/c	concn	$[\eta]$[c]	concn	$[\eta]$
0	2.14	3.9 ± 0.1[d]	2.10	2.3 ± 0.5	15.9	0.25 ± 0.01		
35	0.42	14	0.46	14 ± 4	11.4	1.48 ± 0.04	5.97	0.41 ± 0.10
37	0.36	5.1 ± 2.5	0.39	8.1	11.0	0.94 ± 0.05	5.28	0.30 ± 0.09
57	0.38	20.1 ± 0.8	0.39	34	2.53	3.94 ± 0.01	3.57	2.90 ± 0.10
58–60					2.52	3.25 ± 0.13	2.49	1.70 ± 0.35
64.5			0.36	48 ± 1			2.69	3.82 ± 0.13
70	0.34	22	0.35	29				
72–73	0.37	34	0.39	35	2.64	3.87 ± 0.15	2.52	3.19 ± 0.23
73.9			0.34	64			2.49	5.27 ± 0.09

[a] Degree of methylesterification. [b] Milligrams per milliliter. [c] Deciliter per gram. [d] Standard deviation of triplicates except for DM 35 (H) and DM 72–73, which are duplicates. Single determination if standard deviation not shown.

Table III. Average Radii[a] of Gyration for Pectins[b]

| | number-average | | weight-average | | Z-average | |
DM[c]	Na⁺	H⁺	Na⁺	H⁺	Na⁺	H⁺
0	39 ± 1[d]		45 ± 1		52 ± 1	
35	61 ± 1	42 ± 2	97 ± 2	64 ± 6	155 ± 18	115 ± 14
37	57 ± 1	44 ± 7	96 ± 3	68 ± 17	186 ± 15	108 ± 30
57	107 ± 1	93 ± 2	210 ± 2	176 ± 3	328 ± 5	286 ± 19
58–60	111 ± 1	81 ± 4	208 ± 6	154 ± 6	363 ± 34	259 ± 2
64.5		113 ± 2		211 ± 4		317 ± 12
72–73	109 ± 3	106 ± 2	207 ± 5	207 ± 5	331 ± 18	330 ± 4
73.9		135 ± 8		242 ± 3		349 ± 2

[a] Angstroms. [b] Solvent is 0.05 M NaCl. [c] Degree of methylesterification. [d] Standard deviation of triplicate determination except for DM 35 (H) and DM 72–73 (Na), which are duplicate determinations.

a and b of Figure 8 are typical chromatograms run in water for a series of pectins with differing degrees of methylesterification, whereas parts a and b of Figures 9 are typical chromatograms run in 0.05 M NaCl. Reduced viscosities in water and intrinsic viscosities in 0.05 M NaCl calculated from areas under the excess pressure drop chromatograms are contained in Table II. Even though viscosities in NaCl were essentially intrinsic viscosities, whereas those measured in water were reduced viscosities, pectin viscosities in water are significantly higher than in 0.05 M NaCl.

As indicated by Figure 7, the refractive index trace of pectins chromatographed in 0.05 M NaCl exhibited a large peak eluting at the total retention volume of the column (V_t), whereas this peak was absent in the refractive index chromatograms of pectin eluted in water. A totally included peak in buffered solutions of pectin has been reported previously (Barth, 1980). We have observed this peak in chromatograms measured at 23 °C, but it was much smaller than observed here. No peak was observed at V_t for the comparable chromatogram obtained from the viscosity detector. Since all pectins were dialyzed against four changes of water over 48 h in their preparation, this peak may arise from low molecular weight fragments adsorbed to pectin, but which dissociate under column shear in 0.05 M NaCl.

Radius of Gyration. Previously (Fishman et al., 1986b), for pectins in 0.05 M NaCl, the radius of gyration (R_g) was obtained by assuming that pectins coelute on a size exclusion column with pullulans and dextrans of identical radii. Figure 10 is the calibration curve of log R_g plotted against the partition coefficient, K_{av}, for a series of narrow pullulan and broad dextran standards chromatographed on the bank of fractionating columns employed here. The calibration method has been detailed elsewhere (Fishman et al., 1987). Number-, weight-, and Z-average values for R_g are given in Table III. Comparison of these and previous measurements revealed that the values obtained in this study were about 10–25% lower than values measured in the previous study for comparable pectins
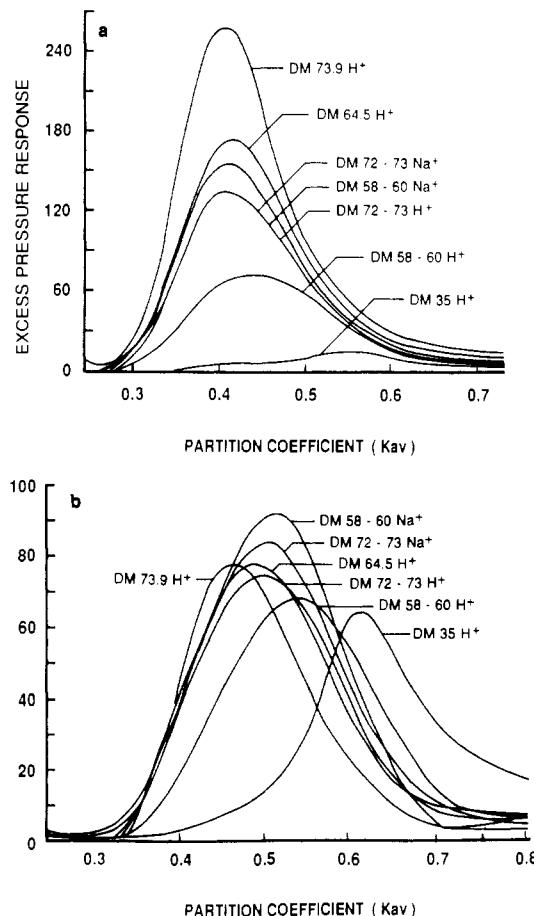

Figure 9. Pectins in 0.05 M NaCl chromatographed on fractionating columns: (a) viscometric detector; (b) refractive index detector. Conditions: injection volume, 100 µL; sample concentrations, DM 35 (H) 5.97 mg/mL, DM 58–60 (H) 2.49 mg/mL, DM 72–73 (H) 2.52 mg/mL, DM 58–60 (Na) 2.52 mg/mL, DM 72–73 (Na) 2.64 mg/mL, DM 64.5 (H) 2.69 mg/mL, DM 73.9 (H) 2.49 mg/mL.

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Table IV. Radius of Gyration[a] at Peak Maximum for Pectins

| | water | | | | | | 0.05 M NaCl | | | | | |
| | Na$^+$ | | | H$^+$ | | | Na | | | H$^+$ | | |
DM[b]	concn	ΔRI[c]	ΔP$_s$ – ΔP$_o$[d]	conc[e]	ΔRI	ΔP$_s$ – ΔP$_o$	concn	ΔRI	ΔP$_s$ – ΔP$_o$	concn	ΔRI	ΔP$_s$ – ΔP$_o$
0	2.14	570 ± 136[c]	2287 ± 671	2.10	540 ± 84	3189 ± 189	15.9	39 ± 1	51 ± 3			
35	0.42	1223	2276	0.46	1248 ± 4	2916 ± 126	11.4	61 ± 1	99 ± 1	5.97	54 ± 5	67 ± 2
37							11.0	60 ± 1	92 ± 3	5.28	58 ± 20	91 ± 38
57	0.38	1684	2775	0.39			2.53	199 ± 3	321 ± 16	3.57	148 ± 6	270 ± 19
58–60							2.52	206 ± 64	295 ± 19	2.49	115 ± 9	231 ± 23
64.5				0.36	1429 ± 5	2198 ± 44				2.69	207 ± 10	310 ± 16
70	0.34	1491	2661	0.35	1392	2802						
72–73	0.37	1628	2682	0.39	1618	3147	2.64	184 ± 4	339 ± 28	2.52	178 ± 10	329 ± 11
73.9				0.34	1770	2485				2.49	280 ± 3	345 ± 18

[a] Angstroms. [b] Degree of methylesterification. [c] Peak maximum of refractive index chromatogram. [d] Peak maximum of excess pressure drop chromatogram. [e] Milligrams per milliliter. [f] Standard deviation of triplicate determination except for DM 35 (H) and DM 72–73 (Na), which are duplicate determinations. Single determination where standard deviation not given.

Table V. Number-Average Degree of Polymerization for Pectins

| | | HPSEC (35 °C) | | HPSEC (23 °C) | | OSM (35 °C)[b] | |
DM[a]	EGT[b]	Na$^+$	H$^+$	Na$^+$	H$^+$	Na$^+$	H$^+$
0	30	27		50	38	51	
35	51	55	38	96	77	126	
37	33	51	39	62	64	94	
57	66	95	83	121	125	186	155
58–60	66	99	72	113	98	196	146
64.5			84				
72–73	60	97	94	118	118	206	171
73.9			115				

[a] Degree of methylesterification. [b] End group titration data from Fishman et al. (1986a). Membrane osmometry data from Fishman et al. (1986a).
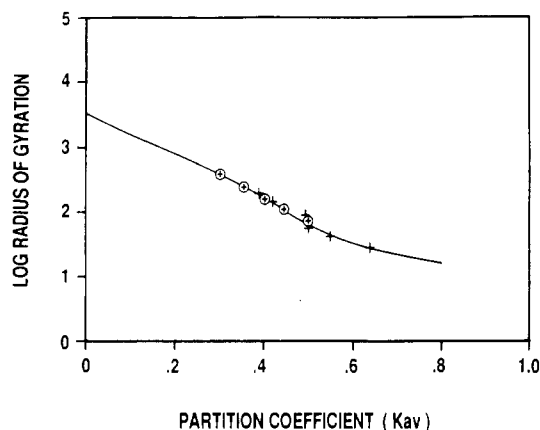

Figure 10. Calibration curve: ⊕ = pullulan; + = dextran. Standards in order of elution volume (P = pullulan; T = dextran): P800, 3.36 mg/mL; P400, 5.33 mg/mL; T500, 17.45 mg/mL; P200, 9.18 mg/mL; T250, 16.21 mg/mL; P100, 13.38 mg/mL; T110, 16.58 mg/mL; P50, 20.86 mg/mL; T70, 19.45 mg/mL; T40, 23.00 mg/mL; T20, 22.51 mg/mL. Injection volume 100 μL.

Figure 11. Dependence of radius of gyration on concentration for sodium pectinate in water. Diamonds are peak positions; squares are number averages; crosses are weight-average values.

(Fishman et al., 1986b). The decreases in R_g were greater for pectic substances with DM 0, 35, and 37 than for those with higher degrees of esterification. Furthermore, the Na$^+$ forms were moderately to slightly larger in R_g than the comparable protonated forms. Previously, HPSEC measurements gave differences in the two forms that were too small to be considered significant. These size differences between the two sets of measurements may have arisen from differences in temperature. Previously, samples were equilibrated and chromatographed at 23 °C whereas in these experiments samples were equilibrated and chromatographed at 35 °C. Earlier results have shown that brief emersion in boiling water tends to dissociate pectins (Fishman et al., 1986a). Furthermore, the intrinsic viscosities of citrus pectins decrease with temperature (Davis et al., 1980).
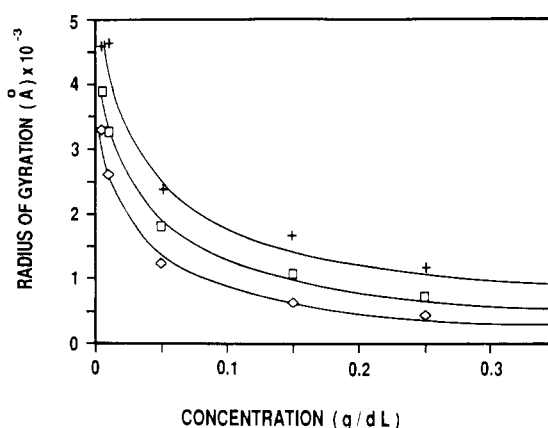
Differences in R_g between Na$^+$ and H$^+$ forms in 0.05 M NaCl are consistent with differences in intrinsic viscosities as examplified by the data in Table IV, which are larger for the sodium form than the protonated form. Comparison of R_g for the two forms at peak maximum (see Table IV), obtained from excess pressure traces such as those in Figure 8a, also revealed generally that R_g is smaller for protonated forms than for sodium pectinates. Nevertheless, differences in the radius of gyration tend to be smaller than differences in intrinsic viscosity as a consequence of eq 8, which shows that intrinsic viscosity is proportional to the radius of gyration raised to a power. This power approaches 2 for rodlike molecules, since in that case x approaches 1 (Yau et al., 1979).

In Figure 11, R_g is plotted against concentration for DM 72–73 sodium pectinate in water. The hyperbolic behavior was similar to that observed for the same material when reduced viscosity was plotted against concentration (cf. Figures 6 and 11). Such behavior is consistent with a rapid
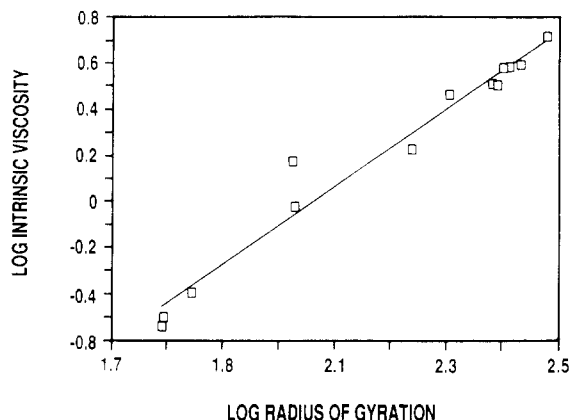
Figure 12. Correlation between log (intrinsic viscosity) and log (weight-average radius of gyration) in 0.05 M NaCl. Pectins in order of elution: DM 73.9, H; DM 64.5, H; DM 57, Na; DM 58–60, Na; DM 72–73, Na; DM 72–73, H; DM 57, H; DM 58–60, H; DM 35, Na; DM 37, Na; DM 37, H; DM 35, H; NaPG (DM 0).

increase in size as pectin concentration approaches zero in water.

In addition to R_g at peak maximum from the excess pressure trace, Table V contains R_g at peak maximum from the differential refractometer chromatograms. Comparison of peak maxima in water for the two detectors revealed that the specific viscosity maxima occurred at much higher values of R_g than concentration maxima (see Figure 9a,b). In 0.05 M NaCl specific viscosity maxima also occurred at higher values of R_g than concentration maxima (see Table IV and Figure 8a,b).

It would appear that the pectins studied here are broad molecular weight distributions. Although smaller species are present in greater concentrations, the large species tend to influence the overall solution viscosity to a greater extent. The relative influence on solution viscosity of the different sized species is more apparent in water than in 0.05 M NaCl. Apparently, in water, the distributions are multimodal, whereas in 0.05 M NaCl the distributions appear unimodal.

Correlations between Intrinsic Viscosity and Radius of Gyration. Figure 12 contains log $[\eta]$ plotted against log (weight average R_g) for pectins in 0.05 M NaCl, with degrees of methylesterification between 0 and 73.9. Linear regression analysis of the data in Figure 12 gave a correlation coefficient of 0.96, a slope of 1.69 ± 0.05, and an intercept of −3.48 ± 0.05. Equating 3 − (1/x) to the slope (see eq 8) gave a value of 0.76 for x. It is predicted that a rod-shaped molecule would give the largest value for x, namely 1, and a value of 2 for the slope of the data in Figure 12. The discrepancy between the experimental value of 1.69 and the theoretical value of 2 appears too large to be explained merely by experimental error. Shapes less extended than a rod, such as a segmented rod, an elipsoid of revolution, or an expanded coil, could give values of x less than 1. Nevertheless, in view of pectins' tendency to aggregate, a more likely explanation is that pectins of different DM could be composed of rods whose total length was not strictly proportional to their degree of polymerization. Aggregated rods, whose aggregation number, in addition to the percentage of overlap between individual rods, depended on the degree of methylesterification, nature of counterions, and nature and placement of side chains could give a value to x less than 1.

Calculation of Molecular Weights Based on the Rodlike Model. Previously (Fishman et al., 1986b), by assuming a rodlike model for pectin and correcting for

neutral sugar side chains, we were able to calculate the number-average degree of polymerization, DP_n, from the number-average radii of gyration, R_{g_n}, which were obtained from HPSEC. The results of similar calculations from R_{g_n} values obtained in this study are in Table V. For purposes of comparison, we have included also values of DP_n from end group titration (EGT), osmometry at 35 °C, and R_{g_n} values at 23 °C. The values from this study, also measured at 35 °C, fall between the osmotic values and those from EGT.

Furthermore, DP_n values obtained from R_g at 35 °C are lower than those obtained at 23 °C. All of these findings are consistent with the hypothesis that pectins undergo aggregation.

CONCLUSIONS

These studies verify that citrus pectins are broad molecular weight distributions of pectic substances that contain a mixture of thermodynamically stable and metastable aggregates. Furthermore, measurement of a single molecular weight related average (e.g., DP_n) on the same sample is subject to variation depending on the concentration range of measurement and possibly on the degree to which the measurement dissociates stable and metastable aggregates. Correlations between R_g and $[\eta]$ obtained by combining HPSEC with online viscosity detection indicated that the results were consistent with a model of aggregated rods. Measurements in water without added salt indicated that a sequential process is responsible for changes in pectin size with concentration. Finally, online viscometry combined with HPSEC is a powerful and useful tool for the elucidation of structural information on a complex ionic polysaccharide such as pectin, in that two independent measures of size are possible from the same experiment.

LIST OF ABBREVIATIONS AND SYMBOLS

DM, degree of methylesterification; DP_n, number-average degree of polymerization; OSM, membrane osmometry; ΔP_o, pressure drop of solvent across capillary; ΔP_s, pressure drop of macromolecular solution across capillary; $\Delta P_s - \Delta P_o$, excess pressure drop across capillary of macromolecular solution over that of solvent; ΔRI, differential refractive index; HPSEC, high-performance size exclusion chromatography; η, kinematic viscosity; $[\eta]$, intrinsic viscosity; η_o, kinematic viscosity of solvent, η_{red}, reduced viscosity of macromolecular solution; η_s, kinematic viscosity of macromolecular solution; η_{sp}, specific viscosity of macromolecular solution; R_g, radius of gyration; R_{g_n}, number-average radius of gyration; x, exponential factor relating molecular weight to radius of gyration.

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Catabolism of L-Canavanine and L-Canaline in the Jack Bean, *Canavalia ensiformis* (L.) DC. (Leguminosae)

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The metabolism of L-canavanine and its primary metabolite L-canaline was investigated in the jack bean, *Canavalia ensiformis* (L.) DC. (Leguminosae). L-[1,2,3,4-^{14}C]Canavanine and L-[U-^{14}C]canaline were synthesized from L-[U-^{14}C]homoserine. After 1.5 h, 35% of the administered radiolabeled canaline was converted to acetone-soluble products; this amount decreased with time to 5.3% at 12 h. This fraction was primarily responsible for respiratory loss of ^{14}C as ^{14}CO$_2$, which reached 52% of the administered canaline after 12 h. The water-soluble, neutral fraction accounted for no more than 10% of the injected canaline. The water-soluble, charged materials contained 35–40% of the ^{14}C at each time period; five compounds were identified within this fraction. They are homoserine, phosphohomoserine, lysine, canavanine, and canaline glyoxylate oxime. Canavanine-administered plants provided a degradation pattern remarkably similar to that of canaline-administered plants, indicating the importance of arginase-mediated hydrolysis of canavanine to canaline in the process of canavanine catabolism.

L-Canavanine, the 2-amino-4-(guanidinooxy)butyric acid antimetabolite of L-arginine, has been isolated from 1200 legumes (Bell et al., 1978). It is the principal nonprotein amino acid of the jack bean, *Canavalia ensiformis* (L.) DC. (Bell, 1958). This potentially toxic secondary plant metabolite can play an important role in legume chemical defense against herbivores, particularly insects (Rosenthal, 1986, 1988). It also functions as a nitrogen-storing metabolite, able to support de novo amino acid synthesis (Rosenthal and Rhodes, 1984; Rosenthal et al., 1988).

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Previous studies of canavanine catabolism in the jack bean reveal that this arginine analogue is cleaved hydrolytically by arginase (EC 3.5.1.5) to L-canaline and urea. The latter compound is degraded by urease (EC 3.5.3.1) to CO$_2$ and NH$_3$. Working in consort, these enzymes are responsible for mobilizing two of the three nitrogens of canavanine's guanidinooxy group (Rosenthal, 1971). But what is the metabolic fate of L-canaline? In 1970, Töpfer et al. reported that [^{3}H]canaline gave rise to tritiated homoserine in germinating seeds of *Caragana spinosa*, but they did not provide data on how significant this reaction was in overall canaline degradation. Thus, an objective of this study is to employ L-[U-^{14}C]canaline and L-[1,2,3,4-^{14}C]canavanine to determine to what extent homoserine functions in the catabolism of these nonprotein amino acids.