Studies of Pectin Solution Properties by High-Performance Size Exclusion Chromatrography

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Pectin, a complex polysaccharide and polyacid, exhibits complicated behavior in solution as demonstrated by high-performance size exclusion chromatography (HPSEC) with E-linear μBondagel columns. Pectins were detected as they emerged from the column by ultraviolet absorption at 206 nm and differential refractive index. By utilization of a variation of the universal calibration principle, the radius of gyration (R_g) was determined by first calibrating the column with a series of dextrans of known R_g. Radii of gyration of pectins depended on such variables as mobile phase, method of purification, chemical modification, and methoxy content. Mechanisms are suggested by which the size of pectins in solution are affected by these variables.

The polysaccharide pectin, is of considerable interest because it is a food fiber (Story and Kritchevsky, 1980; Campbell and Palmer, 1980), a widely used thickening agent for processed foods (Towle and Christensen, 1973), and a major cell wall component in plants (Olson et al., 1969). Depending on the plant source, method of extraction, and chemical treatment after extraction, 78% or more of pectin is composed of α-1,4-linked D-galacturonic acid units with varying degrees of the carboxyl groups methylated. The remainder of pectin is composed of the neutral sugars L-arabinose, D-galactose, and L-rhamnose. Small quantities of D-glucose, D-mannose, and D-xylose have been reported as well (Baig et al., 1980). The chemical diversity of pectin permits it to display solution behavior that is consistent with a delicate compositional balance of hydrophobic/hydrophilic residues. Thus it is a polyacid at low pH (e.g., 2) (Speiser et al., 1945), a polyelectrolyte at high pH (e.g., 7.3) (Pals and Hermans, 1952a), and soluble in mixtures of water and some polar organic solvents (e.g., dimethyl sulfoxide/water mixtures) (Jordan and Brant, 1978).

The molecular size of pectin has been studied by boundry electrophoresis, ultracentrifugation (Barrett and Northcote, 1965), electron microscopy (Hanke and Northcote, 1975), end group analysis (Albersheim et al., 1960), viscometry (Pals and Hermans, 1952a), osmometry (Pals and Hermans, 1952b), light scattering (Sorochan et al., 1971), gel filtration (Davis et al., 1980), and high-performance size exclusion chromatography (HPSEC) (Strubert and Hovermann, 1978; Barth, 1980). No one study has examined the effect of pH, degree of methylation, mobile phase, salt concentration, and sample concentrations on the radius of gyration of pectin (R_g). Here we exploit the capability of HPSEC to measure rapidly the R_g of a series of pectins with a wide range of molecular sizes. Furthermore, dual detection of emerging column effluent with UV absorption at 206 nm and differential refractive index (ΔRI) has given some insight into the relative contributions of galacturonate and neutral sugar residues to the solution properties of pectin. As a result of the study, we were able to resolve some apparent inconsistencies regarding the solution behavior of pectin.

EXPERIMENTAL SECTION

Materials. Pectin samples (Table I) were obtained from Sunkist Grower, Corona, CA, LD-52 (percentage of methyl esterification 57%), and from Bulmers Ltd., Hereford, England, LD-43 and LD-42 (percentage of methyl esterification 72% and 37%). One pectin sample (LT-1) was extracted from fresh grapefruit albedo, according to standard procedures (McCready, 1965). Polygalacturonic acid (>98% pure) was from Sigma Chemical Co., St. Louis, MO. Percentage of esterification was determined by the colorimetric method of Wood and Siddiqui (1971) and by ^{13}C NMR. Dextran standards were from Pharmacia Chemical Co., Piscataway, NJ. The $\bar{M}_w$'s of the dextran standards were as follows: T-2,000, 2×10^6; T-500, 5.32×10^5; T-250, 2.53×10^5; T-110, 1.06×10^5; T-70, 7×10^4, T-40, 4.44×10^4; T-20, 2.23×10^4, T-10, 9.3×10^3. Phosphate buffers and phosphoric acid were from J. T. Baker, Phillipsburg, NJ, reagent grade. Water for mobile phases and solutions was house deionized water, which was further polished by running through a water purifying system containing a mixed-bed ion exchanger, an activated charcoal bed, and a 0.45-μm filter, Continental Water System. Mobile phases were 0.08 M sodium phosphate buffer, pH 3.7 and 7.3, water, and 0.16 M sodium phosphate buffer, pH 7.3.

Apparatus for HPSEC. Solvent was delivered by a Beckman-Altex, Palo Alto, CA, Model 334 chromatograph. The emerging peaks were detected in series: first, by an LKB, Silver Springs, MD, Model 2138 Uvicord S ultraviolet monitor equipped with a 206-nm filter and an HPLC cell and, second, by a Water Associates, Inc., Milford, MA, Model 401 differential refractometer. Data were collected simultaneously on a Houston Instruments, Austin, TX, Model Omniscribe B-5000 dual-channel potentiometric strip chart recorder and a Modcomp Classic computer, Model 7861, Modular Computer Systems, Inc., Ft. Lauderdale, FL. Peak maxima, moments, retention times, retention volumes, and partition coefficients were calculated by computer. High-performance size exclusion chromatography was performed on a Waters Associates, E-linear μBondagel column (30 × 0.39 cm). Results were obtained with three different columns. Random replication on various samples revealed no significant difference among columns with respect to any elution parameters mentioned above.

HPSEC Sample Preparation. Typically, 3–5 mg of lyophilized pectin was weighed out into a glass vial and the volume of solvent pipetted to obtain the desired sample concentration. At concentrations of 1 mg/mL or less, samples dissolved readily. Prior to sample injection, all samples were filtered through a Nucleopore Corp., Pleasanton, CA, 0.4-μm polycarbonate membrane filter.

Chromatographic Conditions. Unless otherwise stated, injection was 20 μL of a 0.9 mg/mL sample, the flow rate was 0.5 mL/min, the chart speed was 2.5 cm/min, the refractive index attenuation was 4 times, and the UV was 0.02 AUFS. As reported in the literature (Barth, 1980),

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Table I. Unmodified Pectins Used in This Study

sample	source	supplier	% E^a	$\bar{M}_n \times 10^{-3}$
PG	citrus	Sigma Chemical Co.	0 [b,c]	6.1 ± 0.4 [d]
LD-42	citrus	Bulmers, Ltd.	37 [b,c]	5.4 ± 0.6
LD-52	lemon	Sunkist Growers	50 [c]-57 [b]	11.6 ± 0.3
LD-43	citrus	Bulmers, Ltd.	72 [b]-73 [c]	10.6 ± 0.7
LT-1	grapefruit	extracted in this laboratory [e]	72 [c]	15.2 ± 0.7

[a] Percentage esterification of D-galacturonate residues. [b] Colorimetric determination. [c] NMR determination. [d] Mean ± SE of end group analyses; four determinations, LD-43, LT-1, LD-42; five determinations, PG; six determinations, LD-52. [e] Procedure of McCready (1965).

pectin concentrations in excess of 1 mg/mL produced peak distortion and a large dependence of peak maximum on sample concentration. At concentrations of less than 1 mg/mL, peak distortion was essentially absent, but there was about an 8% increase in peak retention time over the concentration range 0–1 mg/mL. To prevent carry-over of the sample, the injection valve (Beckman Model 210) was back-flushed with 2 mL of water between runs and 200 μL of sample solution was pushed into the sample loop.

Purification of Pectins. Sunkist lemon pectin and Bulmers citrus pectin were freed of insoluble filter aid residue by dissolving in water and centrifuging at 2200*g* for 30 min. The clear supernatant was filtered through a 0.45-μm Millipore filter and lyophilized to a white product which was designated undialyzed. A portion of the lyophilized material was dialyzed against distilled water for 48 h. This pectin was retained in a dialysis bag with a 12 000 molecular weight cutoff and was designated retentate. In the case of the pectin with 37% degree of esterification, the dialysate was lyophilized for further analysis. The purification of the pectin has been detailed further elsewhere (Pfeffer et al., 1981). Polygalacturonic acid was used without further purification. The content of Mn, Zn, Cu, Fe, Mg, Ca, Na, and K ions was checked by atomic absorption for the 37% OCH_3 and 57% OCH_3 pectins and in no case did the metal concentration exceed 2.5 parts/1000.

Methylation of Pectin and Polygalacturonic Acid. Pectin (72% esterified) was methylated with diazomethane for 12 h as described previously (Pfeffer et al., 1981). Polygalacturonic acid was methylated with diazomethane for $^1/_3$, 2, and 12 h by the same method.

Analysis of Pectin and Polygalacturonic Acid Composition by NMR Spectroscopy. The ratio of uronide to neutral sugar residues in pectin samples, the ratio of methyl esters to free carboxyl groups in pectin samples, and the degree of total methylation (ester plus ether formation) of diazomethane treated polygalacturonic acid samples was determined by ^{13}C NMR spectroscopy on a JEOL FX-60-Q-S spectrometer at 15 MHz.

To determine the ratio of uronide to neutral sugar residues and the ratio of methyl ester to carboxyl groups in pectin samples in solution, the natural abundance ^{13}C NMR spectra of the corresponding gels (250 mg of pectin/2 mL of ^2H_2O) were examined at 65 °C. A typical spectrum required 15 000 transients, a spectral width of 4000 Hz, with 8K data points, a repetition rate of 1.2 s [T_1(carbonyl) = 0.5 s; T_1(anomeric carbon) = 0.1 s], and a pulse angle of 35°. The ratio of the uronide to neutral sugars was established by comparing the areas of the common anomeric C-1 carbon resonances at about 100 ppm to the combined area of the carbomethoxy and carboxyl resonances at 171.3 and 172.8 ppm, respectively. This ratio was established after taking into account the differences in the nuclear Overhauser enhancements observed in pure polygalacturonic acid for the carbonyl (1) and anomeric carbon resonance responses (1.3). The ratio of the carbomethoxy to carboxyl groups was determined from the ratio of the resonances at 171.3 and 172.8 ppm and confirmed by comparison of the 171.3 ppm resonance area with the area of the OCH_3 resonance at 53.7 ppm.

Because of the low solubility of polygalacturonic acid and partially methylated polygalacturonic acid samples, the percentage of methyl ester present was determined on solid samples by ^{13}C cross polarization magic angle spinning (CPMAS) NMR spectroscopy. Standard samples of pure polygalacturonic acid and methylated polygalacturonic acid (350–500 mg) were used to optimize conditions to obtain quantitative magnetization responses of the ^{13}C resonances as a function of pulse delay and C–H contact times. The values for these respective parameters were 1.5 s and 1.5 ms based on the expected ^{13}C magnetization responses of the standard samples. Note that these conditions do not give the maximum resonance responses but those corresponding to the appropriate ratio of the carbons examined. The spectra were obtained with 1000 transients at a spectral width of 8000 Hz, a 90° pulse of 5.2 μs, with 2K data point zero filled to 8K, and 10-G proton decoupling. The total degree of methylation was established by intergrating the OCH_3 resonance area at 53.1 ppm and comparing it with the area of the C=O resonance centered at 171.5 (this area includes the unresolved COOMe and COOH resonances). The difference in area between the OCH_3 region and C=O region gave a measure of the extent of ether formation after correcting for the degree of esterification, which was determined by the colorimetric method of Wood and Siddiqui (1971).

Neutral Sugar Composition of Pectin Retentate and Dialysate. The neutral sugars in each pectin fraction were determined after hydrolysis by gas–liquid chromatography. Thirty milligrams of each pectin fraction was weighed into a glass tube, and 12.0 mL of 2 N trifluoroacetic acid was added. The tubes were heated in an oven for 3 h at 120 °C. The neutral sugars liberated by hydrolysis were then converted to alditol silylates according to the procedure of Bradbury et al. (1981). The levels of the derivatized sugars were determined on a 25-m, OV-101 fused silica capillary column, Applied Sciences, State College, PA, the procedure to be described (Doner and Douglas, 1983). The gas chromatograph was a Hewlett-Packard Model 5880A.

RESULTS AND DISCUSSION

Calibration of Columns. Separation of solute molecules by size exclusion chromatography (SEC) depends on the size distribution of internal pores that are solvent accessible and the hydrodynamic volume of the dissolved solute (Yau et al., 1979a). The partition coefficient, "K_{av}" (Laurent and Killander, 1964), defined by eq 1 is a useful parameter in measuring the percentage of internal pore volume that is accessible to a solute molecule of a given hydrodynamic volume.

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

Here, V_e is the elution volume of the solute under study, V_0 is the void volume or the elution volume of solute that

Table II. Linear Regression Relationships among Size Parameters for Dextran

equation	abscissa[a] range	slope	intercept	correl coeff
$K_{av} = A \log \bar{M}_w + B$	$\leq 7.89 \times 10^4$	−0.30	1.902	−1.000
	$\geq 7.89 \times 10^4$	−0.092	0.8957	−0.999
$\log [\eta] = a \log \bar{M}_w + \log K$	$\leq 8.84 \times 10^4$	0.50	−3.008	0.999
	$\geq 8.84 \times 10^4$	0.31	−1.068	0.994
$\log R_g = a \log \bar{M}_w + b$	$\leq 1.26 \times 10^5$	0.50	−0.538	b
	$\geq 1.26 \times 10^5$	0.45	−0.181	c
$\log R_g = CK_{av} + D$	≤ 0.45	−4.498	3.935	−0.999
	≥ 0.45	−1.681	2.657	−0.999

[a] Range for constants. [b] Constants calculated from viscosity data and eq 7. [c] Constants obtained from Senti et al. (1955).

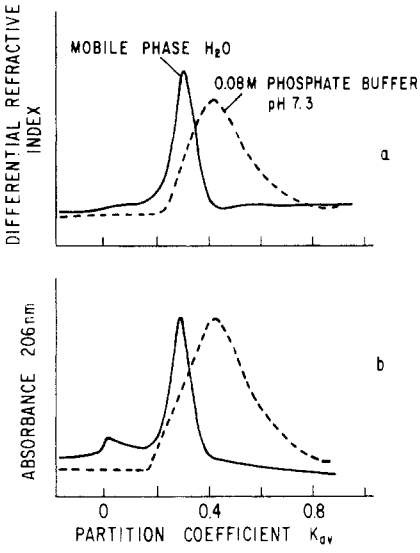

Figure 1. Chromatograms of pectin. Mobile phases: (---) 0.08 M sodium phosphate buffer, pH 7.3; (—) H_2O. Column: E-linear μBondagel (0.39 cm i.d. × 30 cm length). Flow rate: 0.5 mL/min. Injector volume: 20 μL. Detector: RI 4X, UV wavelength 206 nm, 0.02 AUFS. Sample concentration 0.9 mg/mL.

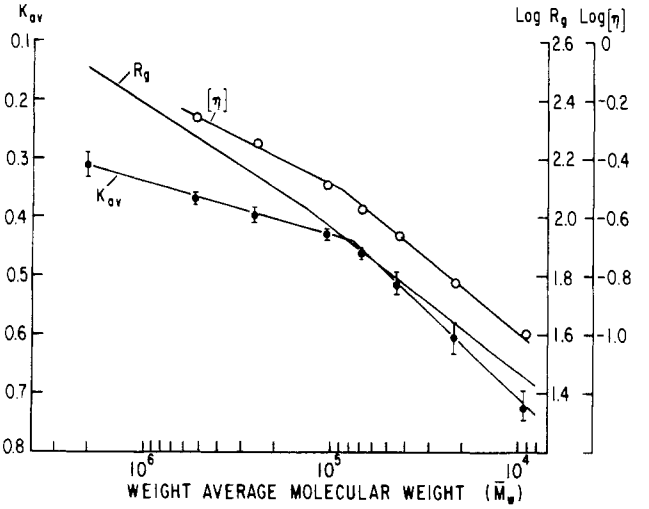

Figure 2. Dependence of dextran hydrodynamic parameters on the weight-average molecular weight, $\bar{M}_w$. K_{av} is the partition coefficient at peak maximum with ΔRI detection; [η] is the intrinsic viscosity (dL/g); R_g is the radius of gyration.

is totally excluded from the internal pore volume of the stationary phase, and V_t is the total volume or elution volume of solute that freely penetrates the solvent-accessible pore volume. All volumes were measured at the peak maxima.

Determination of V_0 by blue dextran was not possible because it fractionates on E-linear μBondagel (Dreher et al., 1979). Several pectins, when eluted with pure water, gave an early peak that disappeared when the mobile phase was phosphate buffer (see Figure 1). Thus, the void volume was determined from the maximum of the first peak detected at 206 nm in pure water (see Figure 1b, solid line, $K_{av} = 0$). At a flow rate of 0.5 mL/min, the maximum occurred about 30 s earlier than the peak given by dextran with a weight average molecular weight ($\bar{M}_w$) of 2×10^6. Typically, V_0 was determined to be 1.18 mL whereas a value of 2.87 mL was found for V_t as determined for glucose. Void volumes for pectins with methyl ester levels of 37%, 57%, and 72% each gave the same value within experimental error. Thus, interactions between the support and pectin that elutes at the void volume (e.g., adsorption or ion exclusion) were not deemed important. Possible causes for pectin eluting at the void volume in water were aggregation of pectin and/or polyelectrolyte expansion.

The partition coefficient K_{av} for a series of well-characterized dextran standards was plotted against log of the weight average molecular weight, $\bar{M}_w$, according to eq 2.

$$K_{av} = A \log \bar{M}_w + B \quad (2)$$

Linear regression analysis indicated that the data was best fit by two straight lines and that the slopes were significantly different ($P \leq 0.05$) (Figure 2). Within the precision of our measurements, the SEC data of Figure 2 indicate that this calibration curve is independent of pH over the range 3.7–7.3 and of ionic strength for phosphate buffer over the range 0–0.16 M. Each point in the SEC data of Figure 2 is the average of at least eight measurements. At least two measurements for each dextran standard were taken in each of the four mobile phase, i.e., water, 0.08 M phosphate buffer at pH 7.3 and 3.7, and 0.16 M phosphate buffer at pH 7.3. Analysis of variance indicated that K_{av} was not significantly different ($P \leq 0.05$) with mobile phase for the series of dextran standards. As an independent check of the K_{av}–log $\bar{M}_w$ relationship for dextran on μBondagel, a Mark-Houwink plot (Onyon et al., 1955) was constructed according to eq 3 from viscosity data supplied by the manufacturer.

$$[\eta] = KM^a \quad (3)$$

The log of the intrinsic viscosity [η] (dL/g) was plotted against log $\bar{M}_w$ (see Figure 2). As found for K_{av} against log $\bar{M}_w$, the data were best fit by two straight lines with significantly different ($P \leq 0.05$) slopes. The Mark-Houwink constants K and a for the lower set of equations (Table II) were in reasonable agreement with those found by Senti et al. (1955), i.e., 9.8×10^{-4} against 8.8×10^{-4} for K and 0.5 against 0.5 for a. Senti's group also found that the curve bent downward but gave no constants for the upper portion of the curves, although it too could have been approximated by a straight line. From eq 2, K_{av} is proportional to log $\bar{M}_w$, whereas from eq 3, log [η] is proportional to log M; thus K_{av} is proportional to log [η]. Furthermore, since K_{av} and log [η] are proportional and in-

Table III. Partition Coefficient (K_{av}) of Pectins

sample	mobile phase: treatment	% M[a]	% E[b]	0.08 M, pH 3.7	H_2O	0.08 M, pH 7.3	0.16 M, pH 7.3
LD-43	retentate	73[d]	72[c]-73[d]	0.34 ± 0.01	0.28 ± 0.01	0.35 ± 0.03	0.37 ± 0.02
LT-1	retentate	72[d]	72[d]			0.33 ± 0.03	
LD-52	retentate	50[d]	50[d]-57[c]	0.32[f]	0.27[f]	0.31 ± 0.02	0.36 ± 0.01
LD-42	retentate	37[d]	37[c,d]	0.40 ± 0.01	0.27[f]	0.40 ± 0.04	0.43 ± 0.01
PG		0[d]	0[c,d]	0.46 ± 0.03	0.27[f]	0.44 ± 0.03	0.48 ± 0.02
LD-42	dialysate	29[d]	29[d]	0.44 ± 0.01	0.28 ± 0.00	0.42 ± 0.03	0.42[f]
LD-43	undialyzed	73[d]	72[c]-73[d]	0.34 ± 0.01	0.29 ± 0.01	0.35 ± 0.01	0.37 ± 0.01
LD-52	undialyzed	50[d]	50[d]-57[c]	0.41 ± 0.01	0.28[f]	0.40[f]	0.39 ± 0.01
LD-42	undialyzed	37[d]	37[d]	0.35 ± 0.02	0.29 ± 0.01	0.34 ± 0.01	0.35 ± 0.01
LD-43	CH_2N_2,[e] 12 h	150[d]	98[c]			0.48 ± 0.01	
PG	CH_2N_2, 0.33 h	45[d]	37[c]				0.48 ± 0.03
PG	CH_2N_2, 2 h	100[d]					0.45 ± 0.03
PG	CH_2N_2, 12 h	150[d]	43[c]				0.46 ± 0.03

[a] Percentage methylation (includes ester and ether). [b] Percentage esterification of D-galacturonate residues. [c] Colorimetric determination. [d] NMR determination. [e] Undialyzed. [f] Error <0.0044.

dependent measures of the radius of gyration (R_g) of a dissolved polymer molecule and behave similarly when plotted against log $\bar{M}_w$, it is concluded that some of the nonlinearity of dextran on E-linear μBondagel is inherent in the molecular size–weight relationship of dextran, even though the internal pore distribution of the packing itself may produce nonlinear curves over the molecular weight range of calibration.

The next step in the calibration procedure was to establish a relationship between R_g and K_{av} for μBondagel, because R_g is a basic molecular parameter of dissolved macromolecular solutes and an approximate universal calibration parameter for SEC as well (Yau et al., 1979b). More specifically, Kato et al. (1983) have shown that a plot of R_g against elution volume gave a single line for dextran, pullulan (a linear polysaccharide), and polyethylene oxide. For $\bar{M}_w \geq 126000$, R_g was calculated from eq 4, which Senti

$$R_g = 0.66\bar{M}_w^{0.43} \quad (4)$$

et al. (1955) obtained from light scattering. For molecular weights below 126 000, for which there was no light scattering data, R_g was obtained from viscosity data. The intrinsic viscosity is related to R_g by eq 5 (Yau et al., 1979a).

$$[\eta] = \phi_0(1 - 2.63\epsilon + 2.86\epsilon^2)6^{3/2}R_g^3/M \quad (5)$$

Here ϕ is a universal constant equal to 2.86×10^{23} and

$$\epsilon = (2a - 1)/3 \quad (6)$$

The quantity, a, is identical with that in eq 3, which is 0.5 (see Table I) for dextran. When a is 0.5, $\epsilon = 0$, and eq 5 reduces to

$$[\eta] = \phi_0 6^{3/2}R_g^3/M \quad (7)$$

Solving for R_g in eq 7, substituting values of $[\eta]$ and $\bar{M}_w$ for dextran standards, yielded eq 8.

$$R_g = 0.29\bar{M}_w^{0.5} \quad (8)$$

A plot of log R_g against $\bar{M}_w$ using eq 4 for $\bar{M}_w \geq 126000$ and eq 8 for $\bar{M}_w \leq 126000$ is shown in Figure 2.

As with $\bar{M}_w$, the K_{av}–R_g relationship is semilogarithmic. As indicated by Figure 3, the data are best fitted by two straight lines with the form of eq 9. Constants for eq 9 are in Table II.

$$\log R_g = CK_{av} + D \quad (9)$$

The accuracy of an R_g for pectin obtained from eq 9 depends upon the accuracy of two approximations. First, dextrans and pectins of identical R_g will coelute, in spite

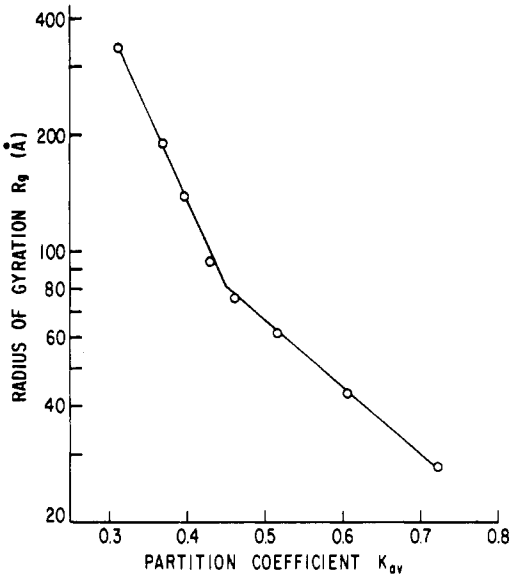

Figure 3. Universal calibration of E-linear μBondagel standards: dextran T2,000, T500, T250, T110, T70, T40, T20, and T10. Mobile phase: H_2O, 0.08 and 0.16 M sodium phosphate buffer, pH 7.3, and 0.08 M sodium phosphate buffer, pH 3.7. See Figure 1 for other chromatographic conditions.

of differences in molecular architecture. Second, for polydisperse samples, the peak maximum of the elution curve can serve to mark the R_g, in place of the point on the curve that corresponds to the second moment on the molecular distribution curve. Both of these approximations could introduce significant systematic errors in our values of R_g for pectin. Thus, R_g values should be regarded as semiquantitative rather than quantitative. Nevertheless, relative size relationships among pectins, the major thrust of what follows, should not be significantly affected by the approximations required to obtain R_g.

SEC Behavior of Pectins. The partition coefficients of a variety of pectins were measured in four mobile phases (see Table III). Partition coefficients were calculated from eq 1, with V_e, the elution volume of the major pectin peak at maximum concentration as detected by ΔRI. Partition coefficients obtained in this way were then used to calculate R_g with the aid of eq 9 (see Table IV). Pectins are in three categories, retentate pectins, undialyzed pectins, and pectins chemically modified after extraction.

The results of these experiments can best be interpreted by accepting the structural models in solution proposed by Rees and co-workers for polygalacturonic acid and pectin (Rees and Wight, 1977; Rees, 1977), namely, that

Table IV. Radius of Gyration (R_g, Å) for Pectins

sample	treatment	mobile phase % M^a	mobile phase % E^b	0.08 M, pH 3.7	H_2O	0.08 M, pH 7.3	0.16 M, pH 7.3
LD-43	retentate	73[d]	72[c]-73[d]	254 ± 11	474 ± 21	229 ± 31	187 ± 17
LT-1	retentate	72[d]	72[d]			274 ± 42	
LD-52	retentate	50[d]-57[c]	50[d]-57[c]	313[f]	525[f]	347 ± 31	207 ± 28
LD-42	retentate	37[c,d]	37[c,d]	137 ± 6.2	525[f]	137 ± 25	100 ± 5
PG		0[d]	0[c,d]	76.5 ± 3.9	525[f]	90.3 ± 12.2	59.7 ± 5.4
LD-42	dialysate	29[d]	29[d]	90.3 ± 4.1	474[f]	111 ± 15	111[f]
LD-43	undialyzed	73[d]	72[c]-73[d]	254 ± 11	427 ± 19	229 ± 10	187 ± 8
LD-52	undialyzed	50[d]	50[d]-57[c]	123 ± 6	474 ± 0	137[f]	152 ± 7
LD-42	undialyzed	37[d]	37[d]	229 ± 21	427 ± 19	254 ± 11	229 ± 10
LD-43	CH_2N_2,[e] 12 h	150[d]	98[c]			59.6 ± 2.7	
PG	CH_2N_2, 0.33 h	45[d]	37[c]				70.8 ± 3.6
PG	CH_2N_2, 2 h	100[d]					79.5 ± 4.0
PG	CH_2N_2, 12 h	150[d]	43[c]				76.5 ± 3.9

[a] Percentage methylation (includes ester and ether). [b] Percentage esterification of D-galacturonate residues. [c] Colorimetric determination. [d] NMR determination. [e] Undialyzed. [f] Error <0.44 Å.

polygalacturonic acid (PG) is a flat helical ribbon and pectins are blocks of these polyglacturonic segments separated by rhamnose inserts acting as tees for branches of neutral sugars. Thus polygalacturonic acid could be considered to be a rod in solution whereas pectins are segmented rods with flexibility at the rhamnose tees. The rodlike nature for PG would be consistent with R_g values in the 60–90-Å range (Table IV). The decrease in R_g for PG with doubling of ionic strength of the buffer at pH 7.3 could be due to a reduction in the helical pitch induced by decreased intramolecular charge–charge repulsions.

Several reports indicate that pectins undergo aggregation in solution (Jordan and Brant, 1978; Sorochan et al., 1971; Davis et al., 1980). A combination of elongated helices at low ionic strength and side by side aggregation with partial overlap of chains could explain the extremely large R_g's found for both PG and pectin in distilled water. For PG in H_2O, hydrogen bonding between carboxyl carbonyls and OH's of galacturonic acid must be responsible for aggregation. In the case of the pectins in H_2O, hydrophobic interactions and the neutral sugar branches also may play a role in the aggregation. In the various buffer solutions, charge–charge repulsions would likely prevent PG from aggregating, whereas the pectins with reduced charge–charge repulsion due to the presence of ester groups as well as interactions involving neutral sugar branches could still undergo aggregation. Thus, although PG and the 37% carbomethoxy ester of pectin (LD-42) have closely similar number-average molecular weights, $\bar{M}_n$, 6.08×10^3 against 5.4×10^3, as determined by end group titration (Albersheim et al., 1960) (Table I), the R_g for LD-42 is 1.5–1.7 times larger than the R_g of PG in all three buffer solutions (Table IV).

As shown by Figure 4a,b,d, a plot of R_g against percentage esterification passes through a maximum in the 57% E range for the pectin retentates at constant ionic strength and pH. As was the case for the PG–LD-43 comparison, it is suggested that the maximum in the R_g–% E function probably arises from changes in the size of the dissolved macromolecules induced by a combination of conformational and aggregational effects rather than from coincidental differences in the degree of polymerization among preparations. If changes in R_g are related to changes in degree of polymerization, an equation in the form of eq 4 or 8 should apply over a limited molecular weight range. Furthermore, the ratio R_g/M^a should be a constant. Since the self-association of pectin is possible, if not likely, only $\bar{M}_n$ from end group analysis would be readily obtained molecular weight average for dissociated pectin. End group analysis for polygalacturonic acid (PG)

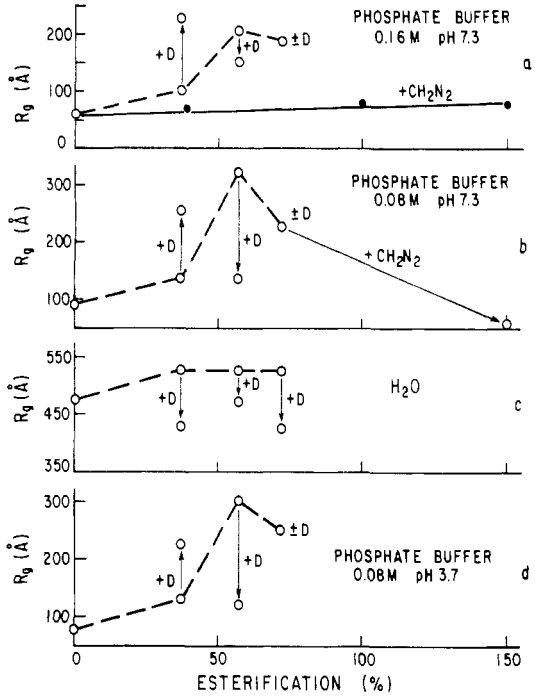

Figure 4. Effect of various parameters on pectin radius of gyration. Radius of gyration calculated from K_{av} at peak maximum. Detection: ΔRI. (---) Connects points represented by retentate. Points represented by +D (with arrow) are for undialyzed pectin (retentate + dialysate). (a) (●) Chemically modified with diazomethane; (b) (→) CH_2N_2 chemically modified with diazomethane.

and four pectin retentates (LD-42, LD-52, LD-43, and LT-1) gave the $\bar{M}_n$ values in Table I. If one assumes the segmented rod model for pectin, the shape factor, a, in the ratio R_g/M^a should be between 0.5 and 1, which are the a values for random coil and rod, respectively (Yau et al., 1979a). Values of R_g/M^a for rodlike and random coil behavior are plotted against % E in Figure 5. For both shapes, a maximum is still obtained. It is noteworthy that LT-1, the 72% carbomethoxy pectin, has a $\bar{M}_n$ value about 1.4 times greater than that of LD-52, the 57% carbomethoxy pectin, and yet the R_g (Table IV) and the R_g/M^a values (Figure 5) are appreciably smaller. Possibly a change in the degree of esterification changes the overall length of the pectin aggregates by changing the amount of overlap between chains and the pitch of the α-D-galacturan helix. Provided $\bar{M}_n$ does not differ greatly among pectin samples, as is the case here (Table I), R_g should be relatively insensitive to molecular weight but not

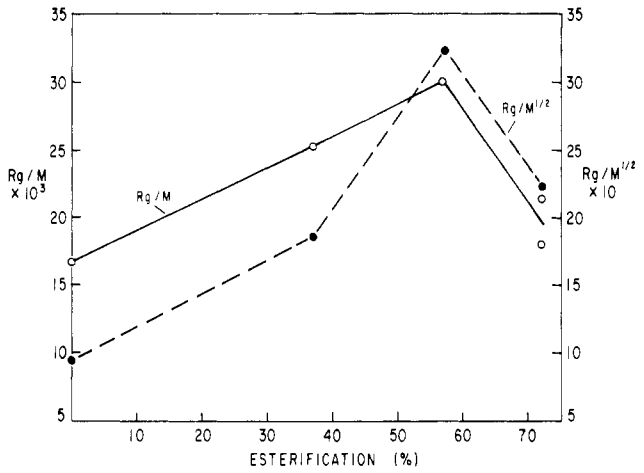

Figure 5. Effect of percentage esterification on the parameter R_g/M^a in 0.08 M phosphate buffer, pH 7.3: (—) rodlike behavior; (---) random coil behavior.

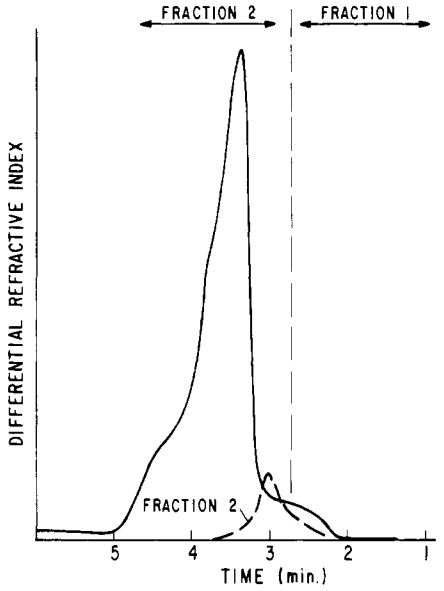

Figure 6. Fractionation of pectin with RI dection: 16× for unfractionated sample, 2× for fraction. (—) Unfractioned; (---) fraction 2. Other conditions were the same as those in Figure 2.

% E. A change in percentage esterification at constant pH and ionic strength could alter the delicate balance between charge–charge repulsions, hydrogen bonding, and hydrophobic interactions in pectins and thus induce changes in R_g.

Davis et al. (1980) have shown that pectins at pH 4.6 in 0.1 M acetate buffer aggregate at high and low ester content. Similarly, we show aggregation for 57% carbomethoxy pectin in distilled water. This was accomplished by injecting pectin onto the column at a concentration of about 6 mg/mL. As shown by Figure 6, two fractions were collected. The second peak when reinjected, showed a leading edge which indicated an increase of the first peak at the expense of the second.

Interestingly, the R_g of pectin can be influenced greatly by the presence of a small amount (2.1% or less by weight) of a dialyzable component in the preparations. Comparison of undialyzed pectin with comparable retentates revealed that R_g for 37% undialyzed pectin was higher than that for the retentates, whereas R_g for 57% undialyzed pectin was lower than that for its retentate and R_g for the 72% undialyzed pectin was practically the same as that for the retentate (Figure 4).

Table V. Percentages by Weight of Neutral Sugars in Undialyzed Pectin and Fractions[a,b]

	undialyzed		retentate		dialyzate	
sugar	% total	% sugar	% total	% sugar	% total	% sugar
galactose	9.09	41.3	8.49	38.6	29.6	46.3
rhamnose	6.31	28.7	7.00	31.8	9.45	14.8
arabinose	2.48	11.3	2.03	9.2	13.55	21.2
glucose	1.51	6.9	2.03	9.2	5.73	9.0
mannose	1.42	6.4	1.32	6.0	2.85	4.5
xylose	1.20	5.4	1.12	5.1	2.71	4.2
total	22.01	100.0	21.99	99.9	63.89	100.0

[a] Determined by gas chromatography (see Experimental Section). [b] Percentage esterification = 37%.

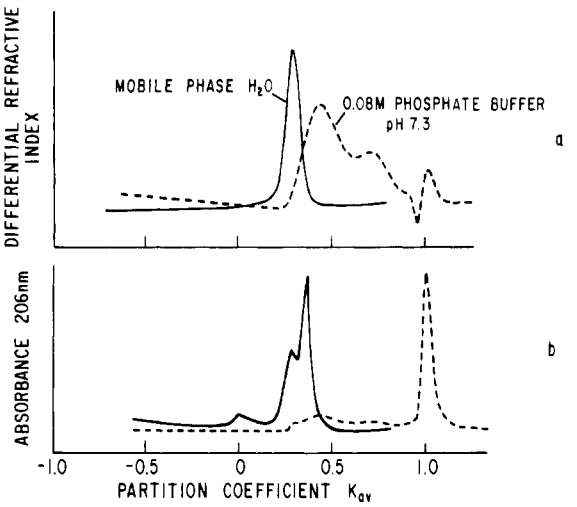

Figure 7. Chromatograms of pectin dialysate. Mobile phase: (---) 0.08 M phosphate buffer, pH 7.3; (—) H_2O. See Figure 1 for chromatographic conditions.

It has been reported that K_{av}'s at peak maximum for 37% and 72% esterified pectins were the same (Barth, 1980). These results were produced in this study only when undialyzed pectin was measured.

Analysis revealed that the dialysate from 37% esterified pectin was composed of the same sugar residues as the retentate but in different proportions (Table V). The pectin was composed of 78% unronate residue and 22% neutral sugars whereas the dialysate was 32% uronates and 64% neutral sugars. The levels of arabinose and galactose were higher in the dialysate than in the retentate, whereas the level of rhamnose was significantly lower. HPSEC of the dialysate on μBondagel with H_2O as the mobile phase (Figure 7) showed that the dialysate reaggregates. As clearly indicated by the 206-nm absorption curve (Figure 7b), a small percentage of the dialysate elutes at the void volume. The major peak by ΔRI has a radius of gyration that is comparable to that of pectin (Table IV). In buffer, as indicated by typical chromatograms in 0.08 M phosphate at pH 7.3 (Figure 7), the degree of aggregation of the dialysate decreases. The fraction rich in uronate residue disaggregated to a size below the nominal 2000 cutoff of the column as indicated by the 206-nm trace (Figure 7b), whereas the neutral sugar was close to polygalacturonic acid in size as indicated by the ΔRI trace (Figure 7a).

The above results show that a pectin-like fragment self aggregates and induces changes in the state of aggregation and/or conformation of pectin and that the effect is highly dependent on % E.

The effect of degree of esterification on the solution properties of the galacturonic acid portion of pectin was

studied by treating polygalacturonic acid (PG) with diazomethane (CH_2N_2) for varying lengths of time. Our results (Figure 4a) show that PG's methylated by treatment with CH_2N_2 have lower R_g's than pectin with equivalent biosynethetic methylation obtained from mild extraction. Apparently, methylation by CH_2N_2 facilitates a maximum overlap between chains. Thus, when pectin with 72% degree of esterification was treated for 12 h with diazomethane, the resultant polymer exhibited a 3.8-fold reduction in R_g over the starting material (Figure 4b) with a final degree of methylation that was 150%. Obviously, some pectin hydroxyl groups are etherified with methanol.

Structural evidence from ^2H NMR and ^{13}C NMR (Pfeffer et al., 1981) measurements verified that CH_2N_2-treated pectin substances undergo etherification in addition to esterification. According to the NMR evidence, both pectin and polygalacturonic acid treated with CH_2N_2 exhibited resonances attributed to methyl ether linkages in addition to resonances attributed to carbomethoxy linkages. In contrast, biosynthetically methylated pectin showed only resonances assigned to carbomethoxy linkages.

CONCLUSIONS

Pectin can undergo changes in R_g in pH 7.3 buffer (this work), in neutral salt (Jordan and Brant, 1978), and also at acid pH (Davis et al., 1980). Since our samples have a fairly narrow range of $\bar{M}_n$'s, the degree of esterification appears to be the most important parameter governing pectin size in buffered solutions (Figure 4a,b,d). Changing the degree of esterification could affect pectin size by affecting the strength of intermolecular ionic repulsions and the character of the attractive forces between chains. At low % E, the attractive forces should be predominantly hydrogen bonds. At high % E, interchain interactions should be predominantly nonpolar with polar groups at the solvent polymer interface where steric requirements permit. The 57% E range may mark the change over from predominantly hydrogen bond interchain interactions to hydrophobic interchain interactions. The hydrophobic interactions marked by smaller R_g's may occur as a result of more complete overlap between aggregated chains. In this way, contact between the hydrophilic solvent and hydrophobic portion of the pectin could be minimized. In unbuffered water, degree of esterification is unimportant in determining pectin size. As indicated by work on the dialysate derived from 37% methoxy pectin, the neutral sugars in pectin can aggregate in water and buffered solutions, whereas uronate residues aggregate only in water or possibly at lower pH than measured here. Furthermore, depending on the degree of esterification, the dialysate can cause the size of pectin retentate to increase, decrease, or remain the same. Moreover, the dialysate is susceptible to denaturation. It has been reported previously (Pippen et al., 1953) that treatment of pectin with diazomethane is an equivalent method of studying the effect of esterification in pectin. Our results, in conjunction with NMR data (Pfeffer et al., 1981), indicate that hydrophobic interactions are overemphasized at the expense of intermolecular hydrogen bonds, due to simultaneous ether- and esterification.

Finally, it is apparent that the ability of pectin to function as a food fiber, thickening agent, or cell wall constituent is not simply related to molecular weight but that degree of esterification is important.

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Registry No. Pectin, 9000-69-5; low methoxyl pectin, 9049-34-7.

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