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Phase Separation of Plant Cell Wall Polysaccharides and Its Implications for Cell Wall Assembly[1]

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Concentrated binary mixtures of polymers in solution commonly exhibit immiscibility, resolving into two separate phases each of which is enriched in one polymer. The plant cell wall is a concentrated polymer assembly, and phase separation of the constituent polymers could make an important contribution to its structural organization and functional properties. However, to our knowledge, there have been no published reports of the phase behavior of cell wall polymers, and this phenomenon is not included in current cell wall models. We fractionated cell walls purified from the pericarp of unripe tomatoes (*Lycopersicon esculentum*) by extraction with cyclohexane diamine tetraacetic acid (CDTA), Na_2CO_3, and KOH and examined the behavior of concentrated mixtures. Several different combinations of fractions exhibited phase separation. Analysis of coexisting phases demonstrated the immiscibility of the esterified, relatively unbranched pectic polysaccharide extracted by CDTA and a highly branched, de-esterified pectic polysaccharide present in the 0.5 N KOH extract. Some evidence for phase separation of the CDTA extract and hemicellulosic polymers was also found. We believe that phase separation is likely to be a factor in the assembly of pectic polysaccharides in the cell wall and could, for example, provide the basis for explaining the formation of the middle lamella.

The primary cell wall of dicotyledonous plants contains three broad classes of polysaccharides: cellulose, hemicellulosic xyloglucans, and pectic polysaccharides, as well as structural glycoproteins. Although it is possible to identify typical structures for the hemicelluloses and pectic polysaccharides of the cell wall, there is increasing evidence of structural diversity within each grouping. For example, the pectic polysaccharides may differ in their neutral sugar content and composition, extent of branching/branch length, and extent of methyl esterification of the D-galacturonic acid in the backbone (Carpita and Gibeaut, 1993). No particular biological significance has yet been attached to this structural diversity, and current models of the primary cell wall still depict a relatively simple arrangement in which partially crystalline cellulose microfibrils are dispersed in a more open polysaccharide matrix (Carpita and Gibeaut, 1993).

One function of the plant cell wall is to perform a complex mechanical role: the cell wall must resist unlimited expansion due to turgor pressure and yet be sufficiently plastic to permit cell growth. After growth there is the possibility of forming more permanent covalent linkages to cross-link the structure, but during cell expansion it is likely that noncovalent interactions make the main contribution to the mechanical properties of the cell wall. For example, on the basis of in vitro experiments, it has been proposed that the hemicellulosic xyloglucan binds and coats the cellulose microfibril (Hayashi and Machlachlan, 1984), and there is evidence that xyloglucan can bridge neighboring fibrils, forming a cross-link (McCann et al., 1990). In addition, in recent experiments on pectic polysaccharides isolated from tomato (*Lycopersicon esculentum*), we have shown that a high-methoxyl pectin can form elastic gels upon the addition of calcium ions (MacDougall et al., 1996). Because extraction of this polysaccharide results in cell separation, a role for ionic interactions involving calcium in cell adhesion is suggested.

In both of the examples given above, the outcome of the molecular interaction is intermolecular binding. However, it is possible for molecular interactions to produce quite a different outcome. Generally, concentrated solutions of polymer binary mixtures exhibit immiscibility and at equilibrium form two phases, each being enriched in one of the polymers. In contrast, miscibility of different polymers is rather unusual (Krause, 1972). Thermodynamically, the phase behavior of polymer mixtures is influenced by entropic and energetic factors. Usually the entropic gain of forming a homogeneous solution (which decreases as molecular weight is increased for a given C of solution) is balanced by opposing energetic factors and immiscibility results. No general theoretical treatment is available for polymer mixtures in an aqueous solution that might predict their tendency to phase-separate from their chemical structure (Zaslavsky, 1995). However, theoretical models exist that allow the energetics of the interaction between different species to be characterized by the experimental determination of interaction parameters. These parameters can then be used to predict phase behavior (Krause, 1972). Small differences in the size of the interaction between the different species can have a major impact on the observed behavior. Because the effect is due to a balance of weak interactions, immiscibility is generally observed in concentrated polymer solutions when

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Abbreviations: C, concentration; CDTA, cyclohexane diamine tetraacetic acid; $[\eta]$, intrinsic viscosity, η_{sp}, specific viscosity; Rha, rhamnose.

polymer domains overlap and the polymer chains become entangled.

Because phase separation is of great importance in the field of applied polymer chemistry, the behavior of a number of different polymers has been studied in several different solvents (Krause, 1972). In experimental biochemistry this phenomenon has found widespread application in the separation and purification of cells and organelles (Albertsson, 1986; Walter and Johansson, 1994). Although there is evidence to suggest that the phenomenon is relevant to the assembly of biological membranes (Israelachvili, 1992) and to the organization of the cytoplasm (Clark, 1994), and although the subject has been discussed in relation to cell wall assembly (Jarvis, 1992), to our knowledge there has been no published report of the phase behavior of plant cell wall polymers and relatively little information is available concerning carbohydrate polymer mixtures in general. Kalichevsky et al. (1986) demonstrated that dextran and amylose phase-separate, as do amylose and amylopectin (Kalichevsky and Ring, 1987). More recently, Garnier et al. (1995) demonstrated the phase separation of dextran and a galactomannan extracted from locust bean.

As part of our continuing research on the interactions between the cell wall polysaccharides, we now report the results of experiments in which we examined the phase behavior of fractions isolated from the cell walls of the pericarp of unripe tomatoes. In the absence of previously published work, we adopted a broad approach, combining cell wall fractions known to contain a mixture of different polymers and examining the outcome.

MATERIALS AND METHODS

Preparation of Cell Wall Material and Cell Wall Extracts

The preparation of cell walls from unripe tomatoes (*Lycopersicon esculentum*) and the extraction of a CDTA-soluble pectic polysaccharide have been described previously (MacDougall et al., 1996). Subsequent extraction with 50 mM Na_2CO_3 (at 1 and 20°C) with 0.5 and 4 N KOH was carried out on approximately 25 g of cell wall material under the conditions described by Redgwell and Selvendran (1986). After neutralization with acetic acid, cell wall extracts were diafiltered with 8 volumes of distilled water in an ultrafiltration system (CH2, Amicon, Beverly, MA) incorporating a fiber cartridge (H1P10-20, Amicon). To de-esterify part of the CDTA extract, 50 mL of a 0.1% (w/w) solution was adjusted to 50 mM Na_2CO_3 at 1°C and left overnight. After neutralization with acetic acid the material was concentrated to 20 mL and diafiltered with 8 volumes of distilled water in a pressure-filtration cell fitted with a PM10 membrane (Amicon). All extracts were stored frozen at −20°C and were not freeze-dried.

Analysis of Residual CDTA

A method described by Bermejo-Martinez (1976) was used to assay CDTA spectrophotometrically. With the final pH maintained at 2 ± 0.05, cuvettes were prepared by the addition of 20% (w/v) 5-sulfosalicylic acid (100 μL), 1 mg/mL Fe(III) ammonium sulfate acidified with H_2SO_4 (100 μL), 2 M sodium acetate (45 μL), and sample/water (755 μL). The A_{520} was compared with a blank lacking the sample. ^{1}H NMR was carried out on a 1% (w/w) solution of polysaccharide in a 400-MHz spectrometer (X400, Jeol) at 50°C, and data were collected for 16 h.

Ion-Exchange Chromatography of Cell Wall Extracts

Solutions of polysaccharide were dialyzed overnight against 25 mM potassium phosphate buffer, pH 6.5. Following centrifugation to remove insoluble material, the solution was passed through a 1.6- × 9-cm column of DEAE-Trisacryl M (Pharmacia). The column was washed with 25 mM potassium phosphate buffer, pH 6.5, and then 25 mM potassium phosphate buffer, pH 6.5, containing 1 M NH_4Cl. Five-milliliter fractions were collected and analyzed for total sugar and uronic acid content. Selected fractions were then pooled and their sugar composition was analyzed.

Sugar Analysis and Determination of the Degree of Methyl Esterification

All samples for sugar analysis were freeze-dried and dispersed in 72% H_2SO_4 for 3 h at room temperature. After dilution to 2 N H_2SO_4, samples were hydrolyzed for 1 h at 100°C prior to uronic acid assay by the colorimetric method (Blumenkrantz and Asboe-Hansen, 1973) and for 2.5 h at 100°C prior to the assay of neutral sugars by reduction, acetylation, and analysis by GC (Blakeney et al., 1983; Englyst and Cummings, 1984). Total sugars in fractions from the ion-exchange chromatography were determined by the method of Dubois et al. (1956). The degree of methyl esterification of the uronic acids was determined as described previously (MacDougall et al., 1995).

Viscosity Measurements

To reduce the effect of ionic interactions, all viscosities were measured in the presence of 100 mM NH_4Cl after adjustment of the pH to 6.5 with ammonia. Viscosities were determined from the efflux time of measured volumes through fine-bored tubing using Ubbelohde-suspended level viscometers at 25°C (Billmeyer, 1984). Specific viscosity (η_{sp}) was determined as $(\eta - \eta_{water})/\eta_{water}$, where η and η_{water} are the efflux times for the sample solution and water, respectively. Intrinsic viscosity ([η]) was determined by extrapolation to 0 C of a plot of η_{sp}/C against C.

Preparation of Solutions for Examination of Phase Behavior

Solutions of cell wall fractions of approximately 1 mg/mL were converted to the potassium salt by adding ion-exchange resin (Dowex AG 50W-X8, Bio-Rad) at 3 g/100 mL solution, swirling for 15 min, filtering through a 10-μm nylon mesh, and adjusting to pH 6.5 with KOH. Solutions were concentrated by rotary evaporation under reduced pressure at 30°C and were then centrifuged for 20 min at 13,000g. The volume of the resulting concentrates was typically between 2 and 5 mL. For the initial assess-

ment of phase behavior the C was assessed by freeze-drying. The C of the solutions used for a more detailed analysis was determined from the total content of anhydrosugars. Initial assessments of the tendency to phase-separate were carried out in small glass test tubes (400-μL capacity). Equal weights of two solutions were mixed in the tube with a Pasteur pipette and, after standing for 24 h at room temperature, samples were centrifuged at 13,000*g* for 30 min. For the main experiments (examining phase separation at a series of dilutions), equal amounts of the two solutions were weighed into 1.5-mL microcentrifuge tubes, appropriate amounts of distilled water were added, and the samples were mixed by repeated inversion. After standing for 48 h at room temperature, the samples were centrifuged for 30 min at 13,000*g*. The lower phase was sampled by pushing a syringe needle of 0.8 mm in diameter on a 1-mL syringe through the side of the microcentrifuge tube; the upper phase was sampled with a pipette. Weighed amounts of each phase were freeze-dried and analyzed for sugar composition or stored at −20°C prior to saponification and methanol determination.

RESULTS AND DISCUSSION

Chemical and Physical Characterization of Cell Wall Fractions

Removal of CDTA from Extracted Polysaccharides

Because it is a powerful chelating agent CDTA has proved to be useful in cell wall extraction, releasing ionically bound pectic polysaccharides. However, difficulty has been experienced in removing the CDTA from the extracted polysaccharide (Mort et al., 1991). Imidazole has been suggested as an alternative, particularly for spectroscopic studies of the cell wall (Sene et al., 1994), but solutions of imidazole do not cause cell separation in unripe tomato fruit as readily as CDTA does, and therefore we have continued to use CDTA. We have found that pressure-filtration systems are very effective in removing CDTA from pectic polysaccharides, provided a sufficient volume of additional water is passed through the system. Initially we used an assay to detect contaminating CDTA based on decolorization of a complex between Fe(III) and 5-sulfosalicylic acid. With adequate control of the pH this method reliably detects CDTA at 7 μg (20 nmol) in the assay cuvette. The assay was unaffected by the presence of 0.5 mg of polygalacturonic acid (2.8 μmol of GalA equivalents) and 1.25 μmol of $CaCl_2$. Contamination greater than 2% (w/w) was therefore detected without difficulty. More sensitive detection is possible through the use of derivatization and analysis by GC (MacDougall et al., 1996), but in the current work the purity of the final preparation of the CDTA-extracted pectin (after the ion-exchange step) was determined with ^1H NMR, and no peaks attributable to CDTA could be found. Deliberate addition of CDTA at 1% (w/w) with respect to the pectin gave two new, unobscured characteristic multiplets at approximately 1.82 and 3.25 ppm, which were readily detected above the background noise. From this we estimate a level of contamination less than 0.5% (w/w).

Composition of Cell Wall Fractions

The sugar compositions of the different fractions isolated are given in Table I; the data are similar to those previously published on the composition of cell wall fractions isolated from tomato (Seymour et al., 1990). The CDTA and Na_2CO_3 extracts are mainly composed of uronic acid, Gal, Ara, and Rha, which are typically found in pectic polysaccharides. These fractions contain very small amounts of Glc, Xyl, Fuc, and Man, suggesting that little hemicellulosic material is present. The CDTA-extracted material has been previously subjected to gel-filtration chromatography (MacDougall et al., 1996) and was shown to be polydisperse, with little variation in the proportion of neutral sugars between materials of different sizes. Ion-exchange chromatography of a similar cell wall fraction from unripe kiwifruit suggests that there are subpopulations in this class of pectic polysaccharide that vary in their degree of methyl esterification but not in their monosaccharide composition (Redgwell et al., 1991). The fractions extracted with Na_2CO_3 have a higher level of neutral sugars than the CDTA-extracted material, indicating a higher degree of branching. The 0.5 and 4 N KOH fractions contain a much wider range of monosaccharides. Subfractionation using ion-exchange and gel-filtration chromatography has revealed that extracts prepared under similar conditions from tomato and other parenchymatous tissues contain both pectic and hemicellulosic polymers (Seymour et al., 1990; Redgwell et al., 1991).

Table I. *Sugar composition of extracts prepared from cell walls isolated from mature green tomatoes*

Fraction	Yield	Monosaccharide Composition									Degree of Methyl Esterification
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic Acid	Total	
	%	*μg/mg dry wt*[a]									%
CDTA	7	10	0	34	3	1	94	8	697	850	60
Na_2CO_3 (1°C)	4	7	2	43	2	2	120	8	543	726	–[b]
Na_2CO_3 (20°C)	1	8	6	59	1	1	186	17	348	626	–
0.5 N KOH	6	7	3	62	100	15	256	189	213	846	–
4 N KOH	9	4	1	55	97	161	171	344	79	911	–

[a] Determined as the anhydrosugar. [b] De-esterified during extraction.

In the phase separations discussed below it was necessary to be able to determine the distribution of the different polymers from the monosaccharide composition of the phases. This analysis is complicated by the fact that individual sugars often occur in more than one polymer (e.g. Gal, Ara, and Xyl are present in both pectic polysaccharides and in hemicellulosic xyloglucans). Partial fractionation was therefore carried out on the 0.5 and 4 N KOH fractions, with the aim of determining the sugar composition of the pectic polysaccharides in these fractions and identifying appropriate marker sugars that could be used to distinguish the pectic from the hemicellulosic polysaccharides. The elution profiles for total sugars and uronic acids from ion-exchange chromatography are presented in Figure 1, and the sugar composition of pooled fractions is given in Table II.

Ion-Exchange Separation of Component Polysaccharides in the 0.5 N KOH Extract

Complete separation of the neutral and acidic polysaccharides was not obtained by ion-exchange chromatography of the 0.5 N KOH-extracted material. Approximately

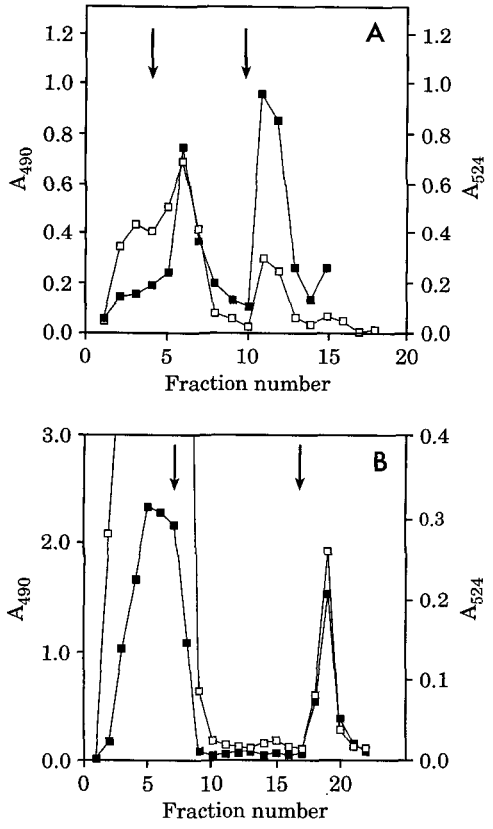

Figure 1. Ion-exchange chromatography of selected fractions. A, 0.5 N KOH extract; B, 4 N KOH extract. Chromatography was performed on DEAE-Trisacryl M. The column was loaded and washed with 50 mM potassium phosphate buffer, pH 6.5, and eluted with 1 M NH_4Cl in the same buffer. The arrows from left to right mark the end of loading and the change to a buffer containing 1 M NH_4Cl, respectively. Fractions were assessed for their total sugar and uronic acid content. ■, Uronic acid (A_{524}); □, total sugars (A_{490}).

20% of the applied material eluted as a partially retained fraction in the loading buffer (fractions 6–9). The sugar composition of this material shows that it contains a mixture of neutral and acidic polysaccharides (Table II). Nevertheless, the main observation of relevance from these data is that the neutral polysaccharide (eluted in fractions 1–5) is low in Gal and high in Glc and Xyl, whereas the main pectic polysaccharide (eluted with 1 M NH_4Cl) has a high content of Gal and contains little Xyl. A conservative but reasonable estimate of the amount of pectic polysaccharide in the 0.5 N KOH extract can therefore be made from the sum of the uronic acid, Rha, and Gal content. A similar estimate of the amount of xyloglucan can be made from the Glc and Xyl content. Ara is more evenly distributed between the fractions, reflecting the observation that this sugar is a relatively minor component of the more highly branched pectic polysaccharides in tomato (Seymour et al., 1990) and that, like other solanaceous plants, tomato contains an arabinoxyloglucan as the major hemicellulosic polysaccharide (York et al., 1996). The 0.5 N KOH extract can be estimated using the marker sugars identified above and the sugar composition given in Table I to be made up of approximately 60% pectic polysaccharide and 40% xyloglucan.

Ion-Exchange Separation of Component Polysaccharides in the 4 N KOH Extract

The bulk of the 4 N KOH-extracted material applied to the column eluted with the loading buffer (fractions 2–10). This fraction appears to contain xyloglucan, a Man-containing polymer, and a small amount of pectic polysaccharide (Table II). Failure of the DEAE-Trisacryl (Pharmacia) to retain the latter may be due to the high degree of branching with neutral sugars in this pectic material, which could hinder the uronic acid from interacting with the column. The acidic material eluted with 1 M NH_4Cl contains little Glc, Xyl, or Man, suggesting that it is predominantly composed of pectic polysaccharides. The conclusions to be drawn from these data are that the level of pectic polysaccharide is best estimated from the uronic acid content alone. The level of Gal in the neutral fraction appears too high to be solely due to contaminating pectic material, and Gal therefore appears to be present as a component of the neutral polysaccharide. In addition, Xyl is likely to be the only component of xyloglucan that is not present to an appreciable extent in other polysaccharides in the extract, and therefore it is the only reliable guide to the xyloglucan distribution in mixtures with other cell wall fractions. It is probable that a significant proportion of both the Gal and Glc are associated with Man. The 4 N KOH fraction is therefore a complex mixture of neutral and acidic polysaccharides. The pectic polysaccharides in both the 0.5 and the 4 N KOH extracts differ from the pectic polysaccharide in the CDTA extract in being de-esterified and containing a much higher level of neutral sugars.

Entanglement Cs of Cell Wall Fractions

Phase separation is associated with mixtures of polymers at Cs high enough to permit significant molecular interac-

Table II. *Sugar composition of fractions separated from the 0.5 and 4 N KOH extracts by ion-exchange chromatography on DEAE-Trisacryl M*

Extract	Fractions	Description	Weight Recovered	Monosaccharide Composition								
				Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic Acid	Total
			%	μg/mg dry wt[a]								
0.5 N KOH	–	Precipitate[b]		7	4	58	5	3	264	5	212	557
	1–5[c]	Unbound	15	0	0	38	151	39	68	323	32	651
	6–9	Loading acidic	17	4	1	48	93	24	159	158	114	601
	11–16	1 M NH_4Cl eluted	48	8	10	63	37	<1	293	14	222	648
4 N KOH	2–10	Unbound	69	4	0	34	87	189	116	383	22	835
	20–23	1 M NH_4Cl eluted	10	8	2	50	12	11	213	12	189	497

[a] Determined as the anhydrosugar. [b] Precipitate formed during dialysis of the extract against starting buffer (50 mM potassium phosphate pH 6.5). [c] Fraction numbers refer to Figure 1.

tion. Therefore, before looking for evidence of phase separation it is important to ensure that the minimum concentration at which polymer entanglement occurs is known for the materials under study. This concentration can be determined directly, from the relationship between η_{sp} and C, or may be estimated from $[\eta]$. In Figure 2 viscosity data for the 4 N KOH extract over the C range 0.4 to 3% (w/w) are presented as a double-logarithmic plot. The shape of the curve is typical for similar plots obtained for other carbohydrate polymers (Kalichevsky et al., 1986; Axelos et al., 1989; Gidley et al., 1991). At the lowest Cs (up to approximately 0.7% [w/w]), η_{sp} is proportional to C, with an exponent of 1.3. In this section of the plot, the polymer molecules are considered to be at too low a C for entanglement to occur, and the viscosity depends on the polymer size (Ferry, 1980). Above approximately 2% (w/w), η_{sp} is proportional to C, with an exponent of 5.0. The change in the relationship between η_{sp} and C arises from polymer entanglement, which introduces additional contributions to viscosity (Ferry, 1980). Between the two extremes of the graph there is a transitional region. For a polymer that behaves as a fully flexible coil, an exponent of 3.3 for the more concentrated solutions is typical (Morris et al., 1981). The exponent for the region of higher C shown in Figure 2 is much larger. As discussed above, the 4 N KOH extract is a complex mixture of polysaccharides but contains a significant proportion of xyloglucan. A similarly large exponent has been observed for a storage xyloglucan extracted from tamarind seed (Gidley et al., 1991). Morris (1995) argued that the higher-than-expected exponent is due to weak intermolecular binding, but other explanations are possible, including side-chain entanglement and stiffness in the polymer backbone. The main conclusion to be drawn from examining Figure 2, however, is that to test for evidence of phase separation in mixtures of the 4 N KOH extract and other cell wall fractions, a minimum C of 0.7% (w/w) of the former is required, since this is the C at which entanglement effects begin to be observed. A similar double-logarithmic plot has been published for the CDTA-extracted material (MacDougall et al., 1996), and in this instance the minimum C for entanglement was found to be approximately 0.2% (w/w). The difference in the C required for these two cell wall fractions reflects the different sizes of their component polysaccharides.

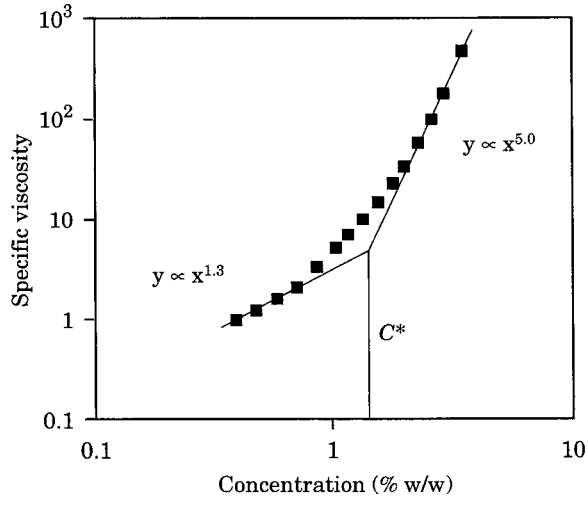

Figure 2. Double-logarithmic plot of specific viscosity versus C for the unfractionated 4 N KOH extract. Measurements were carried out at pH 6.5 and 25°C in the presence of 100 mM NH_4Cl. Extrapolation of the lines drawn through either end of the viscosity profile until they cross gives C^*, the coil overlap C (see text).

The $[\eta]$ of a compound is defined as η_{sp}/C at infinite dilution and is determined experimentally by extrapolation. The units are volume per unit weight, and this parameter is related to molecular size. Morris et al. (1981) have demonstrated that for a range of different polysaccharide solutions plotting η_{sp} against the dimensionless parameter $C[\eta]$ results in the alignment of the data on a single master curve, with the center of the transitional region occurring at $C[\eta] = 4$. Consequently, this relationship can be used to estimate C^*, the coil overlap C (Ferry, 1980), which is the notional transitional point between the two C dependencies on a viscosity profile (Fig. 2). Axelos et al. (1989) found a similar alignment of the viscosity profiles for pectin from different sources when η_{sp} was plotted against $C[\eta]$. These authors also noted that the start of the transitional region, and therefore the minimum C at which entanglement effects could be observed, consistently occurred at $C[\eta] \simeq 0.8$. Comparable figures for the 4 N KOH extract and the CDTA extract (using data from MacDougall

et al., 1996) are 1.1 and 1.3, respectively. These values are sufficiently consistent to justify using this relationship to estimate the minimum C at which entanglement occurs. This is convenient for samples containing only small amounts of material. For instance, in the work presented here, [η] was estimated from less than 15 mg of material, whereas the preparation of Figure 2 required approximately 300 mg. Table III gives values for [η] and estimates for the minimum entanglement Cs for the cell wall extracts we prepared based on a value of C[η] = 1.3, together with the Cs of the stock solutions prepared for use in the experiments described below. The values for [η] are high for all of the extracts, demonstrating that they are composed of high-molecular-weight polymers. The data show that the stock solutions were prepared at Cs that ensured that there was a significant overlap of the constituent polymers in the mixtures formed and that we could be confident of detecting phase separation should it normally occur between the different cell wall fractions under investigation.

Preliminary Results

Precipitates Formed during C of Extracts and Appearance of the Concentrated Solutions

A small amount of precipitate formed on neutralization of the 0.5 and 4 N KOH extracts, but this accounted for less than 3% (w/w) of the total material. After C to the levels given in Table III, the CDTA extract (both untreated and de-esterified) remained clear, whereas subsequent cell wall fractions were increasingly opalescent. The 4 N KOH extract was turbid at 3.2% (w/w). Cloudiness in the solutions suggests that some form of intermolecular association was occurring, but, with the exception of the 0.5 N KOH extract, little material precipitated during rotary evaporation and C. Centrifugation of the concentrated 0.5 N KOH extract at 13,000*g* yielded a pellet that occupied a large volume but this accounted for less than 5% of the weight of the total extract.

Initial Observations on Mixtures of Concentrated Extracts

No phase separation was observed in the concentrated stock solutions. Table IV presents the results of mixing several different pairs of cell wall fractions. When phase separation occurred readily, the mixed solution took on a frosted appearance within 1 h (when viewed with a hand lens), and the two phases could be separated by centrifugation 2 to 3 h later. Mixtures that separated slowly formed two layers over 72 h without centrifugation. The most marked phase separation was found for the mixture of the CDTA extract with the 0.5 N KOH extract (Fig. 3). Some mixtures, notably the CDTA extract and the 4 N KOH extract, formed a pellet on centrifugation rather than a mobile lower layer. However, dilution with water gave two mobile phases, suggesting that this effect was due to the concentration of the lower layer rather than outright precipitation. At all Cs in which immiscibility of the CDTA extract with the de-esterified CDTA extract was observed, the de-esterified CDTA formed a diffuse precipitate. Piculell et al. (1995) have shown how relatively small changes in the degree of neutralization and salt content of solutions of ionic polymers can have a marked effect on their phase behavior. In these initial observations we found evidence that the addition of 50 mM KCl affected the relative volumes of the phases and also encouraged phase separation where it might not otherwise have occurred (Table IV). However, effects of this type were not investigated further, since the main aim of this work was to demonstrate that phase separation can occur in mixtures of cell wall fractions and to identify the polymers involved. Consequently, the analysis presented below is restricted to mixtures of the fully neutralized potassium salts of the different cell wall extracts in the absence of additional salt.

Table III. *Estimates of [η] and the minimum C at which entanglement occurs obtained from the relationship C[η] ≃ 1.3, together with the C of stock solutions of cell wall fractions prepared for examination of their phase behavior*

Cell Wall Fraction	[η]	Minimum Entanglement C	C of Stock Solutions
	mL/g	% (w/w)	
CDTA	810[a]	0.2	1.0
CDTA (de-esterified)	401	0.3	0.7
Na_2CO_3 (20 °C)	309	0.4	1.9
0.5 N KOH	159	0.8	1.7
4 N KOH	111	1.2 (0.7[b])	1.6

[a] Value obtained from MacDougall et al. (1996). [b] Value obtained from inspection of Figure 2.

Table IV. *Outcome of mixing pairs of cell wall fractions at Cs above the minimum entanglement C*

Solutions were prepared as the potassium salt by ion-exchange and neutralization with KOH. The Cs of the stock solutions are given in Table III. Equal weights were mixed, left standing for 24 h at room temperature, and then centrifuged for 30 min at 13,000*g*.

Cell Wall Fractions	Outcome
CDTA +	
Na_2CO_3 (20 °C)	Two phases.
0.5 N KOH	Two phases and a small pellet. Addition of 50 mM KCl led to a marked reduction in the volume of the lower layer.
4 N KOH	Two phases, with the lower layer forming a pellet unless mixture diluted.
De-esterified CDTA +	
CDTA	De-esterified CDTA forms a diffuse precipitate[a].
0.5 N KOH	Miscible. Addition of 50 mM KCl led to the slow formation of two phases.
4 N KOH	Two phases.
Na_2CO_3 (20 °C) +	
0.5 N KOH	Miscible.
4 N KOH	Two phases, with the lower layer forming a pellet unless mixture diluted.

[a] Determined from the degree of methyl esterification of the supernatant and precipitate.

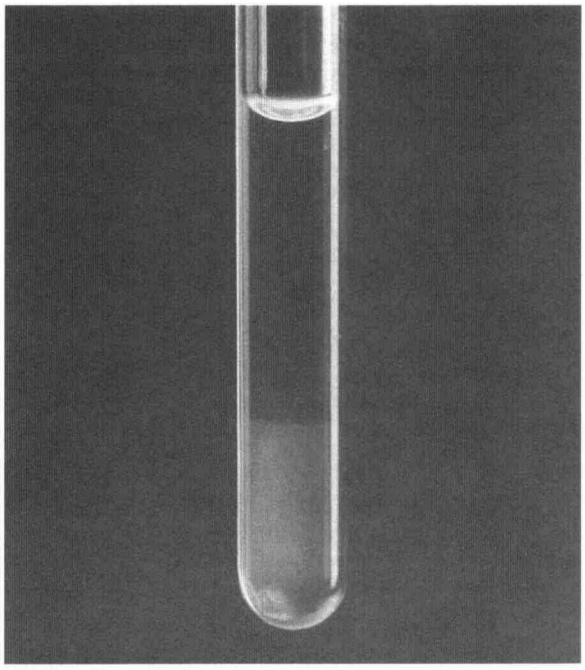

Figure 3. Phase separation observed between the CDTA-extracted material and the unfractionated 0.5 N KOH extract at average *C*s of 0.64 and 0.52% (w/w), respectively. The CDTA extract predominates in the upper layer. The lower layer appears opaque under the lighting conditions used for the photograph but seen against a light background is a clear, yellowish solution.

Phase Separation of Polymers in the CDTA and 0.5 N KOH Extracts

The composition of the starting solutions, the average *C* of the dilutions, and the composition of the two phases (excluding the small pellet formed) for mixtures of the CDTA and 0.5 N KOH extracts are given in Table V. Phase separation was not observed for a solution with an average *C* of 0.32% (w/w). In the most dilute solution in which phase separation was observed, the CDTA pectic polysaccharide was present at an average *C* of 0.17% (w/w). It is notable that this is close to but slightly lower than the *C* at which entanglement effects are observed for this polysaccharide in isolation (Table III), the difference presumably being due to the contribution that both extracts make to entanglement in the mixed solution. The data in Table V also show that the lower phase is markedly more concentrated than the upper phase and that its *C* exceeds that of either of the starting solutions. This behavior is a reflection of the differing affinities of the polysaccharides for water.

The nature of the polysaccharides present in each of the two phases was determined from the data presented in Table V on the basis of the content of methanol and of the marker sugars identified above. The CDTA-extracted pectic polysaccharide, which is esterified and has a low neutral sugar content, is concentrated in the upper phase, whereas the pectic polysaccharide from the 0.5 N KOH extract, which is unesterified and has a high neutral sugar content, is concentrated in the lower phase. This is especially apparent from the Gal distribution, which is highly concentrated in the lower phase. An inequality in the distribution of Glc and Xyl is also apparent, with these sugars being

Table V. *Analysis of phase separation of the CDTA extract and the 0.5 N KOH extract*

Solution	Phase	Relative Volume[a]	Monosaccharide Composition								d.o.m.[b]	*C*
			Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic Acid		
		%	*mg/g solution*[c]								%	% (w/w)
CDTA extract			0.16	0.00	0.54	0.05	0.02	1.51	0.12	11.18	60	1.36
0.5 N KOH extract			0.19	0.07	1.42	2.65	0.31	5.61	4.06	3.60	0	1.79
1:1 Mixture			0.18	0.03	0.98	1.35	0.17	3.56	2.09	7.39	30	1.58
Separated phases (average *C* in % [w/w])												
1.58	Upper	69	0.11	0.00	0.55	1.21	0.13	1.68	1.31	7.49	56	1.25
	Lower	31	0.35	0.08	2.15	1.77	0.24	8.97	2.70	7.04	11	2.33
1.26	Upper	74	0.09	0.00	0.45	0.99	0.13	1.39	1.04	5.91	54	1.00
	Lower	26	0.24	0.17	1.78	1.34	0.18	7.46	2.43	6.48	10	2.01
0.95	Upper	77	0.08	0.00	0.38	0.84	0.10	1.20	0.86	4.35	51	0.78
	Lower	23	0.25	0.18	1.40	0.81	0.12	5.97	1.34	5.06	12	1.51
0.63	Upper	88	0.05	0.00	0.28	0.56	0.07	0.92	0.75	2.92	49	0.56
	Lower	12	0.19	0.06	1.09	0.48	0.00	4.70	0.93	3.98	11	1.14
0.47	Upper	94	0.05	0.01	0.23	0.42	0.06	0.81	0.65	2.18	48	0.44
	Lower	6	0.13	0.11	0.84	0.36	0.06	3.66	0.84	3.23	nd[d]	0.92
0.39	Upper	95	0.04	0.02	0.22	0.35	0.06	0.78	0.45	1.81	46	0.37
	Lower	5	0.13	0.00	0.72	0.35	0.00	2.97	0.85	2.61	nd	0.76

[a] Calculated from the *C* of the two phases and the average *C* of the mixture. [b] d.o.m., Degree of methyl esterification. [c] Determined as the anhydrosugar. [d] nd, Not determined.

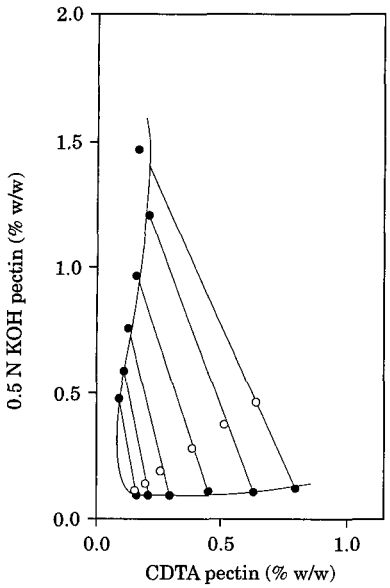

Figure 4. Phase diagram for the separation of two classes of pectic polysaccharides in the CDTA extract and the 0.5 N KOH extract. The diagram was computed from the data in Table V using the Gal content of the phases in the second of two simultaneous equations, as described in the text. ●, Composition of the phases; ○, average composition of mixtures. The line joining the closed circles is the binodal line within which all mixtures will phase-separate. Tie lines connecting data points for the upper and lower phases in individual separations are included.

more concentrated in the lower phase. These data suggest that phase separation may also occur between the CDTA-extracted pectic polysaccharide and the xyloglucans in the 0.5 N KOH extract.

Presentation of the Data as a Phase Diagram

The separations reported here are complex because of the number of different polymers involved and are not strictly comparable with experimental systems dealing with two polymers in solution. Nevertheless, it is helpful to present the data as a phase diagram for the main separation observed between two classes of pectic polysaccharides, so that a general comparison can be made with other systems. To calculate the amounts of the two different pectic polysaccharides in individual phases, it was assumed that they were both sufficiently homogeneous in composition to behave uniformly during phase separation. The amounts of each were then determined from the solution to two equations, the first equating the total weight of pectic monosaccharides to the combined weights of the two polymers and the second relating the weight of an individual monosaccharide to the weight of each polysaccharide multiplied by a separate factor. The factors were determined from the proportion of the pectic polysaccharide in each of the starting solutions accounted for by the chosen monosaccharide. The validity of this approach is supported by the fact that very similar results were obtained when either uronic acid or Gal was used as the monosaccharide in the second equation or when the methanol content was used in a similar way to determine the level of the CDTA-extracted pectic polysaccharide directly.

Data from calculations using Gal as the monosaccharide in the second of the two equations are presented in Figure 4 as an x-y plot (Albertsson and Tjerneld, 1994) and demonstrate the marked difference in the composition of the two phases. Tie lines joining related upper and lower phases are shown. Initial solutions containing the relative proportions given by any point on a particular tie line will phase-separate to yield identical phase compositions (although the relative volumes will differ). The fact that in Figure 4 data points for the average composition of mixtures lie close to the tie lines suggests that the assumptions made in preparing the phase diagram were reasonable. The curve joining the data points is the binodal curve, and all compositions within this boundary may be expected to phase-separate. The tie lines are skewed toward the y axis because of the high C of the lower phase compared with the upper phase.

Table VI. *Analysis of phase separation of the CDTA extract and the 4 N KOH extract*

Solution	Phase	Monosaccharide Composition								C
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc	Uronic Acid	
		mg/g solution[a]								*% (w/w)*
CDTA extract		0.16	0.00	0.54	0.05	0.02	1.51	0.12	11.18	1.36
4 N KOH extract		0.09	0.04	1.69	3.65	6.02	5.39	12.90	1.70	3.15
1:1 Mixture		0.13	0.02	1.12	1.85	3.02	3.45	6.51	6.44	2.25
Separated phases (average C in % [w/w])										
2.25	Upper	0.10	0.01	1.00	1.90	3.19	3.11	6.56	6.57	2.24
	Lower	0.20	0.00	1.95	2.12	4.16	7.73	8.31	5.93	3.04
1.80	Upper	0.08	0.01	0.82	1.49	2.46	2.41	5.07	5.26	1.76
	Lower	0.17	0.00	1.52	1.66	3.18	5.73	6.36	4.95	2.36
1.35	Upper	0.06	0.00	0.61	1.09	1.78	1.88	3.63	4.09	1.31
	Lower	0.11	0.00	1.11	1.18	2.33	4.36	4.61	3.58	1.73

[a]Determined as the anhydrosugar.

Phase Separation in Mixtures of the CDTA Extract and the 4 N KOH Extract

The data presented in Table V suggest that there is phase separation between the pectic polysaccharides of the CDTA extract and the xyloglucans in the 0.5 N KOH extract. The phase separation between the polysaccharides of the CDTA extract and the 4 N KOH extract was therefore investigated to determine whether further evidence of immiscibility between pectic polysaccharides and xyloglucans could be obtained. The data from this analysis are presented in Table VI. The relative volume of the lower phase formed by the most concentrated mixture was less than 10%, and although phase separation was observed down to 0.45% (w/w) overall C, below 1.35% (w/w) the increasingly small volume of the lower phase prevented the collection of accurate data. There was a marked difference in the level of Gal in the two phases, although this is not a reliable marker sugar for any particular class of polysaccharide in this extract (as discussed above). Lesser differences were noted in the levels of Ara, Xyl, Man, and Glc. We therefore did not find evidence for a marked phase separation between the pectic polysaccharide and the xyloglucan. There appear to be several polymers in the 4 N KOH extract that show some degree of phase separation from the CDTA-extracted pectic polysaccharide, including pectic material, xyloglucan, and a Man-containing polymer.

Relevance of the Results to the Cell Wall in Vivo and Implications for Cell Wall Assembly

Phase separation is an equilibrium condition for mixtures of polymers. Because there are features of the plant cell wall that will prevent equilibrium from being attained, it might be argued that the observations we have made are not relevant to the assembly of the cell wall in vivo. In particular, the experiments described were carried out in the absence of calcium ions, which would cause gelation of the pectic polysaccharides and inhibit the formation of separate phases. In most cell walls the level of calcium is high, and in cell walls from unripe tomato, which were the subject of this study, MacDougall et al. (1995) showed that the calcium level is equivalent (on the basis of charge) to one-half of the content of unesterified uronic acid. The majority of the pectic polysaccharides that contain the appropriate sequence of unesterified residues to form calcium cross-links would therefore be expected to do so. It is unlikely that the variable distribution of the esterified and unesterified uronic acid found in the cell wall (Knox et al., 1990) can be explained simply on the basis of phase separation of the pectic polysaccharides after incorporation into the cell wall. Phase separation could also be hindered by restricted mobility of the different polymers under the more concentrated conditions of the cell wall.

Nevertheless, the data we have presented indicate that cell wall models should recognize the potential relevance of polymer immiscibility. Phase separation occurred in a large number of the mixtures tested, and detailed analysis showed evidence of immiscibility for both pectic and hemicellulosic polysaccharides. The behavior of the CDTA extract and the de-esterified CDTA extract shows that differences in the degree of methyl esterification are likely to encourage phase separation. But the fact that we found phase separation for mixtures of the de-esterified CDTA extract and the 0.5 N KOH extract under certain conditions suggests that other features of the structure of pectic polysaccharides are also important. Further investigation of the structural basis on which cell wall polymers phase-separate is therefore warranted.

As an example of the possible role of phase separation in cell wall organization, we suggest that this phenomenon could provide the driving force for targeting of newly exported pectic polysaccharides. This material is of high molecular weight and is thought to be highly esterified and not capable of forming ionic cross-links with calcium. Phase separation could occur during the period before enzymic de-esterification leads to incorporation into the gel network, before some other form of cross-linking takes place. The forces driving this organization would come from subtle energetic interactions rather than from specific forms of intermolecular binding. If this view is correct, the accumulation of a specific class of pectic polysaccharide in the middle lamella could, for instance, be controlled solely by the inclusion of particular structural features during biosynthesis.

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