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DEPOLYMERIZATION OF PECTIN WITH DIAZOMETHANE IN THE PRESENCE OF A SMALL PROPORTION OF PHOSPHATE BUFFER

YUKO INOUE, AKIKO YAMAMOTO, AND KINZO NAGASAWA*
School of Pharmaceutical Sciences, Kitasato University, 9-1, Shirokane 5 chome, Minato-ku, Tokyo 108 (Japan)

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ABSTRACT

Treatment of pectin with diazomethane in diethyl ether in the presence of a small proportion of phosphate buffer resulted in considerable depolymerization of the polysaccharide chain and concomitant methylation of the hydroxyl groups. A mixture of the depolymerized products was fractionated by gel-filtration on a Toyopearl HW-40S column to give di- and tri-saccharide fractions, which were subsequently permethylated with diazomethane–boron trifluoride etherate. The resulting permethylated di- and tri-saccharide fractions were separated into two di- and four tri-saccharides by reversed phase l.c., followed by adsorption l.c. The ^{1}H-n.m.r. spectra of the isolated oligosaccharides were studied by homonuclear, spin-decoupling experiments, COSY-45 two-dimensional homonuclear correlation experiments, two-dimensional *J*-resolved methods, and two-dimensional ^{13}C–^{1}H correlation experiments. The structures (**1** and **2**) for the two disaccharides, and those (**3** and **4**) for two of the four trisaccharides were determined. The structures **5** and **6** for the remaining trisaccharides were tentatively deduced from their ^{1}H-n.m.r. spectral data.

INTRODUCTION

In our previous papers, we described the depolymerization of heparin[1], chondroitin 6-sulfate[2], and dermatan sulfate[2] by β-elimination with diazomethane to give, in each case, a mixture of methylated, even-numbered oligosaccharides having a 4,5-unsaturated uronic acid, nonreducing end-group. This reaction was thought to be useful for cleavage of the glycosyl linkage at the β-position of the uronic acid carboxyl group in heteropolysaccharides containing the uronic acid residue.

It was previously reported that pectin is depolymerized by heating[3] at various temperatures (50–95°) in 0.1M phosphate buffer at pH 6.8, or by treatment[4] with diazomethane in diethyl ether at −30°. This was demonstrated by a rapid decrease

*To whom correspondence should be addressed.

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of the viscosity. However, there has been no report that describes the depolymerization of pectin by heating in the buffer solution or by treatment with a diazomethane solution to give oligosaccharides. We describe herein the depolymerization and methylation of pectin by treatment with diazomethane in diethyl ether at 25° for ~26 h, in the presence of a small proportion of phosphate buffer, as well as the structural elucidation of the reaction products.

RESULTS AND DISCUSSION

Commercial pectin from citrus fruits was adsorbed on a DEAE-Sephadex A-50 column. After the neutral polysaccharide had been eluted with water, a pectin fraction was eluted with 3M formic acid. The purified pectin (3M formic acid eluate) contained 90.1 of D-galacturonic acid and 10.5% of neutral sugars (rhamnose, 3.5; arabinose, 1.5; xylose, 0.3; galactose, 4.4, and glucose, 0.8%). The amount of methanol (10.1%) released by treatment with pectinesterase indicated a degree of esterification of ~60%.

A solution of the purified pectin in 0.1M phosphate buffer (pH 8.0) was treated with an excess of diazomethane in diethyl ether with vigorous stirring for ~26 h at 25° (see Experimental). The pH of the water layer was 10.4 at the end of the reaction. The i.r. and ^{1}H-n.m.r. spectra (90 MHz) of the reaction product indicated that most of the methyl ester groups in the starting pectin were removed. The removal of the methyl ester groups must have occurred under the long-period hydrolysis conditions in alkaline medium. Under these conditions, esterification of the released carboxylate anions with diazomethane did not apparently occur in the presence of water. The spectral data obtained also indicated that most of the alcoholic hydroxyl groups in the pectin were methylated. This observation is consistent with the experience reported by Hough *et al.*[5,6], who observed methylation of alcoholic hydroxyl groups with diazomethane in the presence of water as catalyst. As the reaction products in water without prior re-esterification emerged at the void volume, they were converted into methyl esters before fractionation on a Toyopearl HW-40S column (Fig. 1a). Although each permethylated oligosaccharide isolated by l.c. techniques was insoluble in water, as described later, the initial reaction mixture, which consisted of incompletely methylated oligosaccharides, and its re-esterified material were soluble in water, and the fractionation on the Toyopearl HW-40S column was successfully performed with water as the eluent.

Since it has been reported[3] that pectin decomposes rapidly upon heating in 0.1M phosphate buffer (pH 6.8), a solution of the purified pectin in 0.1M phosphate buffer (pH 8.0) was stirred for 20 h at 25° in diethyl ether alone. The gel-filtration, on Sepharose 6B, of the reaction product indicated that little depolymerization of the pectin had occurred (data not shown). It was also found that the amount of phosphate buffer affects the degree of depolymerization of the pectin; the con-

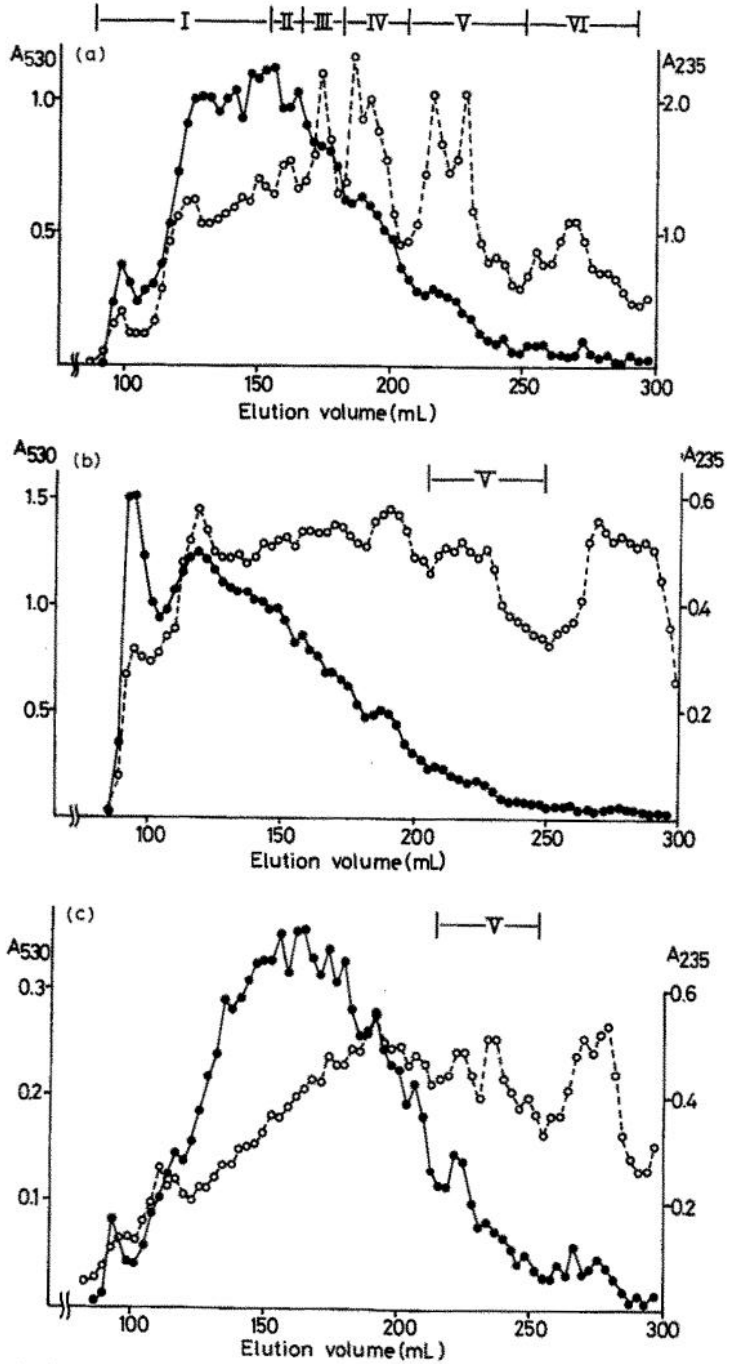


Fig. 1. Profiles of gel-filtration, on Toyopearl HW-40S, of the reaction products (methyl ester) of the purified pectin treated with diazomethane in diethyl ether at 25° under various reaction conditions: A_{530} (—●—●—) and A_{235} (--○--○--). The purified pectin was treated twice with diazomethane (a) in the presence, or (b) in the absence, of 0.1M phosphate buffer (pH 8.0). (8c) The purified pectin in the buffer was treated twice with diazomethane with intermittent esterification.

centration employed in this experiment (25 mg of pectin/mL of buffer) gave the maximum amount of depolymerization.

To evaluate the effect of the electrolytes (disodium hydrogenphosphate and potassium dihydrogenphosphate) on the depolymerization, a solution of the purified pectin in water alone was treated twice with diazomethane as described above. The gel-filtration diagram of the reaction product on Toyopearl HW-40S (Fig. 1b) shows a degree of depolymerization of the polysaccharide lower than that in the presence of the electrolytes, indicating a marked accelerating effect of the electrolytes on the β-elimination induced by diazomethane.

It seemed meaningful to know the difference in the degree of depolymerization between carboxyl- and ester-type pectin. Since complete esterifica-

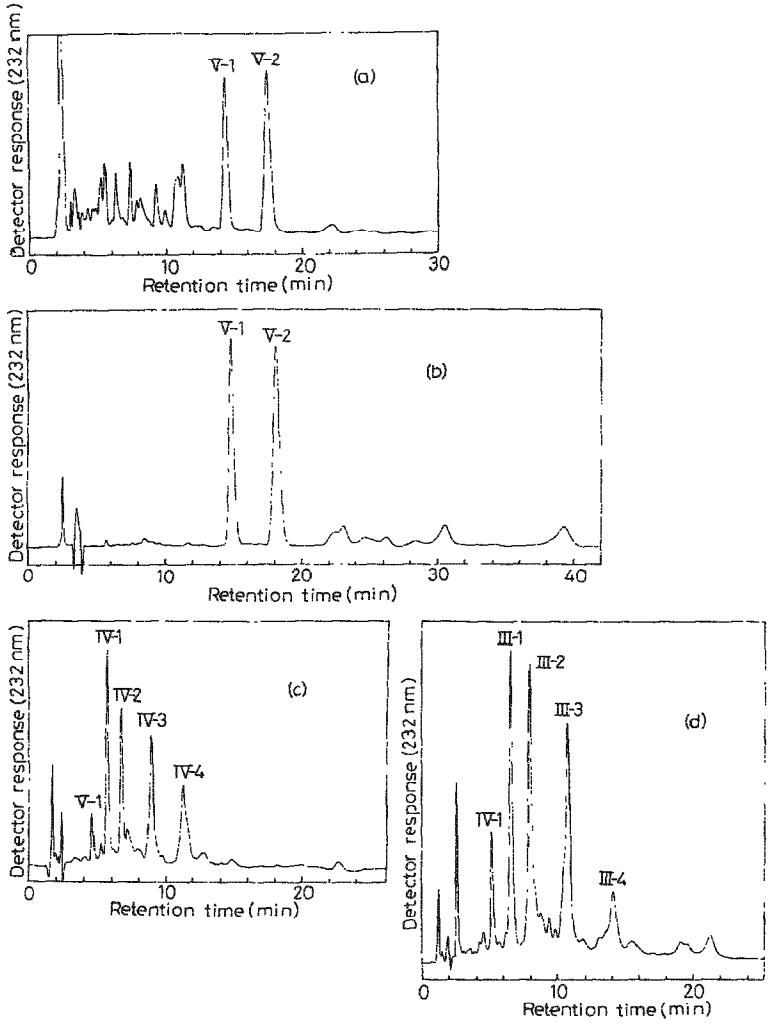


Fig. 2. Liquid chromatography of: (a) Fraction V of Fig. 1a, before permethylation; (b) Fraction V; (c) Faction IV; and (d) Fraction III of Fig. 1a, after permethylation.

tion of pectin without molecular size changes was thought to be difficult, the effect of methyl esterification on the second diazomethane treatment was examined. A solution of the purified pectin in 0.1M phosphate buffer (pH 8.0) was initially treated (~6 h, 25°) with diazomethane in diethyl ether and the reaction product was esterified with diazomethane under anhydrous conditions. The resulting product was again subjected to the diazomethane treatment (~20 h, 25°; see Experimental). The gel-filtration diagram of the final reaction product on Toyopearl HW-40S (Fig. 1c) showed that the methyl ester of the pectin, partially depolymerized by the initial diazomethane treatment, was slightly more effectively depolymerized in the subsequent diazomethane treatment than was the carboxyl type (Fig. 1a). The results illustrated in Figs. 1a and 1c suggested that esterification of the uronic acid residues in pectin is helpful for β-elimination with diazomethane but not essential, in contrast to the conventional base-catalysed β-elimination[7].

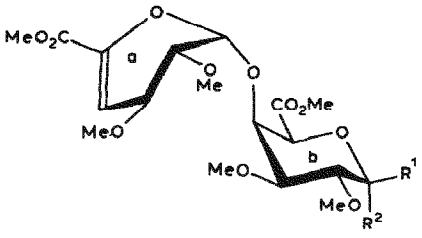
Fraction V (Fig. 1a; a mixture of methylated di- and tri-saccharides) was separated and lyophilized, and an aliquot was subjected to reversed-phase l.c. on a Hitachi 3056 ODS column with 30% acetonitrile–water (Fig. 2a). The remainder of Fraction V was dissolved in dry dichloromethane and permethylated with diazomethane–boron trifluoride etherate. The permethylated product obtained was subjected to reversed-phase l.c. on the same column (Fig. 2b). The methods of Hakomori[8], and Kuhn and Trischmann[9] were found to be unsuitable for permethylation of Fraction V, as indicated by formation of unknown compounds having a low molecular-weight. The results illustrated in Figs. 2a and 2b indicated

TABLE I

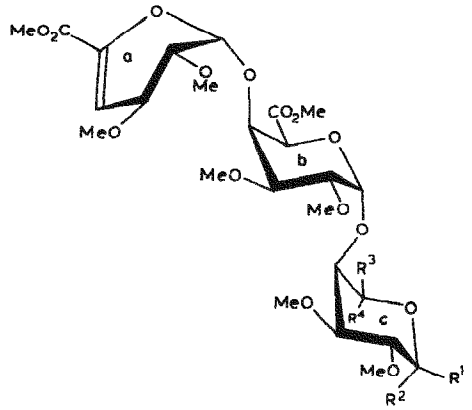
^{1}H-N.M.R. DATA (δ) FOR COMPOUNDS OF PEAKS V-1 (1) AND V-2 (2)[a]

Monosaccharide unit[b]	*H-*	*Compound of Peak*	
		V-1	*V-2*
a	1	5.14 ($J_{1,2}$ 2.4)	5.09 ($J_{1,2}$ 2.0)
	2	3.45 ($J_{2,3}$ 7.8)	3.43 ($J_{2,3}$ 7.4)
	3	4.10 ($J_{3,4}$ 3.0)	4.06 ($J_{3,4}$ 3.0)
	4	6.10	6.10
b	1	4.99 ($J_{1,2}$ 3.2)	4.15 ($J_{1,2}$ 7.6)
	2	3.51 ($J_{2,3}$ 10.5)	3.19 ($J_{2,3}$ 10.0)
	3	3.56 ($J_{3,4}$ 3.0)	3.14 ($J_{3,4}$ 3.0)
	4	4.63 ($J_{4,5}$ 1.2)	4.56 ($J_{4,5}$ 0.8)
	5	4.38	4.05
Methoxyl group[c]		3.42, 3.45, 3.46 3.47, 3.49	3.44 (6 H), 3.49 3.54, 3.59
Methyl ester group[c]		3.78, 3.83	3.79, 3.82

[a]Coupling constants in Hz. [b]See structures **1** and **2**. [c]All the signals showed the intensity corresponding to three protons unless otherwise stated.



1 R^1 = H, R^2 = OMe
2 R^1 = OMe, R^2 = H



3 R^1 = R^4 = H, R^2 = OMe, R^3 = CO_2Me
4 R^1 = OMe, R^2 = R^4 = H, R^3 = CO_2Me
5 R^1 = R^3 = H, R^2 = OMe, R^4 = CO_2Me
6 R^1 = OMe, R^2 = R^3 = H, R^4 = CO_2Me

that diazomethane–boron trifluoride etherate was an excellent reagent for permethylation of these partially methylated oligosaccharides. The recovered Peaks V-1 and V-2 (Fig. 2b) were further purified by adsorption l.c. on a Unisil Q 100-5 column with 1:10:10 (v/v) for Peak V-1 and 3:40:40 (v/v) methanol–ethyl acetate–hexane for Peak V-2 as eluents, respectively. As listed in Table I, the data of ^{1}H-n.m.r. spectral assignment by use of COSY-45, two-dimensional, homonuclear-correlation experiments indicated, for the purified compounds of Peaks V-1 and V-2, structures **1** and **2**, respectively.

A comparison of Fig. 2a (before permethylation, Fraction V in Fig. 1a) with Fig. 2b (after permethylation, Fraction V in Fig. 1a) revealed the disappearance of several small peaks eluted before Peak V-1, in Fig. 2a, and an increase of the peak areas of Peaks V-1 and V-2, and of the peaks eluted after Peak V-2, in Fig. 2b. The peaks eluted after Peak V-2 were assumed to contain permethylated trisaccharides, as judged from the retention times on l.c. Accordingly, the peaks eluted before Peak V-1, in Fig. 2a, probably consisted of mixtures of partially methylated di- and tri-saccharides. Based on these observations, Fraction V, in Fig. 1a, was calculated to be a mixture of disaccharides (61%) and trisaccharides (39%), and 65% of the disaccharides were shown to be fully methylated by the present diazomethane treatment. Assuming that the partially methylated disaccharides contain one free hydroxyl group per molecule, the degree of methylation of the hydroxyl group of the total disaccharides was ~93%.

Comparison of the distribution of the areas of the peaks or peak groups separated by l.c. of Fraction V of Fig. 1b, with that of Fraction V of Fig. 1a (Table II) showed that the degree of methylation in the absence of the electrolytes was lower than that in the presence of the electrolytes. Therefore, the electrolytes

TABLE II

SEPARATION BY REVERSED-PHASE L.C., OF FRACTION V[a] OBTAINED BY DIAZOMETHANE TREATMENT UNDER VARIOUS REACTION CONDITIONS

Fraction V of	*Distribution of area (%)[b] of*			
	Peaks eluted before Peak V-1	*Peak V-1*	*Peak V-2*	*Peaks eluted after Peak V-2*
Fig. 1 (a), before permethylation[c]	53.1	17.0	22.1	7.9
Fig. 1 (a), after permethylation[d]	0	27.0	33.4	39.6
Fig. 1 (b), before permethylation	75.4	3.4	3.5	17.7
Fig. 1 (c), before permethylation	52.5	15.6	17.2	14.7

[a]A mixture of di- and tri-saccharides. [b]Calculated from the area of each peak (or group of peaks) obtained by l.c. of each Fraction V on a Hitachi 3056 ODS column (see Figs. 2a and 2b). [c]See Fig. 2a. [d]See Fig. 2b.

accelerated not only the β-elimination cleavage of the glycosyl linkage (see Figs. 1a and 1b), but also the rate of methylation of hydroxyl groups in the polysaccharide.

Fraction IV (trisaccharides; Fig. 1a) was permethylated with diazomethane–boron trifluoride etherate. Separation by reversed-phase l.c. on a Hitachi 3056 ODS column gave four distinct peaks (Peaks IV-1–4, Fig. 2c) besides a small peak

TABLE III

L.C. AND T.L.C. DATA FOR COMPOUNDS OF PEAKS III-1–4 AND IV-1–4

Peak on l.c.	*L.c. (R_T)[a]*	*T.l.c. (R_F)[b]*
III-1	7.08 (1.00)[c]	0.20 (1.00)[d]
III-2	8.50 (1.20)	0.28 (1.38)
III-3	11.32 (1.60)	0.38 (1.88)
III-4	14.17 (2.00)	0.31 (1.56)
IV-1	5.68 (1.00)	0.33 (1.00)
IV-2	6.76 (1.19)	0.40 (1.20)
IV-3	8.96 (1.58)	0.51 (1.54)
IV-4	11.40 (2.00)	0.45 (1.34)

[a]Retention time (in min) by l.c. on a Hitachi 3056 ODS column, as described in the Experimental section. [b]R_F value by t.l.c. on a silica gel plate with 3:47 (v/v) methanol–ethyl acetate. [c]In parentheses, retention time relative to that of the compounds of Peak III-1 or IV-1. [d]R_F value relative to that of the compounds of Peak III-1 or IV-1.

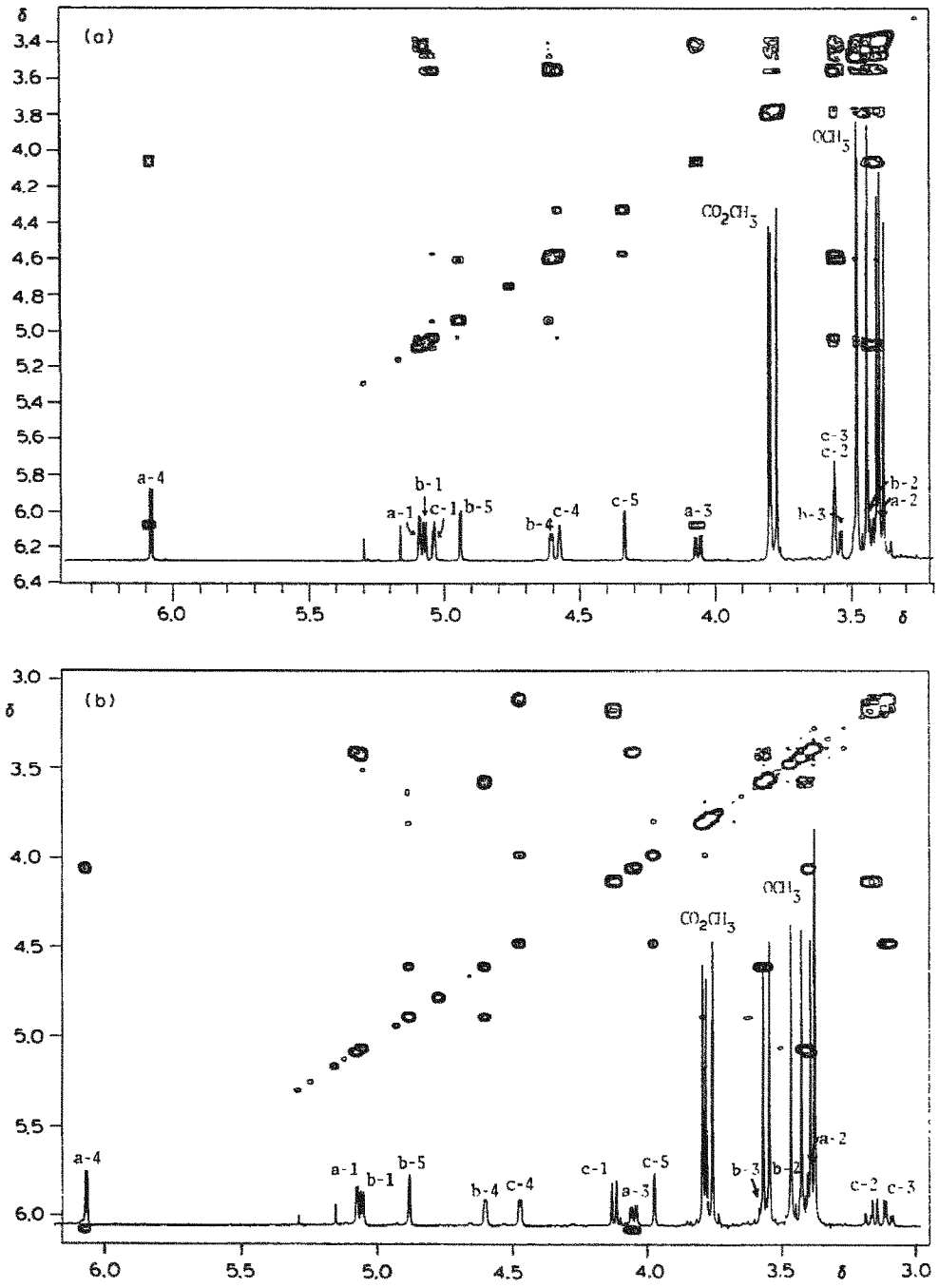

(a)
δ
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
5.0
5.2
5.4
5.6
5.8
6.0
6.2
6.4
OCH3
CO2CH3
a-4
b-1
a-1
c-1
b-5
b-4
c-4
c-5
a-3
c-3
c-2
b-3
b-2
a-2
6.0
5.5
5.0
4.5
4.0
3.5
δ
(b)
3.0
3.5
4.0
4.5
5.0
5.5
6.0
OCH3
CO2CH3
a-4
a-1
b-1
b-5
b-4
c-4
c-1
a-3
c-5
b-3
b-2
a-2
c-2
c-3
6.0
5.5
5.0
4.5
4.0
3.5
δ
3.0

Fig. 3.

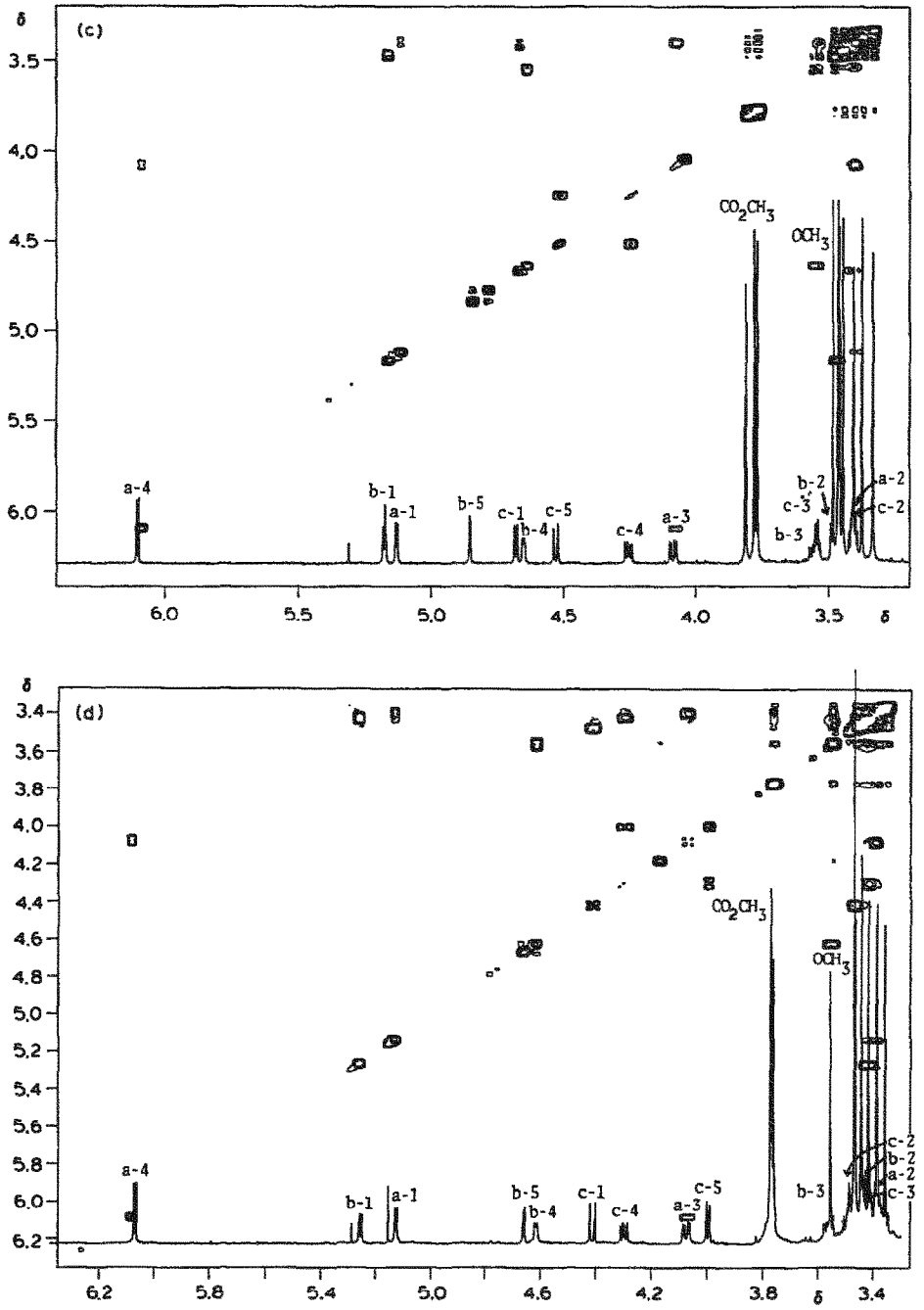


Fig. 3. ^{1}H-shift-correlated, 2-D n.m.r. (COSY) experiments of: (a) compound of Peak IV-1 (**3**), (b) compound of Peak IV-2 (**4**), (c) compound of Peak IV-3 (**5**), and (d) compound of Peak IV-4 (**6**) for solutions in (^{2}H)chloroform at 19°. Chemical shifts (δ) and coupling constants are given in Table IV.

due to Peak V-1 material. Each compound recovered from the column was further purified by adsorption l.c. on a Unisil Q 100-5 column with 1:10:10 (v/v) methanol–ethyl acetate–hexane. Each of the purified compounds migrated as a single spot on a silica gel t.l.c. plate (Table III).

The ^{1}H-n.m.r. spectrum of Peak IV-2 material at 400 MHz was assigned with reference to the signal of the olefinic proton at δ 6.08 by use of a COSY-45, two-dimensional, homonuclear-correlation experiment (Fig. 3b). Assignment of the ring protons overlapping the methoxyl protons and determination of the scalar coupling-constants between the ring protons were successfully performed by use of a two-dimensional, *J*-resolved method. The spectral assignment of Peak IV-2 was consistent with the structure of a trisaccharide (**4**), which corresponds to a homologous trisaccharide containing one more additional D-galacturonic acid residue than disaccharide **2**. The chemical shifts and coupling constants for the ring protons of the monosaccharide units a and b of the compound of Peak IV-1 (Fig. 3a) closely resemble those of the compound of Peak IV-2, and the chemical shifts

TABLE IV

^{1}H-N.M.R. DATA (δ) FOR THE COMPOUNDS OF PEAKS IV-1–4 (**3–6**)[a]

Monosaccharide unit[b]	*H-*	*Compound of Peak*			
		IV-1	*IV-2*	*IV-3*	*IV-4*
a	1	5.09 ($J_{1,2}$ 2.8)	5.09 ($J_{1,2}$ 2.3)	5.12 ($J_{1,2}$ 2.5)	5.14 ($J_{1,2}$ 2.5)
	2	3.40 ($J_{2,3}$ 7.5)	3.41 ($J_{2,3}$ 7.6)	3.41 ($J_{2,3}$ 7.6)	3.40 ($J_{2,3}$ 7.6)
	3	4.06 ($J_{3,4}$ 2.8)	4.07 ($J_{3,4}$ 2.4)	4.08 ($J_{3,4}$ 2.8)	4.08 ($J_{3,4}$ 2.8)
	4	6.08	6.08	6.09	6.09
b	1	5.07 ($J_{1,2}$ 4.0)	5.07 ($J_{1,2}$ 3.9)	5.17 ($J_{1,2}$ 3.5)	5.27 ($J_{1,2}$ 3.6)
	2	3.41 ($J_{2,3}$ 10.5)	3.43 ($J_{2,3}$ 10.5)	3.48 ($J_{2,3}$ 10.5)	3.43 ($J_{2,3}$ 10.2)
	3	3.55 ($J_{3,4}$ 2.8)	3.58 ($J_{3,4}$ 3.0)	3.56 ($J_{3,4}$ 3.0)	3.56 ($J_{3,4}$ 3.1)
	4	4.61 ($J_{4,5}$ 1.2)	4.61 ($J_{4,5}$ 1.2)	4.64 ($J_{4,5}$ 0.8)	4.62 ($J_{4,5}$ 1.2)
	5	4.94	4.89	4.84	4.67
c	1	5.04[c]	4.13 ($J_{1,2}$ 7.2)	4.67 ($J_{1,2}$ 3.8)	4.42 ($J_{1,2}$ 7.2)
	2	3.56 (2 H)[c]	3.18 ($J_{2,3}$ 10.0)	3.41 ($J_{2,3}$ 7.5)	3.48 ($J_{2,3}$ <2)
	3		3.12 ($J_{3,4}$ 3.6)	3.55 ($J_{3,4}$ 3.0)	3.43 ($J_{3,4}$ 6.2)
	4	4.58[c]	4.48 ($J_{4,5}$ 1.2)	4.25 ($J_{4,5}$ 6.4)	4.30 ($J_{4,5}$ 4.2)
	5	4.34[c]	4.00	4.52	4.00
Methoxyl group[d]		3.39, 3.40.3.41	3.39 (6 H), 3.41	3.34, 3.38, 3.41	3.36, 3.39, 3.42
		3.44, 3.45	3.44, 3.48, 3.56	3.45, 3.46, 3.47	3.45, 3.47 (6 H)
		3.48 (6 H)	3.59	3.48	3.56
Methyl ester group[d]		3.77, 3.80 (6 H)	3.77, 3.80, 3.81	3.77, 3.78, 3.81	3.77, 3.78, 3.79

[a]Coupling constants in Hz. [b]See structures **3–6**. [c]Broad singlet. [d]All the signals showed the intensity corresponding to three protons unless otherwise stated.

for the ring protons of the monosaccharide unit c of the compound of Peak IV-1 were similar to those of the monosaccharide unit b of the compound of Peak V-1, although all the ring protons of the monosaccharide unit c of the compound of Peak IV-1 showed broad-singlet signals* (Table IV). Based on these results, the structure of the compound in Peak IV-1 was assigned that of trisaccharide **3**, the α-D anomer of **4**.

The signals of the ^{1}H-n.m.r. spectra of the compounds in Peaks IV-3 and IV-4 were assigned by use of homonuclear, spin-decoupling experiments; COSY-45, two-dimensional, homonuclear-correlation experiments; and two-dimensional, *J*-resolved methods (Figs. 3c and 3d). The chemical shifts and coupling constants for the ring protons of the monosaccharide units a and b of the compounds in Peaks IV-3 and IV-4 were similar to those of the compounds in Peaks IV-1 and IV-2. Among other signals, a doublet at δ 4.67 for the compound of Peak IV-3 (at δ 4.42 for the compound of Peak IV-4) could be assigned to either H-1 or H-5 of monosaccharide unit c. Two-dimensional, ^{13}C–^{1}H-correlation experiments for the com-

TABLE V

^{13}C-N.M.R. DATA (δ) FOR COMPOUNDS OF PEAKS IV-3 (**5**) AND IV-4 (**6**)[a]

Monosaccharide unit	*H-*	*Compound of Peak*	
		IV-3	*IV-4*
a	1	98.93	98.90
	2	77.84	77.86
	3	74.75	74.62
	4	109.50	109.37
b	1	97.86	97.61
	2	76.34	76.9[b]
	3	77.52	77.59
	4	75.30	75.44
	5	70.60	70.58
c	1	100.80	105.17
	2	76.8[b]	79.46
	3	77.98	79.46
	4	74.80	78.10
	5	69.90	80.74

[a]The signals for carboxylic carbon atoms were omitted. The signal for C-5 of the monosaccharide unit a was not observed. [b]Calculated from ^{13}C–^{1}H correlation, because of overlapping with the signal of chloroform.

*In order to explain the appearance of the broad-singlet signals, the ^{1}H-n.m.r. spectra of the compound of Peak IV-1 were recorded at low temperatures (down to −40°) and at high temperatures (up to 50°). The variable-temperature ^{1}H-n.m.r. spectra showed no evidence for a possible conformational equilibrium between $^4C_1$ and $^1C_4$(L) forms for monosaccharide unit c.

TABLE VI

COUPLING CONSTANTS (Hz) CALCULATED FOR $^1C_4$ AND $^4C_1$(L) CONFORMERS OF L-ALTROSE[a]

Anomer	*Conformer*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
β	$^1C_4$	1.3	3.6	3.1	9.8
	$^4C_1$	3.6	10.3	3.1	1.3
	$^1C_4$:$^4C_1$ = 1:1	2.5	7.0	3.1	5.6
α	$^1C_4$	1.8	3.6	3.1	9.8
	$^4C_1$	7.8	10.3	3.1	1.3

[a]Calculated according to the additivity rule derived by Altona and Haasnoot[10].

pounds in Peaks IV-3 and IV-4 were used to facilitate an unambiguous assignment of the signals (Table V). In the spectrum of the compound in Peak IV-3, the signals at δ 97.86 and 98.93, located in the region of the chemical shift for C-1, were correlated with the signals for the anomeric protons of monosaccharide units a and b, respectively. Therefore, another signal at δ 100.80, in the region of the chemical shift for C-1, was assigned to C-1 of monosaccharide unit c, which was correlated with H-1 of monosaccharide unit c at δ 4.67. The remaining signals for monosaccharide unit c were assigned by use of a COSY-45, two-dimensional, homonuclear-correlation experiment (Table IV). The ring protons of monosaccharide unit c of the compound in Peak IV-4 were also assigned in a manner similar to those for the compound in Peak IV-3.

The large values for $J_{4,5}$ of each monosaccharide unit c of the compounds in Peaks IV-3 and IV-4, as compared with the small value for that of the D-galacturonic acid residue, suggested the possible conversion of D-galacturonic acid into L-altruronic acid. To confirm this, the coupling constants for $^1C_4$, and $^4C_1$(L) conformers of L-altrose were calculated according to the additivity rule derived by Altona and Haasnoot[10], although no predictive data for the CO_2^- substituent at C-5 were available (Table VI). The observed coupling constants for the ring protons of monosaccharide unit c of the compound in Peak IV-3 (Table IV) were close to those calculated for a conformational mixture of equal amount of $^1C_4$ and $^4C_1$(L) forms. From these results, the structure of the trisaccharide of Peak IV-3 was tentatively assigned as **5**, an epimer at C-5 of the monosaccharide unit c of trisaccharide **3**, and structure **6**, the α-L anomer of trisaccharide **5**, was likewise assigned to the trisaccharide of Peak IV-4. However, the observed coupling constants for the ring protons of monosaccharide unit c of the compound in Peak IV-4 were inconsistent with the calculated values for any conformational mixture of the conformers. Thus, the monosaccharide unit c of trisaccharide **6** was assumed to be in a boat or twist-boat conformation, based on the observed coupling constants.

Fraction III of Fig. 1a was permethylated with diazomethane–boron trifluoride etherate. Separation of permethylated Fraction III by reversed-phase l.c. on a Hitachi 3056 ODS column gave four distinct peaks (Peak III-1–4), besides

another peak due to Peak IV-1 material (Fig. 2d). The retention times (l.c.) and R_F values (t.l.c.) of Peak III-2–4 compounds, relative to those of the Peak III-1 compound, were similar in relation to the values obtained for those of Peak IV-2–4 (Table III). Furthermore, measurement of field-desorption, mass spectrum indicated a mol. wt. of 886 for the Peak III-2 material. These results suggested that the compounds in Peak III-1–4 were homologous tetrasaccharides; namely, trisaccharides **3–6** plus one D-galacturonic acid residue, respectively. The results also suggested the possible formation, by the diazomethane treatment, of higher-mol. wt. oligosaccharides containing an L-altruronic acid residue, besides a D-galacturonic acid residue, at the reducing end. It was also assumed that the addition of a proton to the carbanion produced by the removal of the proton located at C-5 of the D-galacturonic acid residue resulted in the formation of the L-altruronic acid residue. This conversion would be closely related to the β-elimination cleavage of the polysaccharide chain with diazomethane, as an L-altruronic acid residue was found only at the reducing end of the oligosaccharides.

Di- (Peaks V-1 and -2 of Fig. 2b), tri- (Peaks IV-1–4 of Fig. 2c), and tetrasaccharides (Peaks III-1–4 of Fig. 2d) were isolated only in low yields (1–2 mg of each oligosaccharide from 200 mg of starting material). No monosaccharide could be isolated, and disaccharides containing an L-altruronic acid residue were absent. On the other hand, the color yield of the carbazole reaction on the products of the gel-filtration (Fig. 1a) was much lower than that expected from the amount of pectin employed, even when the reduction in color intensity due to formation of the double bond and methylation of the hydroxyl group in the polysaccharide was considered. On the basis of these results, it was assumed that the glycosyl linkages near the reducing end of the oligosaccharides initially formed by β-elimination cleavage were susceptible to the diazomethane treatment, and that most of the monosaccharides and a part of the disaccharides produced (probably the disaccharides containing an L-altruronic acid residue) had decomposed, before being stabilized by methyl glycosidation, into unknown substances not reacting with carbazole.

The results obtained in the present study provide some information on the mechanism of pectin degradation by diazomethane treatment, although identification of L-altruronic acid is still tentative. The yields of oligosaccharides obtained by the diazomethane treatment were so low (~40 mg from 200 mg of starting pectin) that the usefulness of this procedure as a method for depolymerization of pectin and production of oligosaccharides is rather limited. However, as shown in the results obtained by diazomethane treatment of several glycosaminoglycans[1,2], the yields of methylated, even-numbered oligosaccharides having the hexosamine residue at the reducing end were average (~255 mg from 500 mg of starting heparin). Therefore, it is suggested that diazomethane treatment of some heteropolysaccharides containing both uronic acid and neutral monosaccharide residues may give oligosaccharide blocks by cleaving the glycosyl linkage at the β-position of the uronic acid carboxyl group in the main chain of the poly-

saccharides. It is also emphasized that the cleavage can be carried out in aqueous medium, which is a clear advantage over the conventional β-elimination procedure in organic solvent.

EXPERIMENTAL

Materials. — Pectin from citrus fruits and pectinesterase (E.C. 3.1.1.11) from tomato were obtained from Sigma Chemical Co. (St. Louis, MO 63178). Diazomethane in diethyl ether was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[11], obtained from Tokyo Kasei Kogyo Co. (Tokyo). Boron trifluoride etherate, purchased from Wako Pure Chemical Industries, Ltd., Osaka, was purified by distillation. Toyopearl HW-40S (polymerized hydrophilic vinyl monomer, a packing material for gel-filtration) was purchased from Toyo Soda Kogyo Co. (Tokyo).

Methods. — Uronic acid content was determined by the method of Bitter and Muir[12] using D-galacturonic acid as a standard. Neutral sugar composition was determined by g.l.c. analysis of alditol acetates obtained by hydrolysis (0.5M H_2SO_4, 16 h, 100°), reduction, and acetylation (1:1, v/v, acetic anhydride–pyridine, 1 h, 100°; ref. 13). Methyl ester content of purified pectin was determined by the method of Wood and Siddiqui[14]. T.l.c. was performed on silica gel plates with 3:47 (v/v) methanol–ethyl acetate. The plates were sprayed with 0.5% orcinol in 2M H_2SO_4. G.l.c. was performed with a Shimadzu gas chromatograph GC-4BM equipped with a flame-ionization detector and a glass column (0.4 × 200 cm) packed with 3% of ECNSS-M on Gas-Chrom Q (100–120 mesh), and operated under the conditions described[15]. Liquid chromatography (l.c.) was performed with a chromatographic apparatus equipped with a liquid delivery pump (Milton-Roy, SF-0396, Atto Co., Tokyo) and a variable-wavelength u.v. detector (NS-310A, Nihonseimitsu Co., Tokyo). ^{1}H-N.m.r. spectra were recorded with a Varian XL-400 n.m.r. spectrometer at 19°, for solutions in (^{2}H)chloroform. Chemical shifts (δ) were expressed relative to chloroform as internal standard (δ 7.26). Mass spectra were recorded with a JEOL JMS DX-300 mass spectrometer.

Fractionation of commercial pectin. — A solution of commercial pectin (1 g) in water (150 mL) was applied to a DEAE-Sephadex A-50 (HCO_3^-) column (5 × 46 cm). The column was eluted at 4° successively with water, M formic acid and 3M formic acid, and 0.1M HCl (1.5 L each). Carbohydrate content was monitored by the phenol–H_2SO_4 method[16]. Fractions eluted with 3M formic acid were pooled, dialyzed against tap water for 48 h at 4°, and then against distilled water for 24 h at 4°. Lyophilization of the dialyzate gave 620 mg of purified pectin. The purified pectin was dissolved in water (40 mL) and the solution was poured dropwise into ethanol (160 mL) at 4° to give a fine powder. The powder was collected by centrifugation, dried in air, and then *in vacuo* (P_2O_5).

Depolymerization and methylation of purified pectin with diazomethane in the presence of electrolytes (KH_2PO_4 and Na_2HPO_4). — To a solution of purified pectin

(50 mg) in 0.1M phosphate buffer (pH 8.0; 2.0 mL), in a reaction vessel equipped with a condenser through which cold water (~10°) was circulated, was added a solution of diazomethane in diethyl ether (~60 mL) freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (10 g). The mixture was vigorously stirred at 25° until the yellow color of the reagent had disappeared (for ~6 h). Both diethyl ether and water were evaporated, and the residue, dissolved in water (1.0 mL), was treated again with the diazomethane solution (~60 mL) for ~20 h. After evaporation of diethyl ether, the residue was extracted successively with methanol (5 × 5 mL) and water (2 × 5 mL). The combined extracts were concentrated to a small volume, *in vacuo*, and applied to a Dowex 50W-X2 (H^+) column (1.0 × 13 cm). The column was eluted with water and the eluate was lyophilized. The residue was dissolved in dry methanol (10 mL) and esterified with diazomethane in diethyl ether (15 mL) with stirring for 1 h at −3°. After evaporation of the solvent under reduced pressure, the residue was dissolved in water (~2 mL) and the solution applied to a Toyopearl HW-40S column (2.6 × 60 cm). The column was eluted with water at a flow rate of 0.8 mL/min. The eluate was collected in 3.0-mL fractions, each fraction was analyzed for uronic acid content and for absorbance at 235 nm, and the fractions were pooled as indicated in Fig. 1a. Each of Fractions I–VI (obtained from four experiments on the scale just described) was separately pooled and lyophilized.

Permethylation of Fractions III–V with diazomethane–boron trifluoride etherate[17]. — To a solution of Fraction V in dry dichloromethane (1.0 mL) were added a solution of diazomethane in dichloromethane (6 mL), freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (1 g), and 0.1 mL of a catalyst stock solution which contained freshly distilled boron trifluoride etherate (0.1 mL) in dry dichloromethane (10 mL). The solution was stirred for 5 min at −5°. Excess diazomethane and dichloromethane were evaporated, and the residue was dissolved in methanol (1.0 mL) and applied to a Sephadex LH-20 column (1.2 × 82 cm). The column was eluted with methanol. The eluate was collected in 2.0-mL fractions and each fraction was analyzed for carbohydrate by t.l.c. The fractions positive to the orcinol–H_2SO_4 reagent were combined and concentrated to dryness under reduced pressure.

Fractions III and IV were permethylated by the same procedure as described above.

L.c. separation of permethylated Fractions III–V. — Each of the permethylated Fractions III–V was separated into its component by l.c. using a column (4.6 × 250 mm) packed with Hitachi 3056 ODS (4–6 μm), with 30% acetonitrile in water for permethylated Fractions V, 40% acetonitrile in water for permethylated Fraction IV, and 43% acetonitrile in water for permethylated Fraction III, at 50°, with a flow rate of 1.1 mL/min. Peaks V-1, 2 and IV-1–4 obtained by separation of permethylated Fractions V and IV, respectively, were further purified by l.c. using a column (4.6 × 250 mm) packed with Unisil Q 100-5 (5 μm), with 1:10:10 (v/v) methanol–ethyl acetate–hexane for Peaks V-1 and IV-1–4, and 3:40:40 (v/v)

methanol–ethyl acetate–hexane for Peak V-2, respectively, at room temperature, with a flow rate of 0.9 mL/min. The eluates were analyzed for absorbance at 232 nm.

Depolymerization of purified pectin with diazomethane in the absence of electrolytes. — To a solution of purified pectin (25 mg) in water (1.0 mL) was added a solution of diazomethane in diethyl ether (~30 mL) freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (5 g). The mixture was treated by a method virtually identical with that described for the reaction in the presence of phosphate buffer.

Repeated diazomethane treatment of pectin, accompanied by intermittent esterification. — Purified pectin (25 mg) in 0.1M phosphate buffer (pH 8.0; 1.0 mL) was initially treated (~6 h, 25°) with diazomethane in diethyl ether (~30 mL) according to the procedure described in *Depolymerization and methylation of purified pectin with diazomethane in the presence of electrolytes.* Diethyl ether was evaporated and the residue applied to a Dowex 50W-X2 (H^+) column (1.0 × 6.0 cm). The column was eluted with water. The eluate was lyophilized and the residue dissolved in dry methanol (10 mL). The solution was stirred with diazomethane in diethyl ether (15 mL) for 1 h at −3°. After evaporation of the solvent, the residue was dissolved in water (1 mL) and the solution applied to a Sephadex G-15 column (1 × 62 cm). The column was eluted with 10% ethanol in water, and the fractions having absorbance at 235 nm were combined and lyophilized. The residue was dissolved in 0.1M phosphate buffer (pH 8.0; 0.5 mL) and treated again (~20 h, 25°) with the diazomethane solution (~30 mL) in a manner similar to the first diazomethane treatment.

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