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Specific degradation of pectins via a carbodiimide-mediated Lossen rearrangement of methyl esterified galacturonic acid residues

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Abstract

A specific, chemical degradation of the methyl esterified galacturonic acid residues of pectins is described. These residues are converted, with hydroxylamine, to hydroxamic acids, and then, with a carbodiimide, to isoureas; the latter undergo a Lossen rearrangement on alkaline hydrolysis. The isocyanates formed are hydrolysed to 5-aminoarabinopyranose derivatives, which spontaneously ring open to give 1,5-dialdehydes. The latter are reduced, in situ, to avoid peeling reactions, with sodium borohydride to give substituted arabitol residues. Thus, overall, partially esterified pectins are specifically cleaved to generate a series of oligogalacturonic acids bearing an arabitol residue as aglycone. Analysis of oligomers so generated discloses the pattern of contiguous nonesterification in a variety of pectins of differing degrees of esterification. Other potential applications are described.

Keywords: Pectins; Specific degradation; Galacturonic acid; Lossen rearrangement; Methyl esterification; Carbodiimide

1. Introduction

Specific chemical degradation of polysaccharides has been a useful tool in structural determination.[1–3] The selective derivatisation and cleavage of uronic acid residues, and in particular of galacturonic acid residues, has received much attention, as these sugars are a major constituent of pectins that occur in plant cell walls. Pectins are important in cell wall assembly,[4] and in isolated form have proved useful in the food industry. An improved knowledge of their structure and its relationship to their physical properties would be of considerable application.

Selective cleavage of galacturonic acid residues in pectins allows, in particular, two problems to be addressed. Firstly, if the methyl galacturonic acid esters are selectively cleaved, the arrangement of unesterified residues can be determined, both in native pectins and in pectin methyl esterase-treated samples. This distribution is important, as it is thought to determine the gelling properties of the pectin.[5] Secondly, selective cleavage of unesterified galacturonic acid residues allows sidechains, known to be composed predominately of arabinose and/or galactose, to be released from the molecule.

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In principle, three properties of galacturonic acid residues might be exploited to selectively cleave pectins: (a) the resistance to hydrolysis of their glycosidic linkages compared with neutral sugars; (b) the propensity of esterified galacturonic acid residues to β-eliminate their 4-substituents; (c) their ability to undergo derivatisation selectively at their carboxyl groups. In principle, such derivatisation could promote ring opening and attendant glycosidic cleavage.

All three approaches have been applied to pectins. Mort[5] reduced the methyl esterified residues of pectins to galactose, which were then selectively removed by hydrolysis with anhydrous hydrogen fluoride (HF) at -20 °C to produce unesterified blocks of galacturonic acid, terminated by galactose. Analysis of the resulting mixtures gave the distribution of non-esterified residues. Although highly effective, the method has several disadvantages. Firstly, HF is a highly corrosive material which requires specialised, stringent handling procedures, and thus use of the method has been highly restricted. Secondly, the selectivity of the method is kinetic in nature; low levels of nonspecific galacturonic acid cleavage might be expected in long, acidic blocks, such as might be produced in studies of enzyme hydrolysates. Lastly, long sequences of α-(1 → 4)-linked galactose are rather insoluble.

Selective β-elimination of uronyl ester-containing molecules has been much studied and reviewed.[6] Unfortunately, ester hydrolysis competes under the basic conditions required and, with methyl esters, β-elimination is never quantitative (although conditions to maximise it have been developed[7]). Thus, repeated cycles of reesterification and hydrolysis are needed. β-Elimination is thus unsuited to determinations of unesterified residue distribution, but has been used, with some success, to liberate sidechains.[8]

So far, the most successful degradation, based on targeting the carboxyl groups of underivatised pectins, is a reductive cleavage using lithium in ethylenediamine. This was used to liberate sidechains from rhamnogalacturonan I (RG-1).[9] If esterified galacturonic residues were first reduced to galactosyl residues,[5] lithium in ethylenediamine would liberate blocks of galactose corresponding to the esterified regions of the pectin. However Lau et al.[10] demonstrated, using model compounds, that up to 10% of neutral glycosidic linkages are also cleaved by lithium in ethylenediamine, and a method of block distribution based on this scheme has not been reported.

At present, no enzymes are available to specifically degrade all of the galacturonic acid residues, either esterified or unesterified, in a pectin. For example, rhamnogalacturonases require the prior degradation of sidechains,[11] and the binding requirements of pectin and pectic lyases and galacturonases[12] preclude a direct assessment of acid-block distribution. However, a method for the characterisation of non-esterified galacturonic acid sequences, which uses endopolygalacturonase, has recently been described.[13] But as not all non-esterified residues are removed by the enzyme, and because the degradation products obtained reflect the binding propensity of the surrounding regions, interpretation is complicated.

A truly specific degradation of galacturonic acid residues was thus clearly a desirable tool. A Lossen rearrangement[14,15] seemed attractive (i.e., a chemical degradation of type (c) above), as it would, in principle, require no prior protection of the pectin. (Hydroxyl protection, usually by methylation or acetylation, is essential to the majority of classical chemical degradations of polysaccharides.[1–3]) Hoare et al.[16] reported the quantitative conversion of simple hydroxamic acids, via a Lossen rearrangement, to amines under mild conditions. This facility suggested that the procedure might be used to selectively degrade acidic polymers such as pectins.

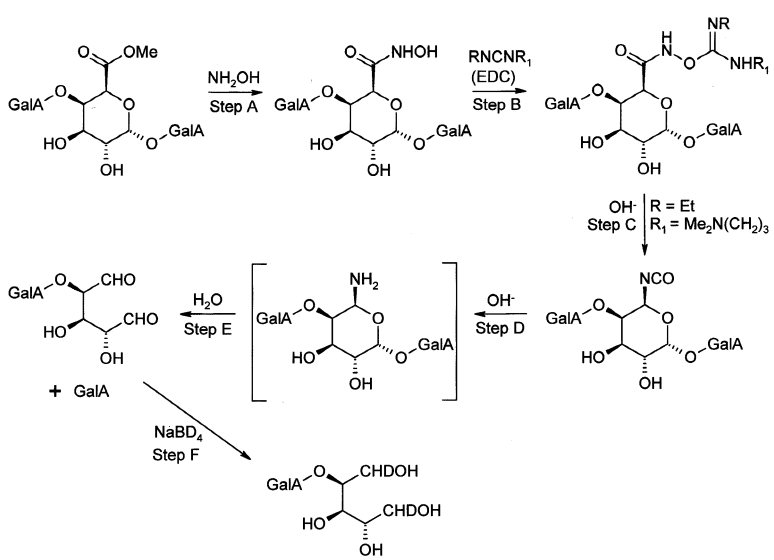
We envisaged generation of contiguous unesterified acidic blocks from pectins as follows (Scheme 1). Methyl esters would be converted into hydroxamic acids (Step A, Scheme 1). Simple methyl esters[17] and methyl esterified pectins[18] have been reported to give hydroxamic acids when treated with hydroxylamine under alkaline conditions. We anticipated (correctly, see below) that the latter would promote extensive β-elimination of pectins, and at least some deesterification (both pro-

cesses are base-catalysed[8]). However, we felt that successful substitution might be possible at, or near, neutral pH, with concentrated hydroxylamine solutions and extended reaction times, for two reasons. Firstly, uronyl esters are activated, by electron withdrawal by O-5, to nucleophilic attack. (For example they are reduced by sodium borohydride; the latter is incapable of reducing unactivated esters.[19]) Secondly, hydroxylamine is a powerful nucleophile, activated by an α-effect.[20]

After hydroxamic acid formation, we would remove excess hydroxylamine by dialysis. (This would prevent destruction of added carbodiimide in the next stage,[21] and undesired conversion of unesterified galacturonic acid residues to hydroxamic acids or *O*-acylhydroxylamines.[16]) Treatment with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) would, we anticipated, lead to an isourea derivative (Step B, Scheme 1), which would undergo Lossen rearrangement to an isocyanate (Step C, Scheme 1). Hydrolysis would be expected to cleave the latter to a hemiaminal (Step D, Scheme 1), which should spontaneously ring open and release the aglycone, to yield a 1,5-dialdehyde (Step E, Scheme 1). The 1,5-dialdehyde could then be protected from base degradation by reduction, in situ, with borohydride (or borodeuteride; Step F, Scheme 1).

2. Results

Conversion of methyl esterified galacturonic acid residues to hydroxamic acid derivatives.—Alkaline treatment of pectins with hydroxylamine led to extensive β-elimination; but also to total deesterification. Treatment of a citrus pectin (Sigma), which had a degree of esterification (dm) of 89%, gave no detectable hydroxamic acid (i.e., no colour change with $FeCl_3$–HCl[18]); and methanol analysis of the product (i.e., hydrolysis of the material and quantification of the methanol produced by gas chromatography (GC)[22]) gave a dm of 0%. In contrast, treatment of a dm 60 citrus pectin (Sigma), in the Na form, with a 1 M solution of $NH_2OH{\cdot}HCl$, pH 7 for 4–5 days at 20 °C, yielded, after diafiltration, a cloudy white mixture which gave a colour reaction with $FeCl_3$–HCl, displayed an infra red (IR) absorption at 1664 cm^{-1} (ν_{CONHOH}), and contained $< 1\%$ residual methyl ester. This indicated quantitative conversion, as pectic esters are stable to hydrolysis at pH 7.[8] The degree of hydroxamic acid conversion (dh) was confirmed by titration of the product, in its acid form, with aqueous NaOH, to give its free galacturonic acid content; from this, a dh of 61% was calculated. (The dh was defined as the percentage of galacturonic residues converted to hydroxamic residues; if quantitative, it would equal the original dm.)



Scheme 1. Derivatisation and carbodiimide-promoted Lossen rearrangement of a methyl esterified galacturonic acid residue.

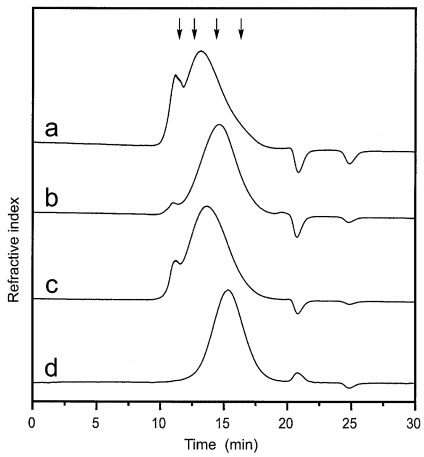


Fig. 1. Comparative HPSEC data (Shodex SB-804HQ column) for a 60 dm citrus pectin and some derivatives (all samples were desalted by diafiltration before examination). Pullanan standards (P-82, Shoua Denko) are, left to right 38.0×10^4, 18.6×10^4, 4.8×10^4, and 1.22×10^4. (a) 60 dm pectin; (b) after treatment with hydroxylamine at pH 7; (c) after treatment with hydroxylamine at pH 5.5; (d) after treatment with hydroxylamine at pH 7, and subsequent reaction with EDC.

Table 1
Percentage hydroxamic acid content of samples after hydroxylamine treatment

pH [a]	dm [b]	dh [c]	
		Titration [d]	CHN [e]
5.5	15	18	nd [f]
5.5	30	26	32
5.5	72	72	64
5.5	89	76	79
5.5	60	50	49
7	60	61	45

[a] pH of hydroxylamine used to prepare hydroxamic acid derivative.
[b] Degree of methyl esterification of starting pectin.
[c] Percentage hydroxamic acid content of sample after hydroxamic acid treatment.
[d] Percentage hydroxamic acid content determined by titration.
[e] Percentage hydroxamic acid content determined by combustion analysis.
[f] Not determined.

High-performance size-exclusion chromatography (HPSEC) of the product (Shodex 804HQ) indicated a slight M_r reduction (Fig. 1(a,b)). To minimise this presumed β-eliminative breakdown, we investigated using lower pH and more concentrated hydroxamic acid solutions. A randomly esterified dm 72 pectin (see below), was treated at 20 °C with 4.3 M hydroxylamine at pH 5, 5.5, and 6, and MeOH release was monitored daily by headspace sampling GC. At pH 6 and 5.5 the reaction reached maximum MeOH release after 4 and 5 days, respectively. The pH 5 reaction was only 75% complete after 5 days. The dh of the products was assessed by IR spectroscopy, after removal of hydroxylamine by diafiltration. The relative areas, both at pH 7 and 3, of ν_{CONHOH} at 1664 cm^{-1}, and ν_{COOH} at 1615 and 1740 cm^{-1}, respectively, were comparable for the pH 5.5 and 6 reactions, and greater than the pH 5 reaction even after 21 days.

Randomly esterified pectins, of dm 15, 30, and 72, were prepared by controlled alkaline hydrolysis of a 89 dm citrus pectin (Sigma),[23] and treated with hydroxylamine at pH 5.5 and 20 °C for 7 days. The products were isolated by diafiltration. Portions of each were converted to their acid form, and examined by titration; their dh was also assessed more directly by measuring nitrogen content by combustion analysis (Table 1). (The pectins, before conversion, were nitrogen free.) The data indicated, in general, conversion rates of 90–100%, although less complete for the highest dm pectin (85–89%); an extended reaction time might have been appropriate. Combustion analysis and titration data were generally in good agreement. The behaviour of the (authenticated) 60 dm pectin (Sigma) was anomalous (only 83% conversion by either assessment); the pH 7 conversion of this pectin was checked by combustion analysis and found to have a dh only 45%. This anomalous behaviour was not investigated further. The pH 5.5 product was examined, after dialysis, by HPSEC (Fig. 1(c)). Breakdown was less than at pH 7, consistent with less β-eliminative fragmentation. A final decision on the optimum condition was deferred until comparative fragmentation data was available (see below).

Conversion of hydroxamic acids to their EDC adducts and subsequent Lossen rearrangement. Verification, and characterisation of products.—A solution of the hydroxamic acid prepared from the dm 60 Sigma citrus pectin

at pH 7 was dialysed, and treated with EDC without any pH adjustment. After 1 h, and diafiltration (PM10 membrane, nominal exclusion limit 10 kDa), a $FeCl_3$–HCl test indicated no residual unsubstituted hydroxamic acid. HPSEC revealed some further breakdown, but little oligomeric material (Fig. 1(d)). The dialysate contained a small amount of oligomeric material; it was not further investigated, as fragmentations (described below) of dialysed and undialysed isoureas, were highly comparable (data not shown) and dialysis was avoided.

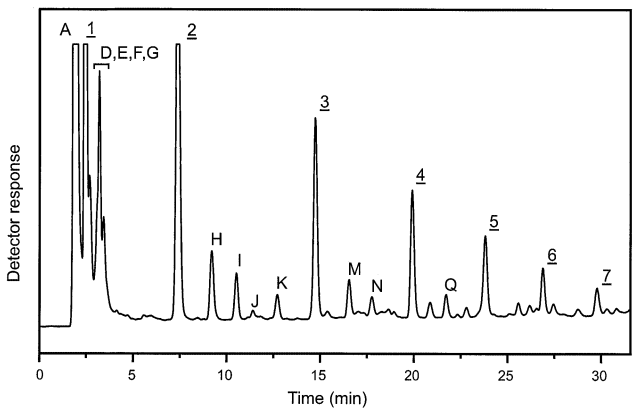


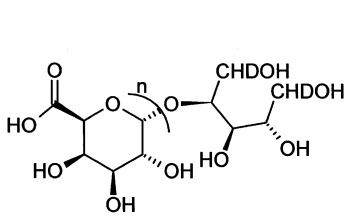
Fig. 2. HPAEC at pH 6 (Gradient A, see Section 4) of Lossen-rearranged and fragmented 60 dm pectin. The precise retention times of the oligomers D, E, F, G (see text, separated by preparative ion-exchange chromatography) was not determined.

Table 2
Observed m/z ratios (negative-ion ESI–MS) of the fragmentation products of a 60 dm pectin shown in Fig. 2

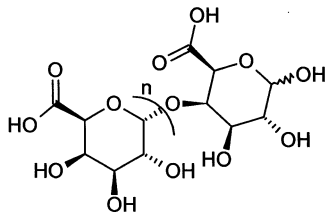
Component [a]	m/z		
	$z=-1$	$z=-2$	$z=-3$
A			
1	329		
2	505	252	
3	681	340	226.5
4	857	428	285
5	1033	516	347
F	193		
G	196		
I	369	184	
N	545	272	181
E	311		
K	487	243	
D	351		
H	527		
M	703	351	
Q	879	439	

[a] **1**–**5**, $(GalA)_n$-1,5-di-D-Araol, $n=1$–5; F, I, N, $(GalA)_n$GalA, $n=0$–3; E, K, GalenA$(GalA)_n$-1,5-di-D-Araol, $n=0$, 1; G, GalA-1-D-ol; D, H, M, Q, data fits series GalenA$(GalA)_n$, $n=1$–4 (but see text).

Treatment of the PM10-dialysed sample with 0.1 M NaOH + 0.2 M $NaBD_4$ at 100 °C gave extensive breakdown; all products which were included on a UHG120 HPSEC column (nominal exclusion limit 2 kDa). After desalting, high-performance anion-exchange chromatography (HPAEC), at pH 6, revealed the oligomeric series shown in Fig. 2. The marked components were separated by preparative anion-exchange chromatography on Q Sepharose, desalted, and examined by electrospray-ionisation mass spectroscopy (ESI-MS). Peak A exhibited the expected $[M+Na]^+$ ion at m/z 177 for a dideuteropentitol residue (Table 2 and Fig. 3). Peak A was acetylated and examined by gas-chromatography–electron impact mass spectroscopy (GC–EIMS). The retention time of the derivative was characteristic of arabitol; and only fragments containing CDHOAc end groups were observed, proving that the material was 1,5-dideuteroarabitol ($n=0$, Fig. 3). This could only have arisen from a Lossen rearrangement. The major series 1,2,3, etc. gave $[M-H]^-$ ions in their ESI–MS spectra corresponding to the series $(GalA)_n$Araol-2D. Multiply charged ions were also observed, corresponding to $m/z=[M-Hz]^{z-}$. The structure of **1** was confirmed by both ^1H and ^{13}C NMR. In particular, the two CHDOH groups of the arabitol residue appeared as triplets in the ^{13}C-spectrum, with a typical $^1J_{CD}$. ESI–MS allowed provisional assignment of some of the minor products. The components labelled E and K gave $[M-H]^-$ ions consistent with the structures GalenA$(GalA)_n$Araol-2-D, $n=0$, 1, respectively (where GalenA is a 4-deoxy-L-*threo*-hex-4-enopyranuronic acid residue). F, I, and N gave ions, and retention times, consistent with the series $(GalA)_n$, $n=1$–3; and G with GalAol-D. These by-products presumably arose from β-elimination reactions. Although the ESI–MS data for D, H, M, Q



A, n=0; 1,2.., n=1,2..



F n=0 ; I n=1; N n= 2. G, GalA-1D-ol.

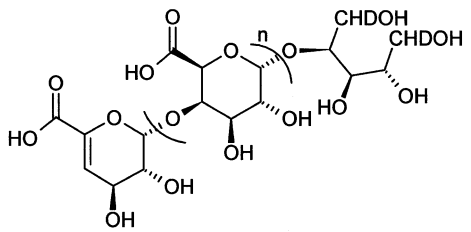


Fig. 3. Structures of selected products obtained by fragmentation of 60 dm pectin.

were consistent with the series GalenA-$(GalA)_n$, $n = 1–4$, or oligogalacturonides with intramolecular lactonisation, neither structure could be confirmed for H by NMR spectroscopy.

Dionex PED response factors.—To assess oligomeric ratios accurately, response factors for the $(GalA)_n$Araol series, eluted from a CarboPac PA1 column with pH 6 sodium acetate, and detected by pulsed-electrometric detection (PED) were required. The concentrations of desalted solutions of weighed samples of the individual oligomers, **1**–**4**, were confirmed by uronic acid analysis. The molar response of the Dionex system is shown in Table 3, relative to GalAAraol **1**. The response to each successive oligomer is seen to rise by a factor of 1.31 ± 0.02; indicating that the true molar ratio of successive oligomers is $0.76 \times$ (observed ratio); this factor was used to correct all subsequent data. The relative response to $(GalA)_2$ is given for comparison. Response factors were not determined for the β-elimination products. A geometric correction factor, of $1/0.93 = 1.07$, was observed by Mort for the series $(GalA)_n$Gal, using pulsed amperometric detection (PAD).

Optimisation of the conversion of hydroxamic acid derivatives to oligomers.—Three connected assessment criteria were identified. Firstly, breakdown to the intended oligomeric products should be maximised. Secondly, the level of by-products should be minimal. And thirdly, the relative ratios of the primary products $(GalA)_n$Araol should accurately reflect the esterification pattern of the degraded pectin. Thus randomly esterified pectins, prepared from highly esterified material by base hydrolysis, were studied; successful degradation would lead to a geometric series of peak areas, where $(GalA)_{n+1}$Araol/$(GalA)_n$-Araol = 1–dm/100.

Hydrolysis pH.—Samples of EDC-treated 60 dh material (Sigma) were hydrolysed, at 100 °C for 1 h, with 50 mM Na_2CO_3, 0.4 M or 4 M NaOH, all containing 0.2 M $NaBH_4$ (Fig. 4(a,c)). All gave results inferior to 0.1 M NaOH hydrolysis (Fig. 2). NaOH (4 M) virtually removed the $(GalA)_n$Araol series (Fig. 4(c)). NaOH (0.4 M) gave increased levels of β-eliminated products (particularly

Table 3
Relative response of PED detector to successive $(GalA)_n$Araol oligomers

n	Response relative to **1**	Ratio n:(n+1)
1	1	
2	1.31	0.76
3	1.69	0.77
4	2.27	0.75
$(GalA)_2$	1.34	

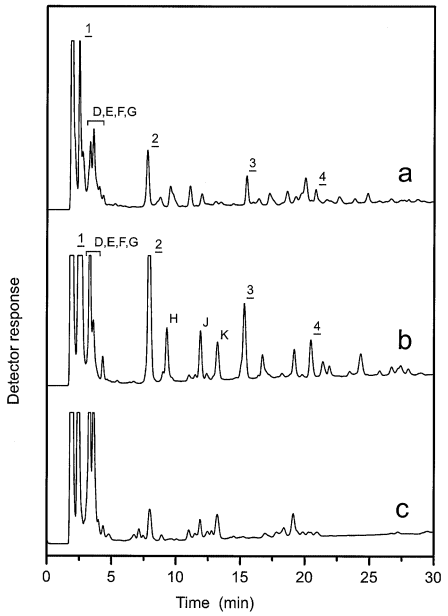


Fig. 4. HPAEC at pH 6 (Gradient A, see Section 4) of products obtained when the EDC-adduct (isourea) of a hydroxamic acid derivative, prepared from 60 dm pectin, was hydrolysed with 0.2 M $NaBH_4$ at 100 °C for 1 h, containing (a) 50 mM Na_2CO_3; (b) 0.4 M NaOH; (c) 4 M NaOH.

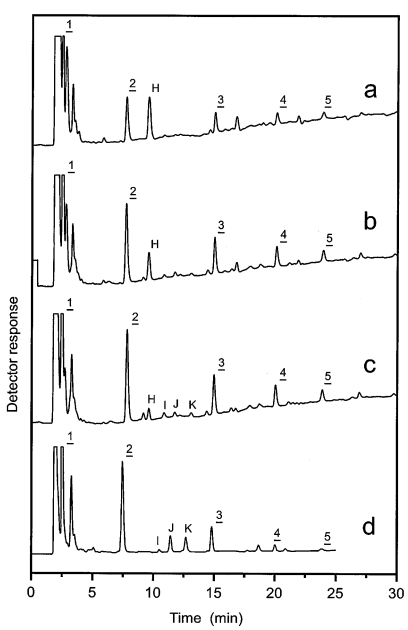


Fig. 5. HPAEC at pH 6 (Gradient A, see Section 4) of products obtained when the EDC-adduct (isourea) of a hydroxamic acid derivative, prepared from 60 dm pectin, was hydrolysed with 0.1 M NaOH–0.2 M $NaBH_4$/100 °C for a variety of times: (a) 10 min; (b) 30 min; (c) 60 min; (d) 120 min (in this case, an additional, equal amount of $NaBH_4$ was added after 60 min).

the uncharacterised J; Fig. 4(b)). Na_2CO_3 (50 mM) lowered $(GalA)_n$Araol levels, and increased levels of β-eliminated products (Fig. 4(a)). HPSEC confirmed less fragmentation in this case (data not shown).

Hydrolysis time, temperature, and borohydride concentration.—The hydroxamic acid prepared from 60 dm (Sigma) pectin at pH 7 was reacted with EDC and fragmented, with 0.1 M NaOH and 0.2 M $NaBH_4$, at 100 °C, for 10, 30 min, 1 and 2 h (Fig. 5(a–d)). The intensity of the $(GalA)_n$Araol series maximised after 1 h. Successive oligomeric ratios, of $(GalA)_2$Araol:$(GalA)_3$Araol upwards, stabilised after 10 min; but, the amount of GalAAraol increased with time, reaching its final value after only 1 h. The relative levels of byproducts changed with time, but were not further investigated. Hydrolysis at 50 °C for 1 h, 20 °C overnight, or with reagent preheated to 100 °C, all gave weak, ill-defined chromatograms. Treatment with 0.1 M NaOH–1 M $NaBH_4$ at 100 °C for 1 h gave unchanged $(GalA)_n$Araol ratios, but diminished the yields of β-elimination products twofold on average (not shown).

EDC-treatment pH and time.—Treatment of dh 89 material with EDC at an initial pH of 3.7 and 5.5 gave closely comparable fragmentation patterns. However, fragmentation of EDC-treated dh 15 at hydroxamic acid at pH 3.7 (Fig. 6(a)) gave high levels of β eliminated products and lowered oligomeric ratios. Adjustment to pH 6 before adding EDC reversed this (Fig. 6(b)); pH 7 gave closely comparable data (not shown), as did an amount of EDC equivalent to only twice the hydroxamic acid residues. Treatment, at pH 4 and 6, with

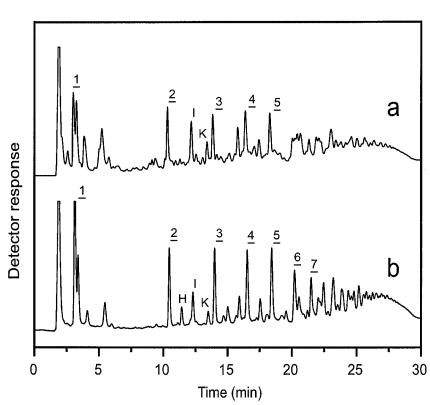


Fig. 6. HPAEC at pH 6 (Gradient B, see Section 4) of a 15 dm pectin, converted to the hydroxamic acid at pH 5.5, and hydrolysed with 0.1 M NaOH–0.2 M $NaBH_4$/100 °C/1 h, after EDC-treatment at (a) pH 4 (b) pH 6.

Table 4
Corrected ratios of successive oligomers obtained by fragmentation of a range of randomly esterified pectins under optimised conditions

$n+1$: n [a]	dm [b]						
	PGA [e] (1.00) [f]	15 (0.85) [f]	30 (0.70) [f]	60 [c] (0.40) [f]	60 [d] (0.40) [f]	72 (0.28) [f]	89 (0.11) [f]
2:**1**		0.82	0.53	0.45	0.50	0.35	0.19
3:**2**	0.90	0.82	0.56	0.44	0.47	0.31	0.22
4:**3**	1.24	0.88	0.66	0.41	0.44	0.33	0.22
5:**4**	0.90	0.77	0.66	0.43	0.53	0.31	0.28
6:**5**	0.58	0.66	0.68	0.46	0.44	0.27	
7:**6**	0.72	0.67	0.51				
8:**7**		0.72					

[a] Ratio of $(GalA)_{n+1}Araol$:$(GalA)_nAraol$.
[b] Percentage methyl esterification of starting pectin.
[c] Hydroxamic acid prepared at pH 5.5.
[d] Hydroxamic acid prepared at pH 7.
[e] Polygalacturonic acid.
[f] Predicted $(n+1)$:n.

10 × the normal amount of EDC (approximately 20 molar equivalents of total GalA) gave dramatically increased byproduct levels, particularly for the pH 4 sample (data not shown).

Effect of pH of hydroxamic acid preparation on composition of fragmentation products.—Samples of hydroxamic acids prepared at pH 5.5 and 7 (see above), treated with EDC and fragmented under the optimised conditions gave highly comparable levels of β-eliminative products. However, the observed ratios of successive $(GalA)_nAraol$ oligomers produced from the pH 5.5 sample were in slightly better agreement with theory (Table 4, averages of duplicate fragmentations).

Test for specificity of the fragmentation protocol-behaviour of polygalacturonic acid.—Unesterified polygalacturonic acid (PGA) should, in theory, be unaffected by any stage of our protocol. A solution of PGA (Na salt) was examined by HPSEC, before and after treatment with hydroxylamine at pH 5 (Fig. 7(a,b)). Virtually no shift in M_r was seen. Direct (i.e., no pre-treatment with hydroxylamine or EDC) NaOH–$NaBH_4$ hydrolysis of PGA, produced a slight shift to lower M_r (Fig. 7(c)). EDC treatment (pH 4), and hydrolysis, again gave only a slight shift in M_r (Fig. 7(d)). But, unexpectedly, when the hydroxylamine-treated material was reacted with EDC and hydrolysed, a considerable shift in

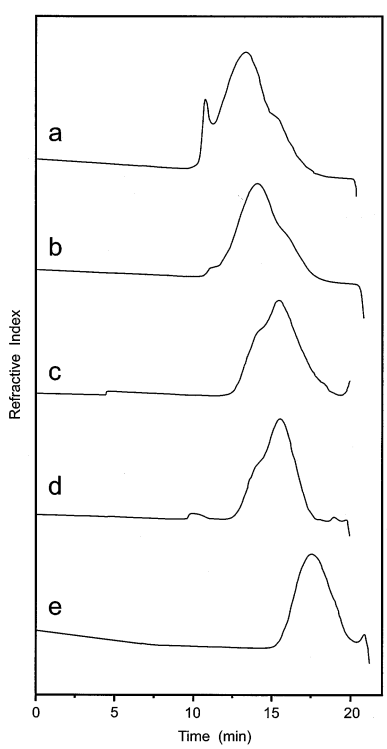


Fig. 7. Comparative HPSEC data (Shodex SB-804HQ column) for polygalacturonic acid and some derivatives (all samples were desalted before examination): (a) sodium polygalacturonate; (b) latter after treatment with hydroxylamine at pH 5.5; (c) effect of direct hydrolysis (0.1 M NaOH–0.2 M $NaBH_4$/100 °C/1 h) of sodium polygalacturonate; (d) effect of EDC-treatment (pH 6) and direct hydrolysis on hydroxylamine-treated polygalacturonic acid; (e) effect of EDC-treatment (pH 6) and direct hydrolysis on polygalacturonic acid.

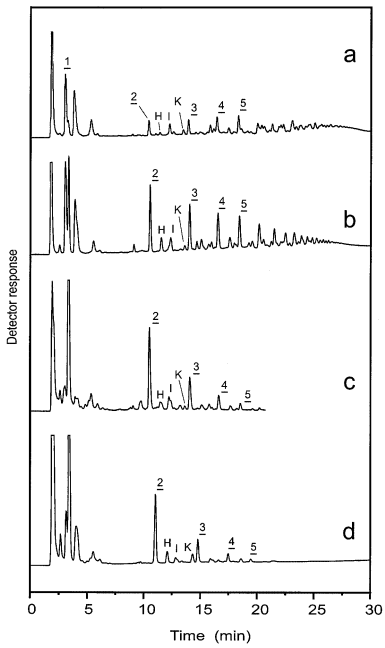


Fig. 8. HPAEC at pH 6 (Gradient B, see Section 4) of Lossen-rearranged and fragmented pectins. (a) Polygalacturonic acid; (b) 30 dm; (c) 72 dm; (d) 89 dm.

M_r was seen (Fig. 7(e)), and HPAEC revealed an approximately equimolar series of $(GalA)_nAraol$ oligomers, together with high levels of byproducts (Fig. 8(a). We feel these results are consistent with a low residual random methyl (or non-methyl?[24]) esterification of the PGA used. Pectic esters become progressively more difficult to hydrolyse as the dm drops, presumably due to a charge effect.[25] Hydroxylamine, as a powerful uncharged nucleophile, might gain access to esters resistant to alkaline hydrolysis. The apparently elevated (and variable) level of GalAAraol remains unexplained.

Fragmentation data.—Application of the above refinements to the randomly esterified pectins gave the corrected oligomeric ratios for the $(GalA)_nAraol$ series shown in Table 4. HPAEC chromatograms, not included in previous figures, are shown in Fig. 8.

Summary of optimised generation of unesterified blocks.—The pectic ester (5 mg/mL) is dissolved, with stirring, in 4.3 M hydroxylamine, pH 5.5 at 20 °C. After 7 days, the mixture is diluted and exhaustively diafiltered (10 kDa cutoff) to the original volume. An aliquot (0.5 mL), adjusted to pH 6, is treated with the carbodiimide, EDC (6 mg), for 1 h, 0.2 M NaOH, containing 0.4 M $NaBH_4$ (0.5 mL) is added, and the mixture is heated on a prewarmed hotblock at 100 °C for 1 h. After cooling and removal of salts, the products are examined by HPSEC.

3. Discussion

Our methodology should prove a useful tool for the determination of pectic structure, as it is straightforward, and requires no special equipment. We have chosen to illustrate its use to generate unesterified blocks from randomly esterified pectins; and have demonstrated that the observed proportions reflect those predicted by theory. But a potential additional advantage of our fragmentation is its versatility. For example, it should debranch hairy pectic regions, though only if the galacturonic residues in the rhamnogalacturonan backbone are methyl esterified; it would thus provide evidence of the latter. Direct cleavage of unesterified galacturonic acid residues in pectins might be achieved. Treatment with EDC, and an *O*-protected hydroxylamine derivative would give *O*-protected hydroxamic acids;[16] deprotection, EDC treatment and hydrolysis would lead to fragmentation. (We have found — unpublished data — that PGA can be broken down to small $(GalA)_n$-Araol oligomers using *O*-tetrahydropyranylhydroxylamine.) If esterified residues were first reduced with borohydride, this could be made selective. We feel sure the method will reward further development; to assist with this process we are posting some reflections (excluded here for reasons of space) on the detailed mechanism of the procedure on our website.[26]

4. Experimental

General methods.—All evaporations were performed in vacuo at 40 °C. Randomly esterified citrus pectin samples, and organic reagents were obtained from the Sigma–Aldrich Company Limited (Gillingham, Dorset).

Conversion of esterified galacturonic acid residues to hydroxamic acid residues

At pH 5.5. Pectin (150 mg) was stirred into solution in aq $NH_2OH{\cdot}HCl$ (4.3 M, 30 mL,

pH 5.5) at 20 °C. The latter was prepared by dissolving $NH_2OH{\cdot}HCl$ in 3/4 the final volume of water, and adjusting the pH to 5.5 at the final volume, with 48% wt aq NaOH, and water. (It is important to adjust to the final pH at 20 °C, as the pH of such a concentrated solution is highly temperature dependent.) After the allotted reaction time (typically 7 days), the mixture was diluted to 200 mL, ultrafiltered to 30 mL, diafiltered (Amicon PM10 membrane, 10 kDa cut-off) against 20 vol of water, and brought to a final volume of 30 mL. (The initial dilution prevented damage to the membrane and poor filtration rates.) The mixture was stored at −20 °C.

At pH 7. Pectin (500 mg) was dissolved in water (125 mL). A solution of $NH_2OH{\cdot}HCl$ (2 M, 125 mL, pH 7) was added at 20 °C, and the mixture was left for the allotted reaction time, typically 5 days. The mixture was concentrated to 100 mL, and diafiltered (Amicon PM10 membrane, 10 kDa cut-off) against 20 vol of water, and brought to a final volume of 100 mL. The mixture was stored at −20 °C.

Preparation of randomly esterified pectins was performed by controlled hydrolysis of a 89% dm citrus pectin (Sigma) as described.[23]

MeOH analyses were determined by hydrolysis and solution analysis,[22] and GC headspace analysis as follows. Assays were carried out in a total volume of 2 mL, containing pectin or cell wall material, 1 mmol propanol as an internal standard, and NaOH at a concentration of 1 M. After standing at rt for 2 h, the headspace was analysed on a Perkin–Elmer Autosystem XL GC (Perkin–Elmer, Cambridge) equipped with a Perkin–Elmer HS 40 XL autosampler. Vials were incubated at 95 °C for 15 min, pressurised to 100 kPa and an injection of 0.05 min made on the GC. The GC column was a 25 m × 0.32 mm ID, 4.0 μm film BP5 column (SGE, Ringwood, Australia), operated at 50 °C. Flame-ionisation detection (FID) was used. Standards containing up to 15 mmol of MeOH were tested and gave a linear response.

Infra red (IR) spectra were recorded on a Biorad FTS-7 spectrometer. Samples of desalted hydroxamic acids were adjusted to either their acid form with Dowex 50W (H^+), or to their anionic form with pH 7 phosphate buffer (1 M). They were then dried down onto CaF_2 plates in vacuo, and examined directly.

Titration of hydroxamic acid preparations.—A solution/suspension of hydroxamic acid (10–50 mg, 5 mg/mL) was treated with Dowex 50W (H^+ form, 5 mL); the latter had been thoroughly washed with water to remove any fine material before use. The resin was removed, and washed several times with water. Decanted material and washings were combined, and evaporated to dryness. The residual solid was dried in vacuo over P_2O_5 overnight, and weighed. The solid was suspended, with stirring, in water (5–25 mL) and titrated against aq NaOH (0.005–0.025 M, depending on predicted dh).

High-performance size-exclusion chromatography was performed on a Shodex SB-804HQ column, with refractive index detection, using 100 mM $HOOCNH_4$ as eluant at 0.5 mL/min.

Combustion analyses were performed on a Carlo Erba 1108 CHN analyser by Satco Ltd. (Hatfield, UK).

Conversion of hydroxamic acid residues to isourea derivatives.—A solution of pectin hydroxamic acid (0.5 mL, prepared as above), in a screw cap culture tube (Soveril SWL 15), was adjusted to pH 6, at 20 °C, with aq NaOH (100 mM). EDC (6 mg) was added, and the mixture was left for 1 h. It was then immediately hydrolysed (see below).

Hydrolysis of isourea derivatives (Lossen rearrangement).—An aliquot (0.5 mL) of a freshly prepared solution of NaOH (0.2 M) containing $NaBH_4$ (0.4 M) was added to a solution of the isourea derivative (prepared as above). The mixture was immediately placed in a preheated hot block (100 °C) for 1 h. The mixture was cooled, and Dowex 50W (H^+) resin (1 mL) was added, with shaking. After 10 min, the supernatant was decanted, the resin was washed with water (1 mL), and supernatant and washings were combined, and evaporated to dryness. Methanol (0.5 mL) and AcOH were added, and the mixture was evaporated to dryness. Methanol addition, and evaporation, were repeated twice. Water (1 mL) was added, and evaporated. The residue was redissolved in water (0.5 mL), prior to analysis by HPAEC.

HPAEC analyses were performed on a CarboPac PA-1 analytical column (4 × 250 mm), Dionex Corporation), equipped with a Carbo-

Pac PA-1 (4 × 50 mm) guard column. A pulsed electrometric detector (PED) was used in conjunction with a 3 mm Au working electrode and a Ag/AgCl reference electrode in integrated amperometric mode (0.2 V, 0 s; 0.2 V, 0.2 s; 0.2 V, 0.40 s; 0.75 V, 0.41 s; 0.75 V, 0.60 s; −0.15 V, 0.61 s; −0.15 V, 1.00 s; integration period 0.2–0.4 s).[19] The column was eluted at 1 mL/min over 30 min with a linear gradient of NaOAc–AcOH, pH 6 (after an initial isocratic period of 1 min. Two gradients were used. Gradient A, 120–600 mM; and Gradient B, 50 mM to 1 M. NaOH (200 mM) was added post-column at 0.5 mL/min. Samples (25–50 μL) were injected, and examined at sensitivities in the range 200 nC–5 μC. Typically, each sample was examined at two sensitivities to allow accurate determination of relative peak areas. The latter were corrected as described in the text.

Large scale preparation of rearrangement products, and preparative ion exchange chromatography.—A hydroxamic acid prepared from a 60 dm pectin (Sigma) at pH 7 (250 mg) was dissolved in water (50 mL). EDC (500 mg) was added, and the mixture was stirred for 1 h. It was then diafiltered (Amicon PM10 membrane, 10 kDa cut-off) against 10 vol of water, and evaporated to dryness. NaOH (0.1 M, 50 mL), containing 0.2 M $NaBH_4$, was added, and the mixture was heated at reflux for 1 h, and cooled. Dowex 50W (H^+, 20 mL) was added, and after 15 min the solution was decanted, and coevaporated with MeOH (3 × 30 mL). The residue was dissolved in 25 mM aq NaOAc (10 mL, pH 5.5).

A column of G Sepharose in 50 mM NaOAc was prepared (45–165 μm beads, 1.5 cm × 1 m). The column was washed with 0.75 M NaOAc, and 50 mM NaOAc (pH 5.5, 5 column volumes each). The sample was loaded and eluted with a gradient of NaOAc (50–500 mM, pH 5.5) at a flow rate of 5 mL/min, and 200 × 9 ml fractions were collected. The fractions were examined by HPAEC, appropriately pooled, and desalted.

ESI–MS analyses were performed, in negative-ion mode, by diffusion of samples in aq MeOH into the electrospray probe of a Micromass Quattro II mass spectrometer.

GC–EIMS of alditol acetates was performed as described.[28,29]

Uronic acid colorimetric analysis was performed as described.[30]

NMR spectroscopic analyses.—^1H and ^{13}C NMR spectra of component **1** (see text and Fig. 3, were obtained using a JEOL GX400 spectrometer, and were consistent with the structure α-D-GalA-(1→4)-L-Araol-2-D. Sample temperature was 27 °C and chemical shifts were determined using acetone (δ 2.217 with respect to TMS) as an internal reference. Dashed assignments are for the arabitol group. All assignments are provisional.

^{13}C NMR (D_2O, pH 3): 174.9 (C-6), 98.2 (C-1), 78.1 (C-2′), 72.5 (C-5), 71.2 (C-4, or C-3), 71.0, 70.3 (C-3′ and C-4′), 70.1 (C-3, or C-4), 68.8 (C-2), 63.4 and 59.8 (2 × t, C-1′ and C-5′).

^1H NMR: 5.25 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1), 4.53 (d, 1 H, $J_{4,5}$ 1.3 Hz, H-5), 4.33 (dd, 1 H, $J_{3,4}$ 3.6 Hz, H-4), 3.78–3.95 (m, 5 H, H-2, H-3, H-2′, H-3′, H-4′), 3.65 (d, 1 H, *J* 5.3 Hz, H-1′ or H-5′), 3.61, 3.65 (d, 1 H, *J* 7.0 Hz, H-1′ or H-5′).

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