Phytochemistry Vol. 49, No. 7, pp. 1879–1890, 1998

0031-9422/98/$ - see front matter

PII: S0031-9422(98)00370-7

*N*α- AND *N*ε-D-GALACTURONOYL-L-LYSINE AMIDES: PROPERTIES AND POSSIBLE OCCURRENCE IN PLANT CELL WALLS

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(*Received* 11 *March* 1998)

Key Word Index—*Lycopersicon* hybrid; Solanaceae; tomato; *Spinacia oleracea*; Chenopodiaceae; spinach; cell cultures; galacturonic acid; lysine; *N*-galacturonoyl amides; isopeptides; pectins; extensins; primary cell wall.

Abstract—Three representatives of a novel class of amide (isopeptide) glycoconjugates have been synthesised: *N*α-D-galacturonoyl-L-lysine and *N*ε-D-galacturonoyl-L-lysine and *N*ε-D-polygalacturonoyl-L-lysine. Galacturonoyl–lysine amide bonds were labile in 2 M trifluoroacetic acid at 120° and in alkali, but relatively stable in cold acid. The amide bonds were resistant to digestion by Driselase, Pronase and trypsin. The polysaccharide backbone of *N*ε-D-polygalacturonoyl-L-lysine was hydrolysed by Driselase to yield two major ninhydrin-positive compounds which were shown by ^{1}H and ^{13}C NMR spectroscopy to be tri- and tetra-α-(1 → 4)-D-galacturonoyl-L-lysines. To investigate the possible natural occurrence of *N*-galacturonoyl isopeptide bonds, we fed cell-suspension cultures of spinach and tomato with D-[6-^{14}C]glucuronic acid, which radio-labels pectic polysaccharides. The radioactive cell walls were digested with, sequentially, Driselase, mild acid, and proteinases. On electrophoresis at pH 2.0, several of the radioactive digestion-products were cathodic. Some of the cathodic products yielded [^{14}C]galacturonic acid upon complete acid hydrolysis. The existence of these products is compatible with the presence of novel *N*-galacturonoyl isopeptide bonds, which could serve as cross-links in plant cell walls.

INTRODUCTION

The primary structures of the major polymers of plant cell walls are known in some detail [1–4]. In contrast, many unresolved questions remain concerning the covalent and non-covalent cross-linking of polymers in the primary cell wall — the means by which the cell wall matrix is assembled [5, 6]. An understanding of the inter-polymeric cross-links is a pre-requisite for a satisfactory working model of the growing cell wall.

The present work was conducted with the aim of testing for covalent linkages between pectins and extensins. Pectins (homogalacturonans and rhamnogalacturonans) are polysaccharides rich in α-(1 → 4)-D-galacturonic acid (GalA) and its methyl and acetyl esters [2, 7, 8]. Extensins are hydroxyproline-rich glycoproteins which are basic owing to a high Lys content [4, 9].

Non-covalent bonds, especially Ca^{2+}-bridges, contribute to the cross-linking of pectins within the cell wall [7]. However, the fact that chelating agents fail to extract a substantial percentage of the wall pectin in most tissues [10] suggests that covalent cross-links may also contribute. Such covalent cross-links could include glycosidic bonds (e.g. xyloglucan → rhamnogalacturonan [11]), oxidatively coupled phenolic side-chains (e.g. pectin–diferulate–pectin [5]) and *O*-galacturonoyl ester bonds [12, 13].

Although newly secreted extensins can often be solubilised from the cell wall in salt solutions, mature extensins cannot be extracted without breakage of covalent bonds. One class of cross-link responsible for the inextractability of mature extensins is the family of tyrosine coupling-products: isodityrosine, pulcherosine and di-isodityrosine [5, 14].

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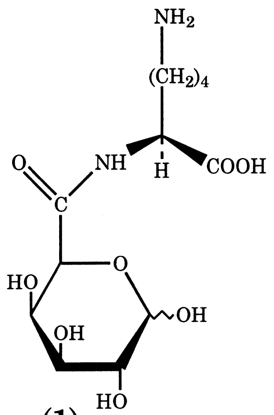

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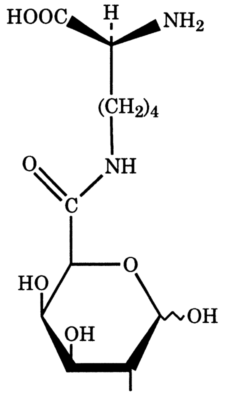

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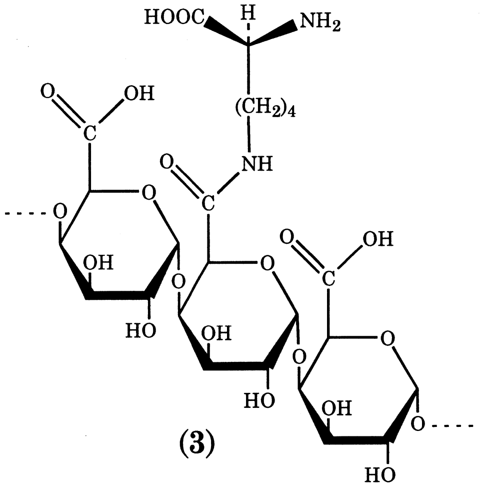

(3)

In addition, pectins and extensins may be linked to each other. A proportion of the pectic polysaccharides often remains with the extensins in the residue obtained after cell walls have been treated with alkali to extract hemicelluloses [15]: this suggests the possibility of a relatively stable bond between pectin and extensin. This idea was supported by the discovery of an apparently covalent bond between rhamnogalacturonan-I and extensin in cotton cell walls [16]. Such a bond could potentially be an amide (isopeptide) linkage between the carboxy group of a GalA residue in pectin and the ε-amino group of a Lys residue in extensin. Related structures exist in the *N*α-glucuronoyl residue that occurs as an *N*-terminal blocking group in a cutinase [17] and the *N*ε-(γ-glutamyl)lysine isopeptide cross-link found in some animal proteins [18]. Furthermore, the occurrence of covalently-bonded polyamines (spermidine, spermine and putrescine) in plant cell walls [19, 20] suggests that these may be amide-linked to pectins.

Brown and Fry [12] reported the preparation and properties of *O*-uronoyl ester model compounds, such as 6-*O*-D-galacturonoyl-D-glucose, and evidence for the occurrence of non-methyl *O*-D-galacturonoyl esters in the pectic polysaccharides of spinach [13]. (The term "uronoyl" denotes a uronic acid moiety linked via its carboxy group.) In the present work, we have adopted an analogous approach to synthesise and characterise *N*-uronoyl amide model compounds. This has enabled a search for the possible natural occurrence in plant cell walls of novel *N*-galacturonoyl amide bonds.

RESULTS AND DISCUSSION

Preparation of Nα- *and* Nε-*D-galacturonoyl-L-lysines* (Nα- *and* Nε-*GalA–Lys*)

Incubation of GalA with L-lysine methyl ester (Lys–Me) in the presence of a water-soluble carbodiimide, followed by removal of the methyl ester group, yielded two Dowex-50-binding products, both of which migrated towards the cathode on paper electrophoresis (PE) at pH 2.0 (Table 1), indicating the presence of a free amino group. Both products stained yellow with aniline hydrogen-phthalate (AHP), indicating the presence of a reducing sugar moiety, and violet with ninhydrin, confirming the presence of a free amino group. The faster-migrating spot was the more abundant, as

Table 1. Chromatographic and electrophoretic properties of *N*α-GalA–Lys, *N*ε-GalA–Lys, and some related compounds

		Mobility (mm from origin) during				Staining with		
		PC in APAW				Ninhydrin		
Compound	*Q*‡	(4:3:1:2, by vol.)	(4:1:3:2, by vol.)	PE† at pH 2.0	(15 min at 20°)	(5 min at 105°)	Aniline hydrogen-phthalate
Lys	1.5+	133	281	252	+	+	–
GalA	0	234	284	11	–	–	+
*N*α-GalA–Lys	0.9+	180	245	117	–	+	+
*N*ε-GalA–Lys	0.5+	180	217	80	+	+	+
Lys–Me	2.0+	338	425	307	+	+	–
GalA–Lys–Me*	1.0+	389	352	127	nd	+	+

nd = Not determined.

**N*α- and *N*ε-isomers; not resolved.

†Distances migrated towards the cathode; GalA, although not ionised at pH 2.0, moves slightly towards the cathode owing to electro-endo-osmosis.

‡*Q* = approximate predicted charge of the compound at pH 2; these values correlate well with mobility on PE.

Table 2. ^{1}H-NMR spectroscopic data for *N*ε-D-galacturonoyl-L-lysine

	Chemical shift (ppm)					Coupling constant (Hz)		
System	δ_1	δ_2	δ_3	δ_4	δ_5	J_{12}	J_{23}	J_{34}
Reducing GalA (α)	5.37	3.82	3.93	4.28	4.53	3.8	10.5	3.3
Reducing GalA (β)	4.65	3.52	3.72	4.22	4.22	8.1	10.0	3.3
	δ_α	δ_β	δ_γ	δ_δ	δ_ε			
Lys	3.73	1.88	1.39, 1.43	1.60	3.25, 3.33			

^{1}H chemical shifts (δ_H) were measured with respect to methyl resonances of TSP taken as $\delta = 0$. Couplings between H-4 and H-5 of the GalA moieties were concealed within the line widths. The Lys resonances appeared as comlex multiplets similar in appearance to those of the free amino acid.

judged by staining with AHP. The compounds were electrophoretically neutral at pH 6.5, indicating equal numbers of free amino and carboxy groups. The formation of both products was dependent on the presence of all three reactants. The products therefore appeared likely to be *N*α-GalA–Lys (**1**) and *N*ε-GalA–Lys (**2**).

The carboxy group of the Lys moiety in *N*ε-GalA–Lys is predicted to have a lower pK_a (≈2) than that in *N*α-GalA–Lys (≈3–4) because of the neighbouring free α-amino group. On PE at pH 2.0, *N*α-GalA–Lys is expected to have a full positive charge on the ε-amino group and only a slight negative charge on the α-carboxy group of the Lys residue, and therefore to migrate more rapidly towards the cathode than *N*ε-GalA–Lys, which should have a full positive charge on the α-amino group and roughly half a negative charge on the α-carboxy group (Table 1). In agreement with the faster-moving spot being *N*α-GalA–Lys, it stained with ninhydrin only after heating, whereas the less abundant, slower-moving spot was revealed by ninhydrin at room temperature, characteristic of an amino acid in which the α-amino group is unsubstituted. The two compounds were also separable by paper chromatography (PC) in the acidic solvent, APAW (4:1:3:2), in which the putative *N*α-GalA–Lys had a higher R_F (presumably because its carboxy group was only slightly ionised) than *N*ε-GalA–Lys. The two compounds were inseparable by PC in the basic solvent, APAW (4:3:1:2), in which both compounds would have their Lys carboxy group almost fully ionised.

The ^{1}H-NMR spectrum (Table 2) of *N*ε-GalA–Lys, obtained in ^2H_2O (pH = 6.94) at 25°, confirmed the presence one reducing GalA group (present as a mixture of α- and β-anomers) and one Lys group. The resonances were assigned by comparison of their chemical shifts and coupling constants with those of free GalA and free Lys, with which there was excellent agreement.

Susceptibility to hydrolysis of N*-galacturonoyl-lysines*

Acid hydrolysis (2 M TFA at 120°) converted *N*α-GalA–Lys and *N*ε-GalA–Lys to GalA and Lys [Fig. 1(a), (b)]. The half-life of *N*ε-GalA–Lys (~50 min) was somewhat longer than that of *N*α-GalA–Lys (~30 min). Each compound also yielded one minor, transitory, ninhydrin-positive, unidentified by-product (Fig. 1). By 16 h the hydrolysis was complete, but the free GalA had been degraded.

Cold alkali [0.16 M $Ba(OH)_2$ at 25°] also released Lys from both amides; two unidentified by-products were also formed but GalA was not detected. The half-life of the amides in cold alkali was about 4 h; some starting material was still detectable after 16 h [Fig. 1(c), (d)]. Hot alkali caused almost complete degradation within 15 min, giving Lys and several alkali-stable, ninhydrin-positive by-products (data not shown).

The enzymes tested (Driselase, Pronase and trypsin) did not affect either *N*α-GalA–Lys or *N*ε-GalA–Lys in a 48-h treatment (Fig. 2). The conclusions about the susceptibility to hydrolysis of *N*-uronoyl amide bonds are summarised in Table 3.

Preparation and susceptibility to hydrolysis of Nε*-polygalacturonoyl-L-lysine*

*N*ε-(Poly-GalA)–Lys (**3**) was synthesised by reaction of α-(1 → 4)-poly-D-galacturonate (poly-GalA) with α-Z-Lys–Me in the presence of a water-soluble carbodiimide followed by removal of the Me and Z protecting groups. After gel-permeation chromatography a high-M_r product was obtained which, unlike the poly-GalA starting material, stained violet with ninhydrin.

Acid hydrolysis of *N*ε-(poly-GalA)–Lys would be expected to cleave both GalA → GalA glycosidic bonds and GalA–Lys amide bonds. In agreement with this, hot acid (2 M TFA at 120°) resulted in the gradual formation of oligogalacturonides and GalA (Fig. 3) as well as Lys (not shown). In ad-

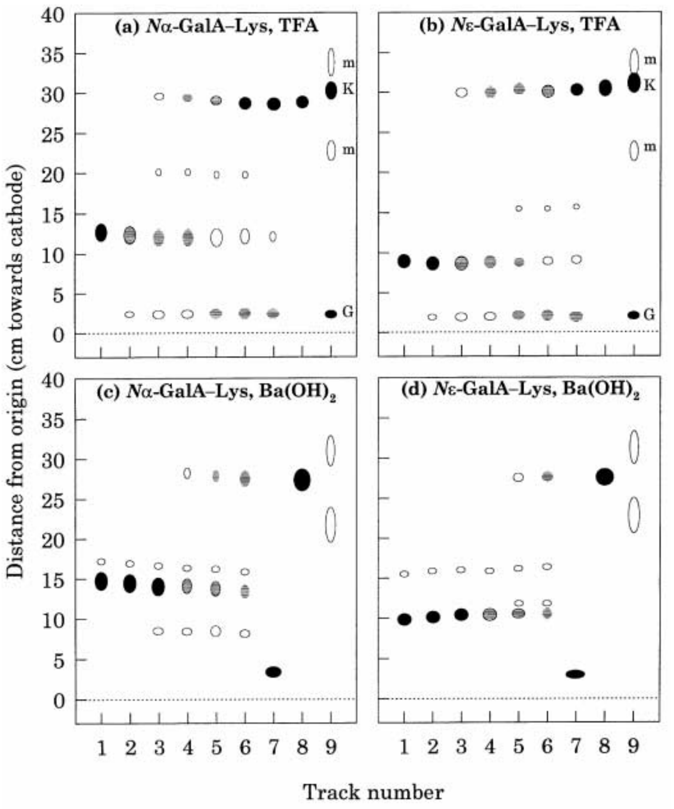

Fig. 1. Effect of hot acid and cold alkali on *N*α-GalA–Lys (a,c) and *N*ε-GalA–Lys (b,d). Samples were subjected to PE at pH 2.0. (a,b): Track (1), GalA–Lys alone; (2)–(8), GalA–Lys incubated in 2 M TFA at 120° for 0, 15, 30, 60, 90, 120 and 960 min; (9), marker mixture showing GalA (G), Lys (K) and methyl green (m; two spots). (c,d): Track (1), GalA–Lys alone; (2), GalA–Lys incubated in a suspension of $BaSO_4$ equivalent to the loadings in tracks 3–6; (3)–(6), GalA–Lys incubated in 0.16 M $Ba(OH)_2$ at 25° for 15, 70, 220 and 960 min followed by neutralisation with H_2SO_4; (7), GalA; (8), Lys; (9), methyl green. Duplicate electrophoretograms were stained with ninhydrin and AHP. The diagrams are composites for both stains. The GalA–Lys starting material stained with both AHP and ninhydrin, GalA stained only with AHP, and all other products stained only with ninhydrin. Shading indicates the relative intensity of staining, as follows: ● > ▨ > ▤ > ○ .

dition, *N*ε-GalA–Lys was detected as an intermediate product, characterised by staining with both ninhydrin and AHP and by its migration between GalA and α-(1 → 4)-D-galacturonobiose ($GalA_2$) (Fig. 3). After 16 h hydrolysis, Lys was the only detectable product, GalA having been degraded.

The amide linkage of *N*ε-(poly-GalA)–Lys was essentially stable to cold alkali (0.5 M NaOH at 25°): no Lys was released within 16 h. Alakine hydrolysis at 60° did release Lys and the half-life of the amide bond was *ca.* 2 h. The amide bond of (poly-GalA)–Lys was thus considerably more stable than that of GalA–Lys. *N*ε-(Poly-GalA)–Lys was resistant to Pronase and trypsin.

Driselase digestion of *N*ε-(poly-GalA)–Lys yielded several products analysed by PC in EAW (Fig. 4) and BPW (not shown). GalA and $GalA_2$ were the most abundant AHP-reactive products. Additional products, A–E, stained with ninhydrin [Fig. 4(b)]; products D and E (and probably A–C, faintly) also stained with AHP [Fig. 4(a)]. The products had retention times on Bio-Gel P-2 increasing in the order A < E. Products C, D and E had R_F values (in EAW) slightly lower than those of $GalA_5$, $GalA_4$ and $GalA_3$, respectively; ninhydrin-positive product B co-migrated with $GalA_6$ (Fig. 4; and other PCs developed for longer times, not shown). These observations suggested that Driselase produced several *N*ε-oligogalacturonoyl–lysines. On PE at pH 3.5 and 6.5, these compounds all migrated towards the the anode, indicating that they possessed more carboxy groups than amino groups. *N*ε-GalA–Lys was not among the products; thus, at least one GalA → GalA glycosidic bond in the immediate vicinity of the Lys moiety of *N*ε-(poly-GalA)–Lys was resistant to Driselase.

NMR study of oligogalacturonoyl–lysines

Products B to E were separated on a larger scale by gel-permeation chromatography on Bio-Gel P-2. By rejecting the fractions in which peaks over-

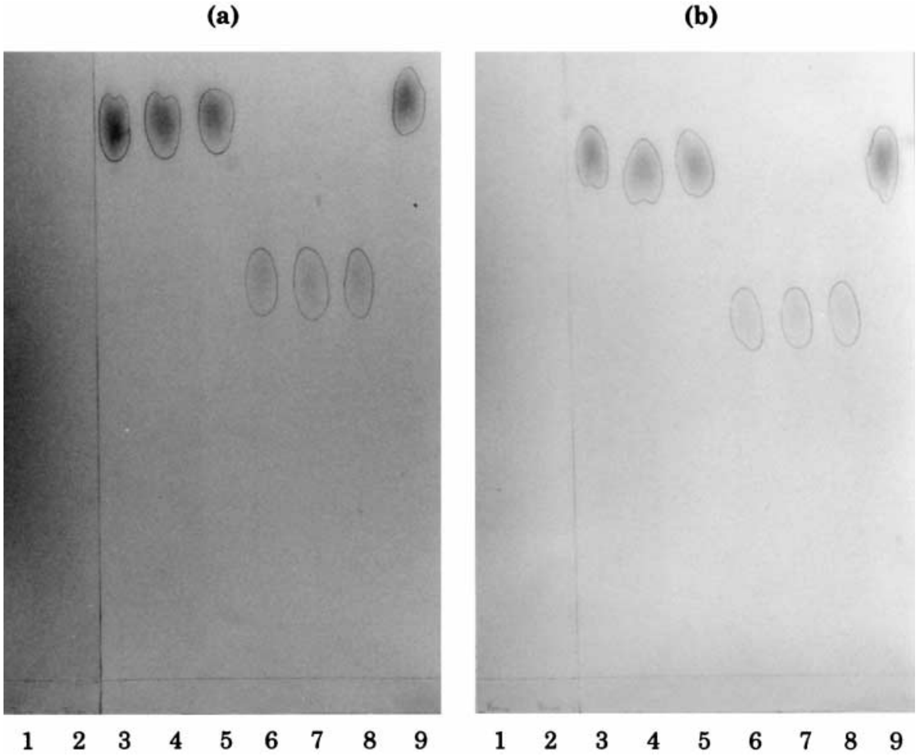

Fig. 2. Effect of trypsin (a) and Pronase (b) on *N*ε-GalA–Lys. Samples were subjected to PC in EAW and stained with aniline hydrogen-phthalate. Track (1), proteinase alone, 0 h; (2), proteinase alone, 48 h; (3), (9), GalA marker; (4), GalA + proteinase, 0 h; (5), GalA + proteinase, 48 h; (6), *N*ε-GalA–Lys alone; (7), *N*ε-GalA–Lys + proteinase, 0 h; (8), *N*ε-GalA–Lys + proteinase, 48 h.

lapped, we obtained four pools of ninhydrin-positive material, each of which gave a single ninhydrin-positive spot on analytical PC in EAW. Yields were: B, 0.56 mg; C, 0.56 mg; D, 1.53 mg; E, 0.96 mg.

The one dimensional ^{1}H-NMR spectra of compounds D and E in ^2H_2O as solvent (pH 3.9), whilst showing the presence of one Lys and several GalA residues, were sufficiently complex that one- and two-dimensional ^{1}H-TOCSY and ^{1}H–^{13}C HMQC correlation spectra were required for their interpretation. In each case the 2D TOCSY spectra (Fig. 5) allowed the number and subspectra of the individual GalA residues to be distinguished and this was confirmed by the clear separation in the HMQC spectrum (Fig. 5) of the anomeric CH signals from remaining GalA signals. The 1D TOCSY spectra allowed the accurate determination of the chemical shifts of the resonances of the individual residues. Compound D (Table 4) was shown to contain one

Table 3. Qualitative summary of the susceptibility to cleavage of bond types discussed in the text

	Susceptibility to cleavage of:			
Method of cleavage	GalA → GalA glycosidic*	GalA–Lys amide†	GalA–Glc ester‡	Xaa–Xaa peptide§
Hot acid	+ + +	+ + +	+ + +	+
Cold alkali	–	–/ + +¶	+ + + + +	–
Hot alkali	±	+ + / + + + + +¶	+ + + + +	+
Driselase	+ + + +	–	–	±
Pronase	–	–	n.d.	+ + +
Trypsin	–	–	n.d.	–/ + + +

– = Essentially resistant.
± = Very slow or partial degradation.
+ = Slow degradation… + + + + + = Very rapid degradation.
n.d. = Not determined.
*Glycosidic linkage in galacturonan.
†*N*-Uronoyl amide bonds in *N*α-GalA–Lys and *N*ε-GalA–Lys.
‡*O*-Uronoyl ester bonds, e.g. in methyl 6-*O*-D-galacturonoyl-β-D-glucopyranoside [12].
§Peptide bond in a polypeptide or glycoprotein.
¶The amide bond in *N*-GalA–Lys was much more labile than that in *N*-(poly-GalA)–Lys.

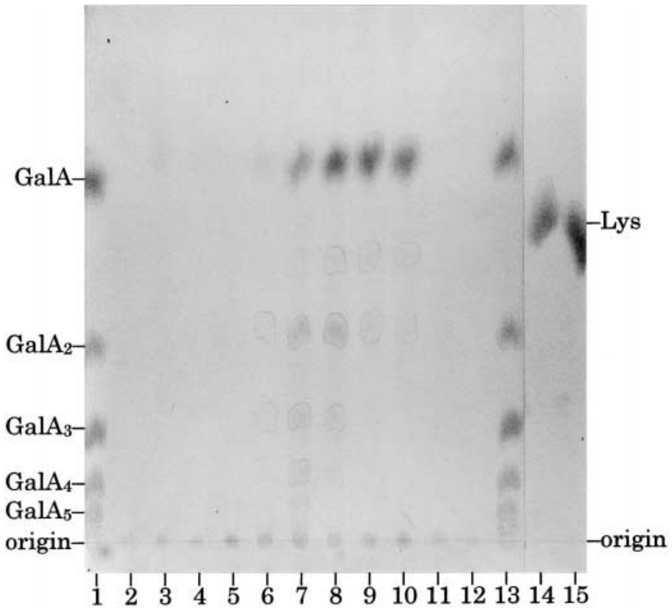

Fig. 3. Effect of hot acid on *N*ε-(poly-GalA)–Lys. Samples were subjected to PC in EAW and stained with aniline hydrogen-phthalate except tracks 14 and 15 (ninhydrin). Tracks (1) and (13), GalA + oligogalacturonide markers; (2), poly-GalA + TFA, unheated; (3), poly-GalA + TFA, at 120° for 24 h; (4), *N*ε-(poly-GalA)–Lys; (5)–(11), *N*ε-(poly-GalA)–Lys + TFA, heated at 120° for 0, 15, 30, 60, 90, 120, 960 min, respectively; (12), poly-GalA; (14), Lys; (15), Lys + TFA at 120° for 960 min.

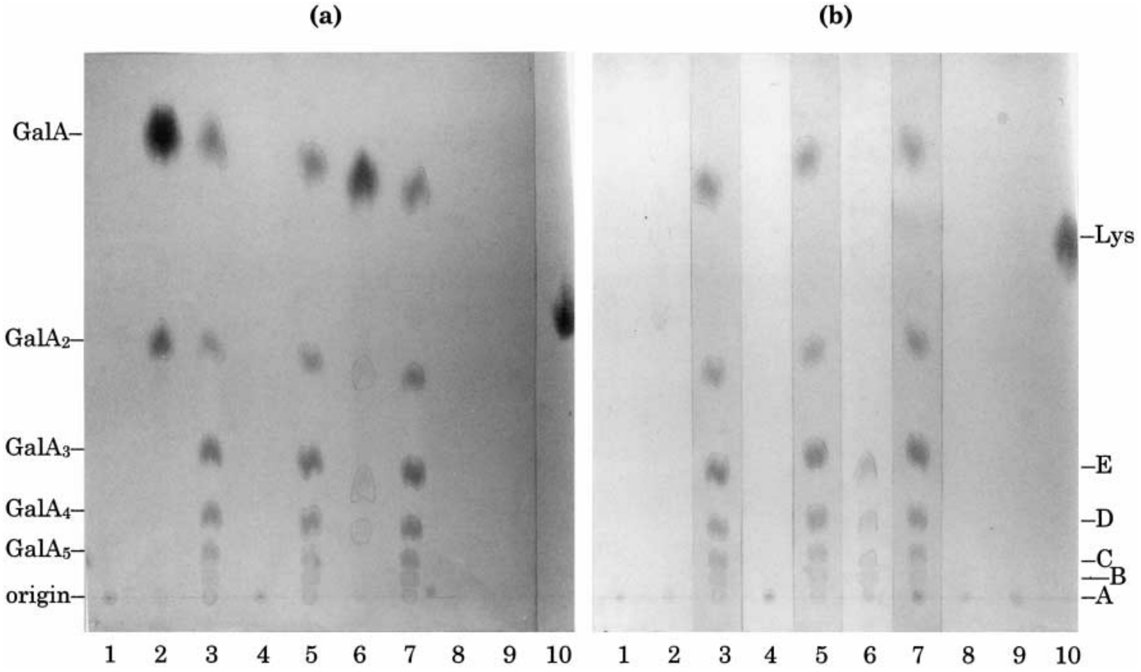

Fig. 4. Effect of Driselase on *N*ε-(poly-GalA)–Lys. Samples were subjected to PC in EAW. Sheet (a) was stained with AHP except track (10) (ninhydrin); sheet (b) was stained with ninhydrin except tracks (3), (5) and (7) (AHP). The two sheets were loaded with identical samples: track (1), poly-GalA + Driselase, 0 h; (2), poly-GalA + Driselase, 48 h digestion; (3), (5), (7), GalA and oligogalacturonide markers; (4), *N*ε-(poly-GalA)–Lys + Driselase, 0 h; (6), *N*ε-(poly-GalA)–Lys + Driselase, 48 h; (8), Driselase alone, 0 h; (9), Driselase alone, 48 h; (10), Lys marker. A–E = *N*ε-Oligogalacturonoyl–lysines formed by Driselase action.

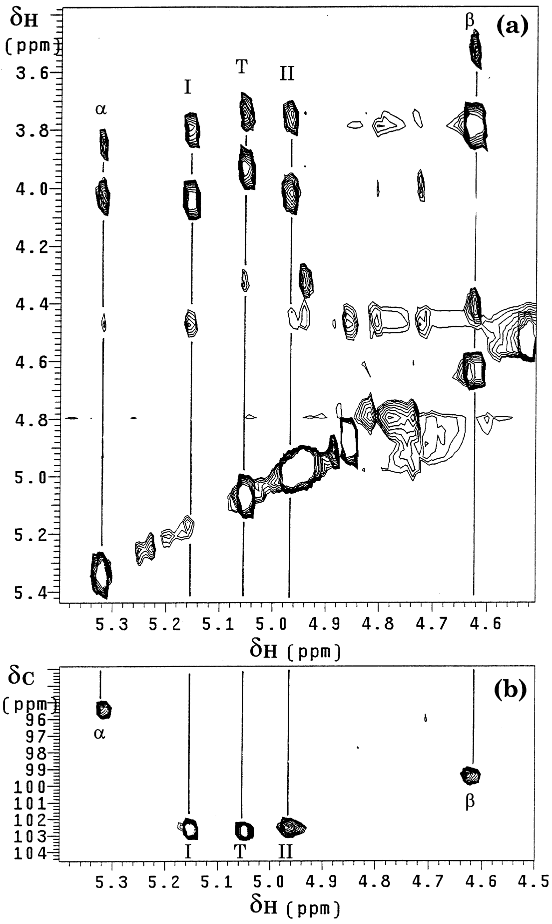

Fig. 5. (a) Part of the 2D TOCSY spectrum of *N*ε-tetragalacturonoyl–lysine (compound D) showing ^{1}H signals from the separate GalA moieties: α = α-reducing, β = β-reducing, I = internal residue adjacent to the reducing terminus, II = internal residue adjacent to the non-reducing terminus, T = non-reducing terminus. (b) Part of the 2D HMQC ^{1}H–^{13}C correlation spectrum of compound D showing anomeric signals from the GalA residues.

Lys residue linked at the carboxy group of one of four GalA residues linked α-(1 → 4). It was not possible to determine to which GalA residue the Lys was attached. This assignment is consistent with its R_F (slightly lower than that of GalA$_4$: see Fig. 4). Compound E (Table 5) was similar but with three GalA residues. The ^{1}H chemical shifts were in good agreement with those of the corresponding unsubstituted GalA oligomers, confirming that no reaction had taken place at the ring hydroxyl groups. The ^{1}H-NMR spectra indicated that products B and C were mixtures containing GalA oligomers bearing Lys.

Possible natural occurrence of N*-galacturonoyl amide bonds in spinach and tomato cell walls*

Information on the susceptibility to hydrolysis of model *N*-galacturonoyl–lysine amides enabled us to design a search for the natural occurrence of novel amide bonds in plant cell walls. Spinach and tomato cell suspensions were fed with D-[6-^{14}C]glucuronic acid. This is taken up by the cells and activated to UDP-GlcA, which is epimerised to UDP-GalA. Hence, radioactivity is incorporated into both GlcA and GalA residues of wall polysaccharides. UDP-Ara and UDP-Xyl do not become radiolabelled because the ^{14}C is lost as $^{14}CO_2$ during decarboxylation of UDP-[6-^{14}C]GlcA. No detectable reduction of UDP-GlcA to UDP-Glc occurs. Thus, the walls of [^{14}C]GlcA-fed cells are efficiently and specifically labelled in their uronate residues [13].

Our working hypothesis was that a GalA residue of pectin was amide-linked to the ε-amino group of a Lys residue in extensin. The radio-labelled cell walls were therefore digested sequentially with Driselase, mild acid, and a proteinase (Pronase or trypsin). None of these treatments causes appreciable cleavage of *N*ε-GalA–Lys amide bonds. Driselase efficiently hydrolyses most of the glycosidic and methyl-ester bonds of the major pectins, homogalacturonan and rhamnogalacturonan-I (S. Aldington and S.C. Fry, unpublished work), while sparing a small number of the glycosidic linkages immediately adjacent to any GalA residue that is amide-bonded to Lys (see above). Driselase does not efficiently hydrolyse extensin, so that a pectic fragment (oligogalacturonide) amide-linked to extensin would be expected to remain in the high-M_r fraction.

Driselase solubilised ~99% of the wall-bound ^{14}C from the tomato cells and essentially 100% of that from the spinach cells. The three radioactive fractions (Driselase-soluble and -insoluble from tomato and Driselase-soluble from spinach) were individually treated with dilute acid to remove Ara*f* side chains from extensin, facilitating digestion by proteinases [21]. Aliquots of each of the three de-arabinosylated fractions were then digested with Pronase or trypsin. The proteinases solubilised some additional radioactivity from the Driselase-insoluble tomato material (*ca.* 0.3% of the initial total wall material).

The combined treatments would be expected to "prune" both the pectin and the protein chains; any *N*-uronoyl amide bond between the pectin and the protein would be left intact within a stable oligosaccharide–oligopeptide or oligosaccharide–Lys conjugate. Such a conjugate would have the highly unusual combination of properties of being (a) radioactive owing to the GalA residues and (b) cationic owing to the free α-amino group.

On PE at pH 2.0, most ^{14}C-labelled products co-migrated with GalA and Glc markers (Fig. 6), indicating a majority of acidic or neutral material, as expected. The slight movement of GalA and Glc towards the cathode is due to electroendo-osmosis. In addition, however, proteinase-digestion yielded

Table 4. ^{1}H and ^{13}C NMR spectroscopic data for *N*ε-tetragalacturonoyl–lysine, compound D

		Chemical shift (ppm)					Coupling constant (Hz)		
System	δ	δ_1	δ_2	δ_3	δ_4	δ_5	J_{12}	J_{23}	J_{34}
Terminal GalA	δ_H	5.05	3.72	3.92	4.30	4.94	3.8	10.6	3.5
	δ_C	102.7	71.2	72.3	73.5	74.5			
Internal GalA-II	δ_H	4.96	3.74	4.00	4.42	4.97	3.9	10.6	3.0
	δ_C	102.6	71.2	71.6	81.6	74.0			
Internal GalA-I*	δ_H	5.15	3.78	4.02, 4.04	4.45	4.84	4.0	10.6	3.4
	δ_C	102.6	71.2	71.6	79.6	74.0			
Reducing GalA (α)	δ_H	5.32	3.83	4.01	4.44	4.18	3.9	10.5	2.9
	δ_C	95.5	71.1	71.6	81.6	73.3			
Reducing GalA (β)	δ_H	4.62	3.50	3.78	4.39	4.16	7.8	10.3	3.0
	δ_C	99.4	74.4	75.2	80.7	76.9			
		δ_α	δ_β	δ_γ	δ_δ	δ_ε			
Lys	δ_H	3.75	1.85, 1.90	1.39, 1.44	1.59	3.20, 3.31			
	δ_C	57.5	33.0	24.9	31.0	41.9			

^{1}H chemical shifts (δ_H), measured from the 1D ^{1}H and 2D TOCSY spectra and ^{13}C chemical shifts (δ_C) measured from the 2D HMQC ^{13}C–^{1}H correlation spectrum, are with respect to methyl resonances of TSP taken as $\delta = 0$. Couplings between H-4 and H-5 of the GalA residues were concealed within the line widths.

*Residue internal-I is linked to the reducing terminus.

3–4 radioactive peaks that migrated further towards the cathode and therefore carried a positive charge, most likely an amino group. The formation of cationic, ^{14}C-labelled products is evidence for a linkage between positively charged molecules and a wall-bound uronic acid.

The major wall-bound uronic acids are GalA, GlcA and 4-*O*-Me–GlcA [1, 29]. Complete acid hydrolysis of several of the cationic fractions (Pronase and trypsin digestion products of Driselase-soluble and -insoluble material) yielded a ^{14}C-labelled product that co-migrated with GalA on PE at pH 3.5 (e.g., Fig. 7), which efficiently resolves these uronic acids. After elution from the electrophoretograms and analysis by PC in BAW, the putative [^{14}C]GalA showed exact co-chromatography with non-radioactive GalA added as an internal marker (data not shown), strongly supporting its identity.

Conclusion

Previous reports described the preparation and characterisation of model *O*-galacturonoyl esters [12] and their use to assist in the design of methods for the detection of non-methyl *O*-galacturonoyl esters *in vivo* [13]. In the present work, we report the preparation and characterisation of model *N*ε-galacturonoyl amides of Lys, the first representatives of a new class of isopeptide glycoconjugates.

The susceptibilities of the novel amide bonds to cleavage by acid, alkali and enzymes (Table 3) suggested a strategy by which to search for such linkages in the plant cell wall. Application of this strategy led to the discovery of ^{14}C-labelled GalA residues in cationic digestion-products. The isolation of such conjugates suggests the existence in the cell wall of a linkage between a pectic polysaccharide and a positively charged molecule. The

Table 5. ^{1}H and ^{13}C NMR spectroscopic data for *N*ε-trigalacturonoyl–lysine, compound E

		Chemical shift (ppm)					Coupling constant (Hz)		
System	δ	δ_1	δ_2	δ_3	δ_4	δ_5	J_{12}	J_{23}	J_{34}
Terminal GalA	δ_H	4.94	3.70	3.90	4.29	4.94	4.0	10.6	3.4
	δ_C	102.8	71.4	72.6	73.9	75.0			
Internal GalA	δ_H	5.16	3.79	4.02, 4.04	4.44	4.85	4.0	10.6	3.1
	δ_C	102.8	71.4	71.8	79.9	74.2			
Reducing GalA (α)	δ_H	5.30	3.83	4.00	4.42	4.48	3.9	10.6	3.2
	δ_C	95.8	71.4	71.8	81.7	73.6			
Reducing GalA (β)	δ_H	4.60	3.50	3.77	4.38	4.12	7.9	10.2	3.2
	δ_C	99.7	74.7	75.5	80.9	77.3			
		δ_α	δ_β	δ_γ	δ_δ	δ_ε			
Lys	δ_H	3.76	1.86, 1.91	1.40	1.60	3.20, 3.31			
	δ_C	58.0	33.1	25.1	31.0	42.0			

^{1}H chemical shifts (δ_H), measured from the 1D ^{1}H and 2D TOCSY spectra and ^{13}C chemical shifts (δ_C) measured from the 2D HMQC ^{13}C-^{1}H correlation spectrum, are with respect to methyl resonances of TSP taken as $\delta = 0$. Couplings between H-4 and H-5 of the GalA residues were concealed within the line widths.

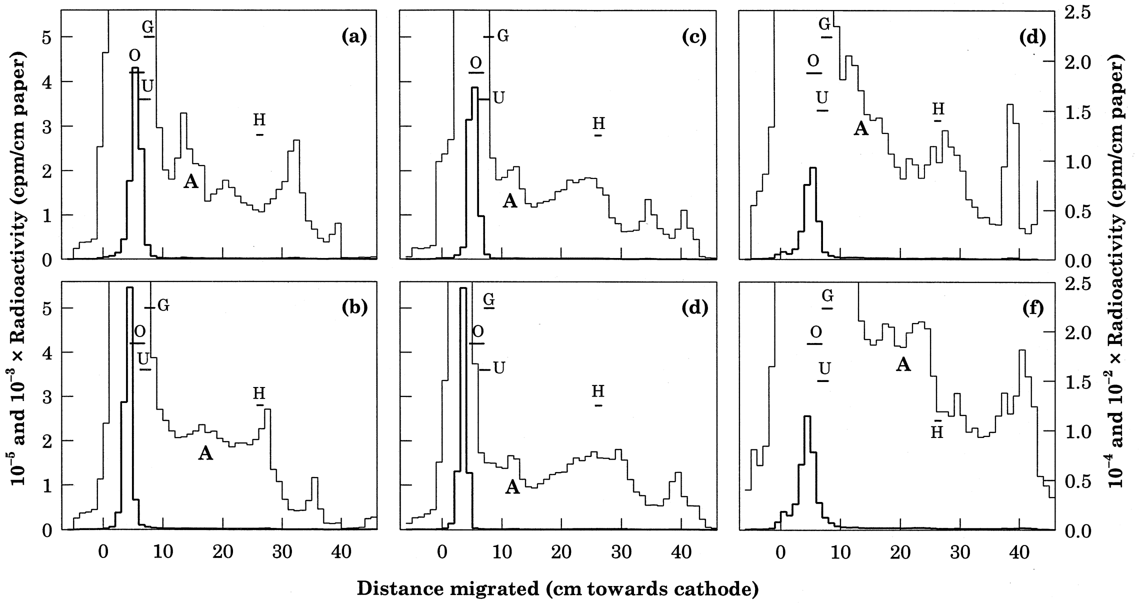

Fig. 6. Radioactive cationic derivatives of oligo-uronides from plant cell walls. The walls of [6-^{14}C]GlcA-fed cells were digested with, sequentially, Driselase, dilute TFA, and a proteinase. Digestion products were analysed by PE at pH 2.0 (2.5 kV for 110 min). The positions of external markers are shown (———; G = glucose, U = galacturonic acid, O = oligogalacturonide mixture of DP 2–6, H = hydroxyproline). (a), (b) = Driselase-soluble material from spinach; (c), (d) = Driselase-soluble material from tomato; (e), (f) = Driselase-insoluble material from tomato. (a), (c), (e) = Digested with Pronase; (b), (d), (f) = digested with trypsin. Cationic material in zones **A** (from each track, 5–9 neighbouring strips of paper, pooled) was analysed further (Fig. 7).

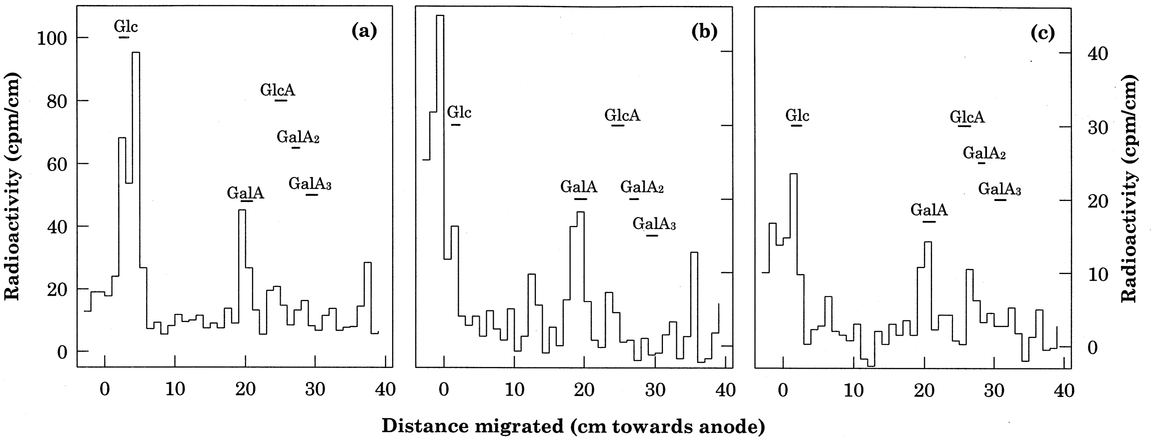

Fig. 7. Radioactive products of acid hydrolysis of cationic derivatives of oligo-uronides from plant cell walls. Selected peaks from the electrophoretograms shown in Fig. 6 were hydrolysed in 2 M TFA (120° for 1 h) and then re-run by PE at pH 3.5. The positions of markers (Glc, GalA, GlcA, $GalA_2$, $GalA_3$) are shown (———). The GalA was an *internal* marker, stained after scintillation counting. (a) = peak **A** from Fig. 6(a); (b) = peak **A** from Fig. 6(d); (c) = peak **A** from Fig. 6(e)

requirement for proteinase digestion suggests that the positively charged group arose from a peptide backbone rather than some other amino compound such as spermidine or glucosamine. Thus, the observed digestion products may represent the core of a pectin–protein bridge.

EXPERIMENTAL

Preparation of Nα- *and* Nε-*GalA–Lys*

To 15 ml of a soln containing GalA monohydrate (0.5 g) and L-Lys Me-ester hydrochloride (1 g) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC; 1 g). The mixture was incubated for 36 min at 25° and then 1.25 ml 10 M NaOH was added to remove the protecting Me ester group. After 15 min, the soln was adjusted to pH 4 by the addition of HOAc, diluted to 50 ml and applied to a 1.5 × 15 cm column of Dowex-50 resin (pyridinium form). Solutes were eluted with a gradient of 0.5–100% buffer (pyridine/HOAc/H_2O, 1:1:3, pH ~ 4.7). Those fractions of the eluate which when spotted on to filter paper stained with both ninhydrin and AHP were pooled and subjected PE at pH 2.0. The two main bands were eluted from the electrophoretogram with H_2O by the method of Ref. [22] and stored as aq. solns frozen at –20°.

Preparation of Nε-*(poly-GalA)–Lys*

To 20 ml of a soln containing sodium polygalacturonate (poly-GalA; 75 mg, from orange) and *N*α-benzyloxycarbonyl-L-Lys Me-ester (α-Z-Lys–Me; 250 mg; Novabiochem) was added EDAC (250 mg). The reaction mixture was incubated for 24 h at 25° and then applied to a column of Bio-Gel P-2 (equilibrated and eluted in pyridine/HOAc/H_2O, 1:1:23). Aliquots (50 μl) of the eluate were assayed for poly-GalA by the *m*-hydroxybiphenyl method [23]. Further aliquots were analysed by PC in BAW (ninhydrin staining) to confirm separation of the modified poly-GalA from lower-M_r reagents. The desalted, modified poly-GalA was incubated in 1 M NaOH at 25° for 1 h to remove the Me ester groups of the α-Z-Lys–Me residues and then neutralised with HOAc and dialysed against H_2O. The Z groups were removed by catalytic hydrogenation, carried out by bubbling H_2 through the soln in 1 M HOAc containing a 2% (w/v) suspn of palladium black at ambient temp and pressure. Removal of the Z groups, monitored by the increase in sensitivity of the modified poly-GalA to ninhydrin staining, was complete within ~8 h. Palladium black was removed by centrifugation (2000*g*) and the *N*ε-(poly-GalA)–Lys soln was freeze-dried and stored at –20°.

Susceptibility of isopeptide bonds to cleavage by acid, alkali and enzymes

Acid hydrolysis of *N*α- and *N*ε-GalA–Lys and *N*ε-(poly-GalA)–Lys was carried out in 2 M CF_3COOH (TFA) at 120°. Hydrolysis products were analysed by PE at pH 2.0 or by PC in EAW.

Alkaline degradation of the compounds was carried out in 0.16 M $Ba(OH)_2$ or in 0.5 M NaOH at 25° or 60°. Degradation in $Ba(OH)_2$ was stopped by neutralisation with H_2SO_4 followed by pelleting of the insoluble $BaSO_4$. Degradation in NaOH was stopped by the addition of 2 equivalents of HOAc. Products were analysed by PE at pH 2.0 or PC in EAW.

Further samples of the compounds were incubated with the following enzymes: (a) 0.5% Driselase (a mixture of polysaccharide hydrolases from *Irpex lacteus*; partially purified [24]) in buffer A (pyridine/HOAc/H_2O, 1:1:98, pH $\sim$ 4.7; (b) 0.5% Pronase (Type XIV, from *Streptomyces griseus*) in 30 mM NH_4HCO_3 (pH 7.9), containing 1 mM $CaCl_2$ [25]; (c) 0.5% trypsin (Type I, from bovine pancreas) in 100 mM $(NH_4)_2CO_3$ (pH 8.5), containing 1 mM $CaCl_2$ [25]. In each case, the buffer contained 0.5% 1,1,1-trichloro-2-methylpropan-2-ol to prevent microbial growth and digestion was carried out for 48 h at 25°. Products were analysed by PE at pH 2.0 and by PC in EAW, BPW and APAW (4:1:3:2). Prior to PC or PE, Driselase digests were acidified with HCO_2H (to 15%) to prevent digestion of the chromatography paper.

Radiolabelling of cell cultures

Suspension-cultures of spinach (*Spinacia oleracea* L.) and tomato (*Lycopersicon esculentum* × *Lycopersicon peruvianum*) were maintained as described before [24, 26]. At 4–5 days after sub-culture, 0.2-g (fresh weight) portions of the cells were transferred aseptically into 60-ml polycarbonate beakers (Sterilin) containing 100 μCi of D-[6-^{14}C]glucuronic acid (55 Ci/mol) (synthesised by the method of Ref. [27]) and incubated for 48 h at 25°. The cells were then filtered and washed briefly with H_2O.

The radiolabelled cells were stirred in 2×8 ml of PhOH/HOAc/H_2O (2:1:1, w/v/v) at 25° for a total of 48 h, and the insoluble (cell wall-rich) material was collected by centrifugation, washed repeatedly in buffer A to remove all detectable PhOH, and then shaken gently in 200 μl of 5% Driselase in buffer A. The digest was centrifuged (800*g* for 5 min) and both the supernatant and pellet were dried *in vacuo* and treated with mild acid (0.1 M TFA for 1 h at 100°) to remove the arabinofuranose side chains of extensin [21]. This acid treatment would be expected to cleave very few *N*-GalA–Lys amide bonds, which had been shown to have a half-life of 30–50 min in 2 M TFA at 120°. The hydrolysed samples were re-dried *in vacuo* and digested with Pronase or trypsin, as above; products were analysed by PE at pH 2.0. The two proteinases were selected on the basis of their different specificity: trypsin cleaves preferentially at Lys or Arg residues whereas Pronase is less specific [25].

The positively charged products (which migrated towards the cathode relative to a neutral marker, glucose) were eluted from the electrophoretogram [22] and hydrolysed in 2 M TFA at 120° for 1 h. The monosaccharides produced were analysed by PE at pH 3.5 to resolve different uronic acids [28]. Putative [^{14}C]GalA was eluted from the paper and re-analysed by PC in BAW after the addition of GalA as an internal marker.

Paper chromatography (*PC*) *and paper electrophoresis* (*PE*)

Descending PC was performed on Whatman 3MM (57-cm lengths) using APAW (Me_2CO/pyridine/HOAc/H_2O, 4:1:3:2 or 4:3:1:2) for 20 h; BAW (BuOH/HOAc/H_2O, 12:3:5) for 18 or 24 h; EAW (EtOAc/HOAc/H_2O, 10:5:6) for 28 h; or BPW (BuOH/pyridine/H_2O 4:3:4) for 48 h.

PE was performed on Whatman 3MM or No. 1 paper, in buffer of pH 2.0 (HCO_2H/HOAc/H_2O, 1:4:45, usually at 3 kV for 50 min); pH 3.5 (HOAc/pyridine/H_2O, 10:1:189, 3 kV for 70 min); or pH 6.5 (HOAc/pyridine/H_2O 1:33:300, 3 kV for 50 min). The papers (40×57 cm) were kept at $< 30°$ by white spirit during electrophoresis. Detection was effected with 0.5% ninhydrin in acetone for amino compounds or AHP for reducing sugars [29].

Assay of radioactivity

Dry samples (strips cut from paper electrophoretograms or chromatograms) were placed in 2 ml of OptiScint "HiSafe" scintillant (Fisons, Loughborough) and assayed in a liquid scintillation counter at about 73% efficiency. Radioactivity in aq solns was assayed (at about 86% efficiency) after the addition of 10 volumes of OptiPhase "HiSafe" 3 (Fisons).

NMR spectroscopy

The NMR spectra were measured on ^2H_2O solns with a Varian INOVA 600-MHz spectrometer operating at 599.9 MHz for ^{1}H and 150.9 MHz for ^{13}C nuclei.

1D TOCSY [30] ^{1}H spectra were obtained using the sequence: D1–180°sel–90°–D2–90°–D3–90°–AQ with D1 = 2 s (pre-saturation of residual HOD signal), 180°sel = 300 ms (= 20 Hz), D2 = 0.12 s (pulsed spin lock [31]), D3 = 0.01 s (z-filter) AQ = 2.56 s. Other parameters were SW = 7000 Hz; 32K data points. A shaped (IBURP) [32] selective spin inversion pulse was applied on- and off-resonance on alternate scans and the FID's alternatively added and subtracted to give a "difference" FID which, on transformation, gave spectra showing only TOCSY responses.

The 2D TOCSY phase sensitive ^{1}H spectra were obtained using the sequence [31]: D1–90°–t1–D2–AQ with D1 = 3 s (pre-saturation) D2 = 0.08 s (spin lock), AQ = 0.294 s. An 8-step phase cycle (hypercomplex acquisition) was used. Other parameters were SW = 7000 Hz; 2K data points; 128 increments. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1-data to 512 data transformation.

The 2D ^{1}H-detected one-bond ^{1}H-^{13}C correlation (HMQC) spectra were obtained using the sequence [33]: D1–90°(^{1}H)–D2–180°(^{1}H); 180°(^{13}C)–D2–90°(^{1}H)–D3–90°(^{1}H)–D2–90°(^{13}C)–$t_1/2$–180°(^{1}H)–$t_1/2$–

90°(^{13}C)–D2–AQ. The delays used were D1 = 1.8 s (pre-saturation), D2 = 3.7 ms [$1/(2^1J_{CH})$] and D3 = 500 ms (to minimise signals from protons bonded to ^{12}C nuclei). The experiment was preceded by 128 dummy scans to establish thermal equilibrium. A 16-step phase cycle (hypercomplex acquisition) was used with ^{13}C broad band decoupling during acquisition of the ^{1}H signals. Other parameters were SW(^{1}H) = 6000 Hz; 2K data points; 256 increments; SW(^{13}C) = 31000 Hz, AQ = 0.171 s. The data were processed using shifted sine-bell squared functions in both dimensions with zero filling of the F_1 data from 256 to 1024 W before transformation.

Acknowledgements—P.P. thanks the European Communities' Biotechnology Programme for the award of a research fellowship within the Project of Technological Priority, 1993–1996.

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