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INCORPORATION OF [^{14}C]CINNAMATE INTO HYDROLASE-RESISTANT COMPONENTS OF THE PRIMARY CELL WALL OF SPINACH

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Key Word Index—*Spinacia oleracea*, Chenopodiaceae, spinach, suspension culture, primary cell wall, cinnamate, phenols, oxidative phenolic coupling, peroxidase

Abstract—[^{14}C]Cinnamate was taken up very rapidly by cultured spinach cells and completely incorporated into low-MW conjugates within 20 min The ^{14}C-labelled products were similar whether the [^{14}C]cinnamate was supplied continuously over a period of hours via a peristaltic pump or instantaneously Radioactivity was slowly recruited from the low-MW pool into aromatic components of the cell-wall fraction Saponification of the radioactive wall fraction yielded, in addition to radioactive ferulate and *p*-coumarate, large amounts of ethyl acetate-soluble radioactive material with the properties of oxidatively coupled phenols The coupled material was associated with the most highly 'Driselase'-resistant fractions of the cell wall In contrast, 'Driselase' released most of the wall's ferulate and *p*-coumarate on disaccharide fragments It is suggested that the oxidatively coupled phenols are formed from simpler phenols by peroxidase and that they cross-link the polysaccharides to which they are attached, making these polysaccharides relatively 'Driselase'-resistant

INTRODUCTION

The primary cell walls of higher plants contain phenolic compounds covalently linked to the structural polymers These phenols are different from lignin and they occur in rapidly-growing cell walls [1, 2] Examples of such phenols that have been chemically identified are protein-bound tyrosine and its dimeric oxidation product isodityrosine [3] and the polysaccharide-bound cinnamate derivatives *p*-coumarate and ferulate [1, 4, 5]

Interest in wall-bound phenols centres around their propensity to undergo oxidative reactions, especially the peroxidase-catalysed coupling of pairs of phenol molecules [3–9] Roles for oxidative phenolic coupling have been proposed in the lowering of wall extensibility [2, 3, 5, 9, 10], the induction of resistance to fungal invasion [5, 11, 12] and the initiation of lignification [1, 5, 13, 14]

Of the cinnamate derivatives, *p*-coumarate and ferulate are easy to study owing to their UV fluorescence [1, 5] Preliminary evidence is, however, also available for other, less fluorescent polysaccharide-bound phenols [5, 15] The present work was designed to verify the occurrence of these other phenols in primary cell walls and to investigate their possible role in wall polymer cross-linking The work is based on *in vivo* radioactive labelling of the phenols by their presumed precursor, [^{14}C]cinnamate, followed by partial enzymic digestion of the cell walls with 'Driselase' This commercial enzyme mixture cleaves polysaccharides to yield limit digestion products still bearing their phenolic groups [4, 5] For example, 'Driselase' releases 60% of the ferulate from spinach cell walls in the form of the feruloylated disaccharides 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose and 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose [4] It was anticipated that the ^{14}C-labelled phenols under investigation in the present work would be similarly solubilized by 'Driselase' as phenol–oligosaccharide complexes amenable to chromatographic study

RESULTS

A large proportion (84 7%) of the [^{14}C]cinnamate supplied to an exponentially-growing spinach culture was taken up from the culture medium within 1 min The initial products were largely methanol-soluble, and methanol extracts taken during a labelling time course were subjected to TLC in system 1 The cinnamate (R_f 0 86) and any free hydroxylation products (*p*-coumarate, caffeate, ferulate, not resolved from cinnamate) were completely metabolized within 20 min yielding polar derivatives, some of which (R_f values 0 80, 0 75, 0 69, 0.60) were themselves further metabolized to products (R_f values 0 51, 0 43) which persisted for at least 20 hr Other, minor, highly polar derivatives ($R_f \sim$ 0 26) accumulated ^{14}C to a plateau within 1 min and maintained a similar level of labelling for at least 20 hr This highly polar material was strongly acidic upon paper electrophoresis (PE) (at pH 3 5, $m_{picrate}$ = 0 7, at pH 2 0, $m_{Bromophenol\ Blue}$ = 0 6), suggesting the presence of phosphate groups and is therefore tentatively identified as (hydroxy)cinnamoyl thioesters of coenzyme-A [16] The other metabolites were not identified individually, but PE (pH 3 5) showed that they included (possible identification in parentheses based on known widely-occurring conjugates) (i) neutral species, $m_{picrate}$ = 0 0 (cinnamoyl and hydroxycinnamoyl glucosides [17, 18]), (ii) at least two weakly acidic species,

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$m_{picrate}$ = 0 2, 0 3 (hydroxycinnamoyl esters of quinate and shikimate [19, 20]), and (iii) a minor basic species, $m_{picrate}$ = −0 2 (hydroxycinnamoyl amide of putrescine [21])

Secreted and wall-bound metabolites

Concurrently with this metabolism, there was a gradual recruitment of ^{14}C into a methanol-insoluble pool (hydroxycinnamoyl esters of polysaccharides, etc) such that after 20 hr, 9 % of the ^{14}C was methanol-insoluble In addition, after 20 hr, *ca* 10 % of the ^{14}C initially taken up had been secreted back into the culture medium in the form of compounds that could be partitioned from the medium into butanol These were probably related to the extracellular phenols X_1–X_5 previously shown to be secreted by spinach cells [2]

The composition of the methanol-insoluble pool was investigated as a function of time (Fig 1) Recruitment into this pool continued for at least 11 days, the rate gradually falling off Very little (less than 1 %) of the methanol-insoluble ^{14}C could be dissolved by sonication in water or 2 M ammonium formate or ethyl acetate (data not shown) The radioactive products of alkaline hydrolysis of the methanol-insoluble material included *p*-coumaric and ferulic acids and the ferulate *p*-coumarate ratio gradually rose (Fig 1) as would be expected from ^{14}C-flow along the pathway cinnamate

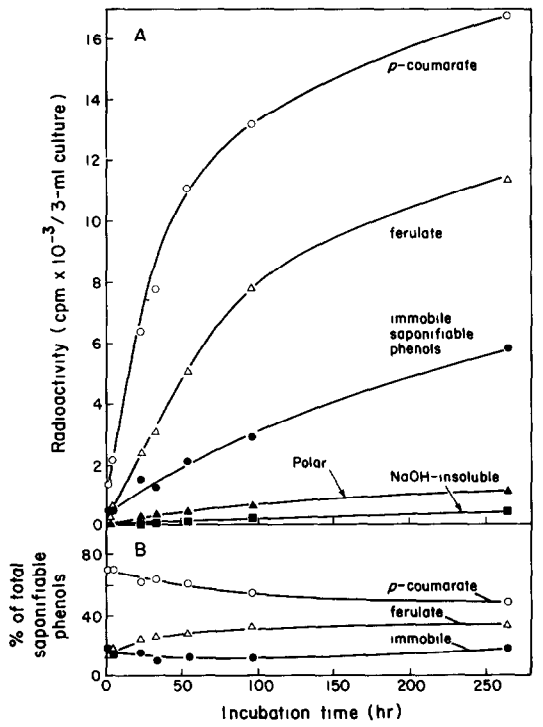


Fig 1 Time course for incorporation of [^{14}C]cinnamate into spinach cell-wall components The walls were saponified and fractionated into (i) *p*-coumarate, (ii) ferulate, (iii) immobile saponifiable phenols, (iv) sodium hydroxide-solubilized material failing to partition into ethyl acetate after acidification ('polar') and (v) sodium hydroxide-insoluble material (A) Absolute cpm in fractions (i) to (v) The total ^{14}C available to the cells was *ca* 140000 cpm/3 ml culture (B) Relative distribution of radioactivity between the saponifiable wall phenols [(i) + (ii) + (iii)]

→ *p*-coumarate → caffeate → ferulate In addition, some unknowns became radioactive, most of which (after alkaline hydrolysis) were immobile on TLC (system 2)

Specificity of labelling

In order to determine whether ^{14}C was escaping from the cinnamate pathway and entering general metabolism (e g via $^{14}CO_2$), incorporation into cellulose was investigated The methanol-insoluble, sodium hydroxide-insoluble, cellulose-rich fraction from the 264 hr time point in the above experiment contained only 1 1 % of the total methanol-insoluble ^{14}C (Fig 1) Furthermore, Saemen hydrolysis of the cellulose-rich fraction [9] followed by PC (system 3) yielded no radioactivity in the hexose spot Thus, none of the ^{14}C from cinnamate was incorporated into wall glucosyl residues after 11 days of metabolism

Validity of feeding method

Since the exogenous [^{14}C]cinnamate was taken up almost instantaneously, the initial mean concentration of free cinnamate in the cells in the above experiments would have been over 20 μM, which may be physiologically abnormal Therefore, detoxification mechanisms may have been in operation, competing with the flow of ^{14}C along the normal cinnamate pathway To test for this possible artefact, [^{14}C]cinnamate was supplied to a shaken spinach culture gradually, by means of a peristaltic pump over a period of 5 5 hr, followed by a further 3 5 hr incubation A control culture was fed an equal amount of [^{14}C]cinnamate instantaneously and incubated under identical conditions for 9 hr Essentially identical 9 hr labelling patterns were seen from the two cultures upon TLC (systems 1 and 2) of (i) methanol-soluble cell material, (ii) methanol-insoluble cell material (after alkaline hydrolysis) and (iii) butanol-extractable material in the culture medium Only minor quantitative differences were noted, and these were attributable to the fact that in the pump-fed cultures the [^{14}C]cinnamate had been undergoing metabolism for 3 5–9 hr, whereas in the controls it had all been undergoing metabolism for a full 9 hr This observation suggests that [^{14}C]cinnamate supplied in relatively large amounts as a single pulse has a metabolic fate similar to that of cinnamate supplied gradually at a rate mimicking the *in vivo* action of phenylalanine ammonia-lyase

Enzymic hydrolysis of cell walls containing [^{14}C]*cinnamate derivatives*

For this work, long-term labelling was used so as to favour the incorporation of ^{14}C into any phenol oxidation products After 33 days of (*ca* eight cell doublings) exposure to [^{14}C]cinnamate, 38 % of the ^{14}C was recovered in the cell-wall fraction Of this wall-bound ^{14}C, 94 5 % was soluble in 0 5 M sodium hydroxide and 54 5 % of this could be partitioned into ethyl acetate after acidification This ethyl acetate solution is referred to as 'saponifiable phenols' The radioactive saponifiable phenols included ferulic and *p*-coumaric acids (Table 1), caffeic, sinapic, cinnamic and diferulic acids were absent or failed to dissolve in the solvent (Poor solubility of diferulic acid in ethyl acetate [22, 23] and a tendency of cinnamic acid to bind to glassware [17] have been reported) However,

Table 1 Saponifiable [^{14}C]phenols from 'Driselase' digestion products of cell walls labelled *in vivo* with [^{14}C]cinnamate

Fractions from 'Driselase' digest	Total cpm	% Composition of saponifiable [^{14}C]phenols		
		p-Coumarate	Ferulate	Other*
Water-insoluble	92000	15 1	6 2	78 7
Water-soluble, methanol-insoluble	126200	14 1	11 9	74 0
Methanol-soluble	160100	31 7	19 1	49 2
Total	378300	21 8	13 6	64 6

*Very largely immobile on TLC (system 2)

ferulic and *p*-coumaric acids accounted for only part of the radioactive saponifiable phenols, most of the remainder was chromatographically immobile (system 2) This immobile material was most abundant (relative to ferulate + *p*-coumarate) in the 'Driselase'-insoluble wall residue, less abundant in the polysaccharides solubilized by 'Driselase' digestion and least abundant in the methanol-soluble products of 'Driselase' digestion (Table 1) Thus the immobile saponifiable phenols appeared to be associated with 'Driselase' resistance

The 'Driselase'-generated, methanol-soluble products were further fractionated on Sephadex LH-20 (Fig 2) In general, there was correspondence between the peaks of UV-absorbing material and of radioactivity, supporting the view that [^{14}C]cinnamate efficiently and specifically labels the aromatic components of the cell wall The digestion products eluting in or near the void volume were enriched in the immobile saponifiable phenols, whereas those eluting last were enriched in ferulic and *p*-coumaric acids (Table 2) Thus again, the immobile saponifiable phenols appeared to be associated with 'Driselase' resistance

Table 2 Saponifiable [^{14}C]phenols from Sephadex LH-20 fractions of the methanol-soluble 'Driselase' digestion products

LH-20 fraction*	Cpm analysed†	% Composition of saponifiable [^{14}C]phenols		
		p-Coumarate	Ferulate	Other‡
A	3900	16 0	11 0	73 0
B	2150	18 3	18 3	63 4
C	3760	25 6	18 1	56 3
D	1070	24 9	13 9	61 2
E	1020	26 3	16 2	57 5
F	1820	29 2	20 2	50 6
G	4250	32 6	29 3	38 1
H	650	41 4	17 6	41 0
I	3290	44 2	30 0	25 8
Total	21910	28 0	20 7	51 3

*See Fig 2

†This represents *ca* 20% of the total ^{14}C in the LH-20 eluate

‡Very largely immobile on TLC (system 2)

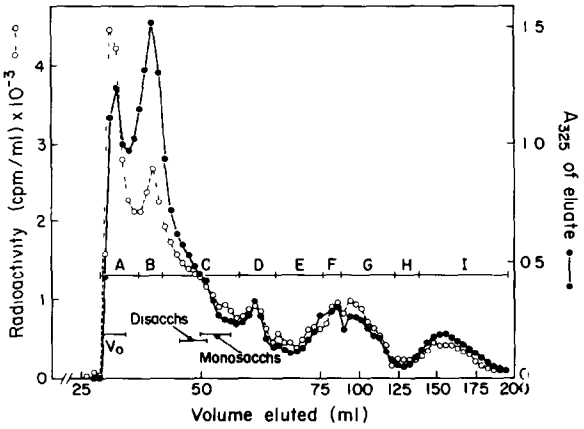


Fig 2 Chromatogram on Sephadex LH-20 of the 'Driselase' digestion products of cell walls isolated from a spinach culture that had been fed with [^{14}C]cinnamate The fractions were pooled as shown (A–I) The void volume (V_o) was previously determined with blue dextran Radioactivity measurements are corrected for background

PC (system 3) showed that Sephadex fraction G (see Fig 2) was predominantly 4-*O*-(6-*O*-feruloyl-β-D-galactopyranosyl)-D-galactose and the corresponding *p*-coumaroyl compound, and that fraction I was rich in 3-*O*-(3-*O*-feruloyl-α-L-arabinopyranosyl)-L-arabinose and the corresponding *p*-coumaroyl compound (Fig 3) [4] Fractions A–F (see Fig 2) had lower R_f values on PC (for both ^{14}C and UV fluorescence) and there was a positive correlation between R_f and Sephadex elution volume (Figs 2 and 3) This supports the view that the compounds eluting earlier from Sephadex were of higher MW

DISCUSSION

The results show that ^{14}C from exogenous cinnamate is rapidly taken up by spinach cells and metabolized only into products bearing a close metabolic relationship to cinnamate, via pathways similar or identical to those occurring naturally The metabolites were found as low-MW conjugates, the major species of which were probably in equilibrium with a small, rapidly turning-over pool of CoA thioesters, providing a steady supply of activated (hydroxy)cinnamoyl groups for transferring to nascent

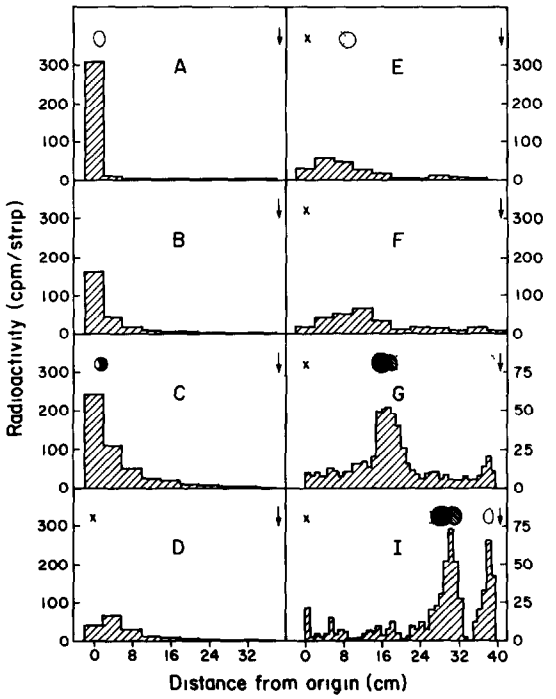
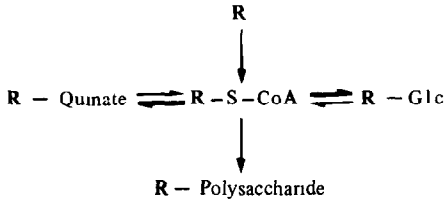


Fig 3 Paper chromatography of fractions (A–I) from the Sephadex LH-20 chromatogram described in Fig 2 For each chromatogram, the ^{14}C-profile is given (corrected for background) together with a drawing of the UV-fluorescent spots Fluorescence was categorized as feruloyl ester-like (●, strong, ○, moderate, , faint) or *p*-coumaroyl ester-like (◍), as described in ref [4] The origin (×) and solvent front (↓) are shown



Scheme 1 **R** is the radioactive aromatic moiety, somewhere along the pathway cinnamate → *p*-coumarate → caffeate → ferulate

polysaccharides [4] during a period of several weeks (Scheme 1)

Since the principal metabolites of cinnamate are phenols, the feeding of [^{14}C]cinnamate should be a useful way of labelling the unidentified phenols believed to be present in the primary cell wall Any phenol oxidation products should also become labelled, some of the oxidation products might not possess free phenolic hydroxy groups [22] and might therefore be difficult to recognize by routine phenol analysis

Evidence was obtained by this method that the primary walls of cultured spinach cells contain quite large amounts of chromatographically immobile saponifiable phenols as well as of ferulate and *p*-coumarate The walls contained *ca* 0 4% (dry wt) ferulic acid, the combined data would suggest *ca* five times as much of the immobile saponifiable phenols (Table 1) if [^{14}C]cinnamate labelled all the wall phenols with equal efficiency

The immobile saponifiable [^{14}C]phenols appeared to be associated with relatively 'Driselase'-resistant regions of the cell wall (Table 1, Fig 2) Table 1 illustrates this from solubility data because it is likely that the water-insoluble products were of highest MW (least digested) and that the products soluble in both water and methanol were of lowest MW (most digested) Fig 2 illustrates the same phenomenon from gel chromatography data on the following grounds Other factors being equal, Sephadex LH-20 separates on the basis of MW by the normal principles of gel chromatography Elution of aromatic compounds may be delayed owing to adsorption to the gel, but compounds do not normally elute much earlier than predicted by their MW [5] Therefore, chromatography on LH-20 can provide a minimum estimate of the actual MW Thus, in Fig 2, fraction A is of MW > 4000, fraction B is of MW > 1000, and the later-eluting fractions are probably of progressively lower MW

Other studies, involving *in vivo* labelling with D-[1-^{3}H]glucuronic acid and L-[1-^{3}H]arabinose (unpublished), have shown that exhaustive 'Driselase' treatment cleaves spinach cell-wall polysaccharides predominantly to mono- and disaccharides It is therefore significant to find in the partial digestions reported here an enrichment of the immobile saponifiable [^{14}C]phenols in the relatively 'Driselase'-resistant polysaccharides and higher oligosaccharides 'Driselase' resistance may indicate polysaccharide cross-linking, leading to molecular domains from which hydrolytic enzymes are excluded The observations reported here are therefore in accord with the idea that immobile saponifiable phenols are themselves involved in cross-linking the polysaccharides

The idea of phenolic cross-links between polysaccharide molecules is strengthened by a consideration of the possible chemistry of the phenolic moiety Solubilization by 'Driselase' suggests association of the ^{14}C with wall polysaccharides The fact that the immobile saponifiable [^{14}C]phenols became ethyl acetate-soluble only after treatment with cold dilute alkali suggests that the phenols were attached to the wall polymers through ester-linkages Their solubility in ethyl acetate but immobility on TLC (system 2) suggests, by analogy with the results of Baumgartner [24], that they were oxidatively coupled [^{14}C]phenols Their proposed role in cell-wall architecture is therefore quite plausible, since the oxidative coupling of two polysaccharide-bound phenols would automatically cross-link the two polysaccharide molecules involved [22]

The precise chemistry of oxidative phenolic coupling is complex and incompletely understood [3, 8, 22, 24, 25] In view of this complexity, it will be difficult to identify the immobile saponifiable [^{14}C]phenols reported in the present work They are likely to comprise a complex mixture of coupled oligomers It is for this reason that in the present work a TLC system was chosen that effects a group-separation of oligomeric phenolic coupling products such that they all have R_f values close to 0

Attachment of monomeric hydroxycinnamoyl groups alone (e g ferulate and *p*-coumarate) can render polysaccharides less digestible by enzymes [26] The present work suggests that the phenol oxidation products are associated with considerably stronger enzyme resistance

Further evidence that coupled saponifiable phenols do contribute to the structural coherence of cell walls comes from the observation that slightly acidified sodium chlorite (which cleaves phenolic coupling products [3]) and methanolic sodium methoxide (which cleaves esters) will solubilize some otherwise water-insoluble wall polysaccharides [27, 28]

Phenols are classic substrates of peroxidase, which is a characteristic cell-wall enzyme whose secretion is controlled hormonally [9] Thus the peroxidase-catalysed oxidative coupling of polymer-bound phenols is a feasible reaction *in vivo* and could play a role in governing the properties of the cell-wall polymers In particular, such coupling could cross-link the phenol-bearing polymers, this could in itself increase the coherence of the cell wall and it could also help to exclude the endogenous hydrolytic enzymes which may be involved in wall-loosening during cell expansion The apparent protection of polysaccharides against 'Driselase' by coupled phenols, reported here, could be a model for the latter idea

EXPERIMENTAL

Materials *trans*-[side-chain-3-^{14}C]Cinnamic acid (60 mCi/mmol) was from Amersham International, U K It was dried from toluene soln in a culture flask, culture medium was added, and the radioactive medium adjusted to pH 4 4 and autoclaved

'Driselase', a crude mixture of basidiomycete enzymes including exo- and endo-polysaccharidases [5], was from Kyowa Hakko Europe G m b H , Dusseldorf It was partially purified as described in ref [4]

Suspension cultures A green cell suspension culture of spinach (*Spinacia oleracea* L , cv Monstrous Viroflay, line G-10 of Dalton and Street [29]) was maintained in hormone-free medium containing 1% sucrose at pH 4 4 under fluorescent lighting [4] and subcultured once a fortnight For the labelling expts, cultures were grown in medium supplemented with [^{14}C]cinnamate at 0 025 μCi/ml (= 0 42 μM) Initial cell densities, determined as in ref [30], were 20–50 μl PCV/ml suspension For the expt where [^{14}C]cinnamate was added gradually, the cells were inoculated into 20 ml medium and then [^{14}C]cinnamate (0 625 μCi in 5 ml medium) was supplied at 0 91 ml/hr by means of a peristaltic pump, while the culture was being incubated on a reciprocal shaker The mouth of the delivery tube (from the pump) was submerged in the cell suspension so that the [^{14}C]cinnamate was added steadily rather than as drops For the long-term labelling expt (Figs 2 and 3, Tables 1 and 2), a 200 ml culture was grown in the presence of 5 μCi [^{14}C]cinnamate for 15 days and then the cells were aseptically collected by filtration on muslin, resuspended in 800 ml of fresh non-radioactive medium and grown for a further 18 days Yield, 1 3 g cell walls

Analysis of phenols For MeOH extraction, the cells were harvested by filtration on muslin, weighed, transferred into 5 vols MeOH and shaken overnight at 4° in the dark Samples of the extract were subjected to TLC (system 1) and PE (system 4) For preparation of cell walls, the MeOH-insoluble residue was washed with 85% MeOH until the washings were no longer radioactive, followed by $CHCl_3$–MeOH–H_2O (10 10 3) The chlorophyll-free residue was dried and sonicated with a Dawe 'Soniprobe' in 2 M ammonium formate to remove ionically-bound proteins The wall residue was washed with H_2O, freeze-dried and stored at −20° Saponification was conducted with 0 5 M NaOH at 20° in the dark under N_2 for 16 hr The saponification products were acidified to pH 2 with H_3PO_4 and partitioned against EtOAc, the organic phase was analysed for phenolic acids by TLC in system 2

For enzymic digestion, cell walls (300 mg) were treated with purified 'Driselase' (160 mg) in 4 ml 25 mM NaOAc buffer, pH 4, containing 1 mM NaCN (as a peroxidase inhibitor), at 20° for 6 hr The partial digestion products were centrifuged at 12 000 *g* for 10 min and the pellet was washed with 3 × 1 ml H_2O The residue was the 'Driselase' insoluble material (Table 1) The pooled supernatants were made up to 85% MeOH and stored overnight at 4° The ppt was collected by centrifugation (12 000 *g* for 10 min), washed with 3 × 1 ml 85% MeOH, redissolved in H_2O and freeze-dried (= soluble polysaccharide fraction) The pooled supernatants were rotary-evapd to dryness and constituted the MeOH-soluble fraction

Chromatography and electrophoresis TLC on silica gel was performed in solvents 1 (BuOH–HOAc–H_2O, 4 1 1) and 2 (C_6H_6–HOAc, 9 1, under continuous exposure to 360 nm UV light [5]) PC was on Whatman No 1, by the descending method, in solvent 3 (BuOH–HOAc–H_2O, 15 3 5) PE was on Whatman No 1, in solvent 4 (HOAc–pyridine–H_2O, 10 1 189, pH 3 5) and solvent 5 (HCOOH–HOAc–H_2O, 1 4 45, pH 2 0), with 'white spirit' at 20–30° as coolant, at 5 kV for 30 min Picric acid and Bromophenol Blue were used as acidic markers at pH 3 5 and 2 0 respectively, and glucose was the neutral marker CC on Sephadex LH-20 was performed in 35% aq MeOH on a gel bed of 65 ml The loading (1 5 ml 35% MeOH soln) contained the MeOH-soluble 'Driselase' digestion products from 260 mg radioactive cell walls For location of mono- and disaccharides, a portion of each fraction was subjected to PC and stained with aniline hydrogen phthalate [31] Phenolic compounds on chromatograms and electrophoretograms were located by fluorescence under 360 nm UV light in NH_3 vapour

Radioactivity ^{14}C in soln was assayed by liquid scintillation counting after addition of 10 vols 0 5% PPO in toluene–Triton X100 (2 1, by vol) Strips from chromatograms were assayed for ^{14}C without elution, by scintillation counting in 0 5% PPO in toluene, at an efficiency of ~ 90% (TLC) or ~ 20% (PC) TLC plates were autoradiographed by exposure to X-ray film

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