Phenoxyacetic Acid Residue Incorporation in Cell Walls of Soybean (*Glycine max*.)

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The metabolism of [^{14}C] phenoxyacetic acid (POA) and the formation of bound residues were studied in soybean leaves and stems. POA was metabolized to 4-HO-POA and to 4-HO-POA glucoside, and a significant fraction of the radioactivity was incorporated in the cell walls (CW). An extraction procedure of CW polymers was developed to specifically isolate the radioactivity associated with each of them. In leaves, the radioactivity showed a preferential distribution into the hemicelluloses and lignins, while pectins and lignins were the most radioactive CW polymers in stems. The identified bound metabolites were 4-HO-POA, POA, and phenolic residues. The latter and POA were essentially incorporated into the lignin fractions and were linked to the benzylic carbons of lignin monomers. 4-HO-POA, the major bound residue, was more evenly distributed in CW polymers. It was esterified to noncellulosic polysaccharides and lignins, but in the latter, contrary to other POA residues, it was mainly linked at the γ-carbon of propanoid side-chains of lignin monomers. That type of linkage suggested an enzymatic incorporation of 4-HO-POA in CW, contrary to others residues which have an opportunistic lignin incorporation. That incorporation of 4-HO-POA in CW polymers looks like that of endogenous hydroxycinnamic acids.

Keywords: *Bound residues; cell wall; Glycine max.; lignin; phenoxyacetic acid; polysaccharides; soybean*

INTRODUCTION

The degradation of pesticides in plants leads to two types of ultimate products: conjugated metabolites, stored in cell vacuoles; and bound residues, also called nonextractable residues, mainly associated with cell walls (CW). Bound residues are most often not bioavailable when fed to animals, because they are linked to nondigestible CW polymers. For that reason, they are usually considered as devoid of the toxicity. However, the bound residues of some specific pesticides have been found to be partially absorbed by animals (Kacew et al., 1992). That bioavailability can be explained by two reasons: the capacity of intestinal microorganisms to partly degrade some CW constituents and the chemical characteristics of some pesticides, leading to a weaker binding to CW polymers.

It is thus important to determine the amounts and the structures of bound residues, the nature of the CW polymers on which they are fixed, and the types of chemical bonds involved. However, these determinations meet with several difficulties. First, most of the CW polymers are hard to solubilize. In addition, the levels and the natures of bound residues vary with the plant−pesticide combination. And finally, the amounts of nonextractable residues detected can change with the extraction method, because these amounts depend on the efficiency of the solubilization of the various CW polymers and on the preservation of the residue-polymer bonds.

In the present study, we have characterized in soybean the bound residues of phenoxyacetic acid (POA), chosen as a model chemical. Contrary to its chloro-substituted analogues, that molecule is devoid of herbicidal activity, and thus does not disturb plant metabolism. Moreover, its metabolic pathway in plants is rather simple, since it gives rise to an hydroxylated metabolite and to the corresponding glucoside, and possibly also to a glucose ester (Hutber et al., 1978; Cole and Loughman, 1982).

The first goal of the present work was to draw up a protocol allowing to correctly and unambiguously release bound residues. Then, with that fractionation method in hand, we have examined the fate of POA in soybean plants and the attachments of its metabolites to the various polymers of the cell walls.

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals and enzymes were purchased from Sigma (Saint Quentin Fallavier, France). [^{14}C]Phenoxyacetic acid (POA) was synthesized by reaction of bromoacetic acid with [*phenyl*-^{14}C(U)]phenol (Melnikov, 1971). It was then purified by TLC and dissolved in acetone. Synthetic POA was characterized by co-chromatography with a commercial standard and by mass spectrometry. The desired specific radioactivity was obtained by addition of non radioactive POA.

General Procedures. Soybean seeds (cv. Weber) were planted in vermiculite trays watered with one-third strength Hoagland's solution and were grown under a 16/8 h light/dark photoperiod. Eight-week-old plants were treated with 3.3 mM [^{14}C]POA (sp act 2.76 μCi·mmol^{-1}) dissolved in nutrient solution. Plants were exposed to the radioactive solution for 24 h and then collected immediately or grown 48 h more in the absence of POA.

Roots, stems, and leaves from two plants were collected separately. They were cut into small pieces, frozen, and ground with a ball grinder for 10 min. Ground tissues were transferred in methanol−dichloromethane−water (2−1−0.8), stored at −20 °C overnight, and then sonicated for 20 min in ice bath.

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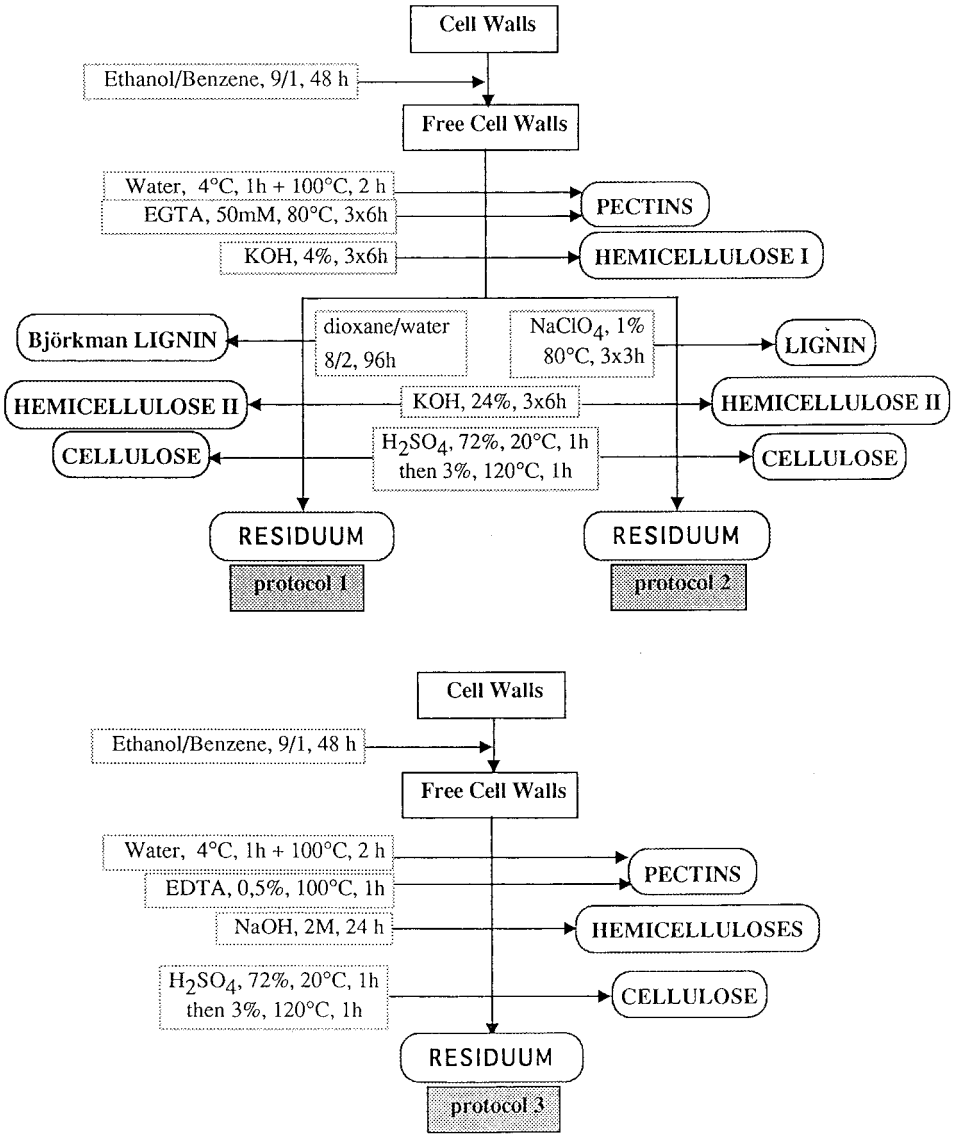

Figure 1. Protocols 1, 2, and 3 for fractionation of FCW containing bound residues of [^{14}C]phenoxyacetic acid.

Insoluble residues were then collected by filtration, and successively washed with the extraction mixture, ethanol, and acetone. Extracting and washing solutions were combined and concentrated by vacuum evaporation at 35 °C to give the cell extract. The remaining CW materials were allowed to air-dry at room temperature for 24 h to remove acetone, and ground with a mortar or a ball grinder. Aliquots were combusted in an oxidizer for radioactivity determination. CW were then Soxhlet extracted with ethanol-benzene (9−1) for 48 h to give the free cell wall fraction (FCW), which was stored under nitrogen in a desiccator.

FCW Fractionation. In a first series of experiments, FCW fractionations were done according to three protocols (Figure 1): protocol 1 after Langebartels and Harms (1985), protocol 2 after Pillmoor et al. (1984) and protocol 3 after Goubet et al. (1994). Taking into account the results of these experiments, a fourth fractionation procedure was then devised, leading to the "final protocol" of Figure 2. In all those protocols, extractions were performed under continuous shaking. After each extraction step, the residues were washed with the extraction mixture or the buffer used for enzymatic digestions, then with water, and all solutions were combined.

Chemical extractions were performed under nitrogen. Alkaline extracts were acidified to pH 2−3 with 12 N HCl at 0 °C before recovering the organo-soluble radioactivity with ethyl ether. The residues solubilized by trifluoroacetic acid (TFA) hydrolysis were also extracted by ether.

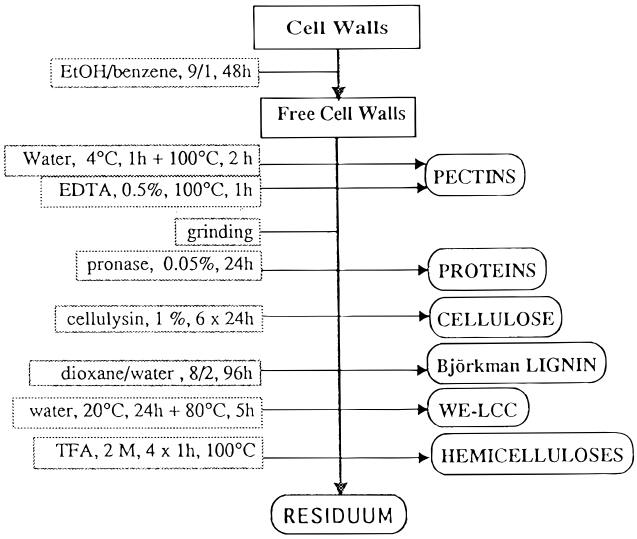

Figure 2. Final protocol for soybean cell wall fractionation and extraction of polymers with their [^{14}C]phenoxyacetic acid bound residues.

Bjorkman lignin was obtained by extraction with a dioxane−water mixture (8/2, v/v). The combined dioxane−water extracts

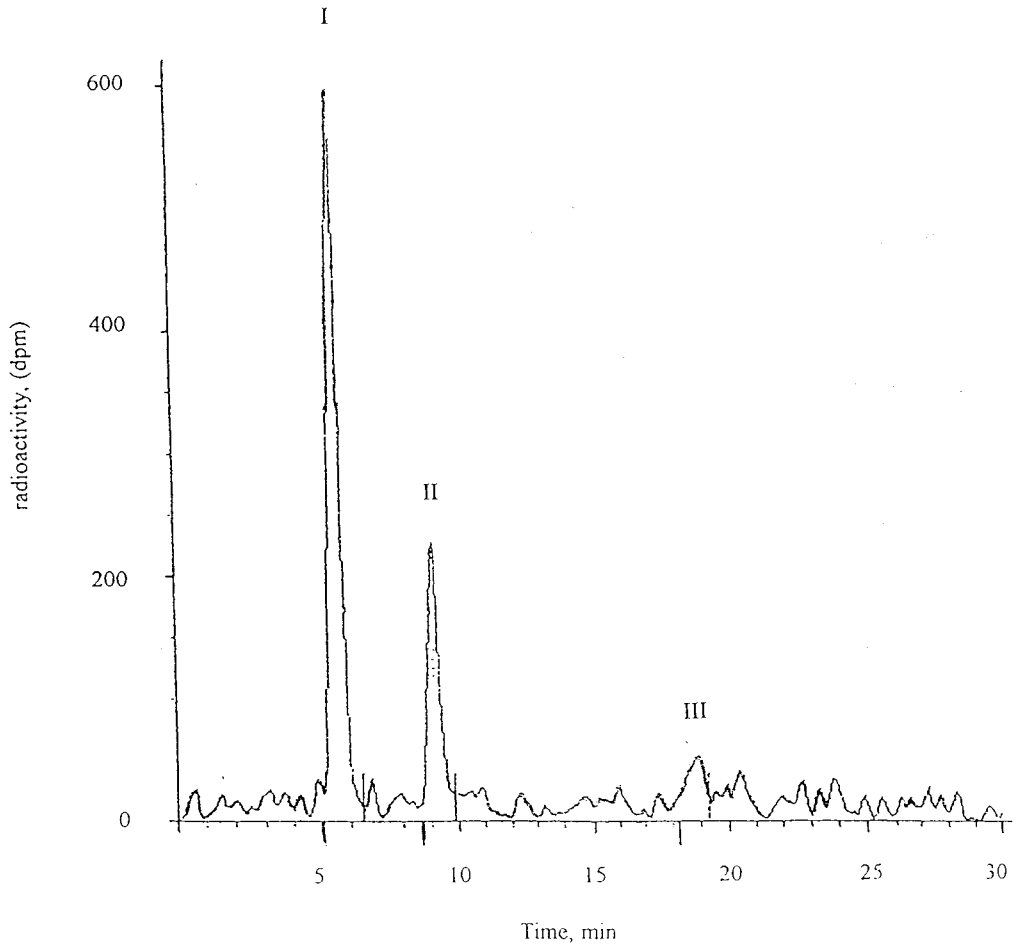

Figure 3. HPLC analysis of soluble metabolites extracted from soybean leaves 72 h after treament by [^{14}C]POA: I, 4-HO-POA-β-D-glucoside; II, 4-HO-POA; III, POA. Solvent A: acetonitrile. Solvent B: 2% (v/v) acetic acid in water. Gradient: hold at 5% A for 10 min, linearly to 50% in 15 min, hold at 50% A for 5 min.

were concentrated under vacuum at 45 °C to give a brown viscous residue. After addition of water, the precipitated Bjorkman lignin was freeze-dried and weighed.

Enzymatic digestions were carried out at room temperature, in reaction mixtures containing 0.05% chlorbutol. Proteins were hydrolyzed for 24 h with 0.05% (w/v) Pronase in pH 2 HCl solution. Driselase was purified, freed of carboxyesterases, and incubated with FCW for 48 h as described by Fry (1988a). Cellulose was hydrolyzed with Cellulysin (Calbiochem), tested to be free of esterase activity, at a concentration of 1% (w/v) in pH 5 acetate buffer, according to Ralph et al. (1994). After 24 h, the cellulysin incubation mixture was centrifuged, the supernatant was recovered, fresh enzyme solution was added and the mixture incubated again for 24 h. The digestion was repeated for a total of 6 days. Glucose was determined in the supernatant with the Glucose [HK] 10 system (Sigma), to follow the progress of cellulose hydrolysis. The total sugar content and uronic acid were analyzed according to Fry (1988b).

Chemical Analyses. Methylation of lignin was performed according to the method of Lapierre and Rolando (1988) over 4 days for Bjorkman lignin and 10 days for the hemicellulose−lignin complex (LCC). Dried methylated Bjorkman lignin was solubilized into dioxane−water and concentrated under vacuum. The viscous residue was stirred in a dichloroethane−water mixture (18: 1, v/v). The mixture was refluxed with an equal weight of dichlorodicyanoquinone (DDQ) at 70 °C for 2 h as described by Watanabe (1989). The DDQ-treated Bjorkman lignin was partitioned between ether and water, and the ether extract analyzed by HPLC. LCC was submitted to TFA/DDQ hydrolysis as described by Imamura et al. (1994). Thioacidolysis of lignins was performed according to Lapierre et Rolando (1988), as its analytical procedure with a sample-to-reagent ratio of 1 mg/mL, for 4 h. Methanolysis was carried out with 1.5 M sodium methoxide in dry methanol.

HPLC. The extracts were analyzed by reversed-phase HPLC with a Spectra-Physics chromatographic system consisting of a P4000 pump, a Rheodyne 7125 injection valve with a 200 μL injection loop, and a model P1000 Spectra-Physics UV detector set at 216 nm. For spectral analysis, the HPLC system was connected to a multichannel detector (Pye-Unicam PU4021 model). Separations were carried out on a C18, 6 μm Bischoff column with a guard cartridge using acetonitrile (solvent A) and 2% acetic acid in water (solvent B) as mobile phases. Elutions were performed at ambiant temperature at a flow rate of 1 mL·min^{-1} using the gradient described in Figures 3 and 4.

The radioactivity of column effluent was monitored on-line with a Packard Flow-one scintillation detector or in fraction aliquots with a Packard C2400 scintillation counter. Some metabolites were identified by cochromatography with authentic standards from Fluka (hydroquinone), Aldrich (resorcinol) and Sigma (catechol), and also on the basis of their UV spectra recorded with the photodiode-array detection system (Bartolomé et al., 1993). HO−POA and its glucoside were compared to synthesized standards on the basis of their retention times. The glucoside was also identified by its sensitivity to β-*O*-glucosidase.

Data Analysis. Values are the average of two determinations for each plant. These results were analyzed for statistical significance by *t* test.

RESULTS AND DISCUSSION

Comparison between Various Extraction Protocols. Bound pesticide residues are usually recovered

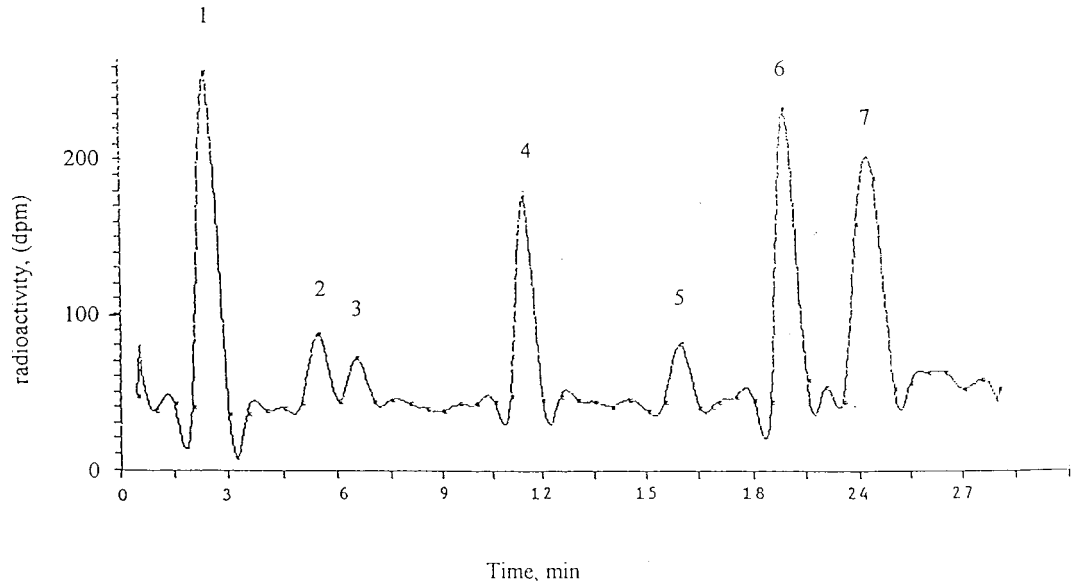

Figure 4. HPLC analysis of a DDQ hydrolysate of methylated stem Björkman lignin: 1, hydroquinone; 2, cathecol; 3, resorcinol; 4, 4-HO-POA; 5, anisole; 6, 4-HO-POA-methyl ester; 7, POA. Solvent A: acetonitrile. Solvent B: 2% (v/v) acetic acid in water. Gradient: 2% A to 50% A linearly in 30 min.

Table 1. Distribution of Radioactivity in Free Cell Wall Fractions from Soybean Plants, 72 h after [^{14}C]POA Application[a]

	protocol 1	protocol 2	protocol 3
pectins	10.0	10.5	17.0
hemicellulose I	6.5	6.5	
hemicellulose II	13.7	20.0	58.0
cellulose	11.0	2.0	1.0
lignin	5.0	56.0	
residuum	50.0	<1.0	12.0

[a] Values expressed as percentage of the radioactivity incorporated in FCW. [b] Björkman lignin in that protocol.

and characterized in plants by sequential extraction of CW polymers (Kearney, 1982). However, a survey of the literature (Huber and Otto, 1983; Pillmoor and Roberts, 1985) shows that the published protocols lead to disparate conclusions about the residues associated with the diverse classes of wall polymers. A first reason for that discrepancy is that structurally different pesticides are prone to react with different classes of wall polymers. Another, less obvious reason is that CW fractionation schemes are devised to selectively and completely extract the various polymers, but not necessarily to keep intact the associated pesticide residues (Huber and Otto, 1983). With these considerations in mind, we have tested three protocols (Langebartels and Harms, 1985; Pillmoor et al., 1984; Goubet et al., 1994) for the extraction of bound POA residues from soybean CW (Figure 1).

After extraction of the soluble fraction, the residual CW was ground with a mortar to a thickness of 40 mesh (protocols 1 and 2) or with a ball grinder to a thickness of 60 mesh (protocol 3). The powders were then Soxhlet extracted to obtain cell walls free from soluble residues, i.e., free cell-walls (FCW). In all cases, the amounts of the residues recovered by Soxhlet extraction were less than 3% of the radioactivity of the CW fraction. The FCW material was then fractionated, and the radioactivity of the various fractions was estimated (Table 1). The three protocols began by the extraction of hydrosoluble polysaccharides, mainly pectins, with water at 0 °C then at 100 °C, and with a Ca^{2+} chelating agent (EGTA in protocols 1 and 2; EDTA in protocol 3). The radioactivity extracted with these pectin fractions was 63 to 65% higher in protocol 3 than in protocols 1 and 2 (Table 1). That higher yield seems to result from a more effective grinding of the CW in protocol 3, rather from the nature of the extracting solutions. Indeed, in all case, more than 97% of the radioactivity was extracted by the same boiling water treatment.

In the three protocols, hemicelluloses were then extracted by alkaline solutions. Recoveries ranged from 20.2 to 57.9% of the FCW radioactivity. In protocols 1 and 2, hemicelluloses I and II were successively extracted by 4%, then 24% KOH solutions (respectively 0.7 and 4.28 M), while in protocol 3, they were recovered together by a single extraction with 2 M NaOH. Moreover, a step of lignin extraction was inserted between the hemicellulose I and II solubilization steps in protocols 1 and 2, whereas in protocol 3 all hemicelluloses were solubilized without any prior lignin extraction. Therefore, the radioactivity extracted with hemicellulose II in protocols 1 and 2 was dependent upon the previous step of lignin extraction. It is nevertheless surprising that the NaOH extraction of protocol 3 released more residues than the KOH extractions of protocols 1 and 2. As already mentioned about pectins, the most likely explanation for that higher yield lies in the finer CW grinding in protocole 3. On another hand, apart from the question of the amounts of the recovered residues, it must be kept in mind that the high sodium or potassium hydroxide concentrations used for the hemicellulose extractions can hydrolyze some residues which were bound on other polymers.

Lignin extractions released very different quantities of residues in protocols 1 and 2 (4.9 and 56.3%, respectively). The dioxane-water mixture used in protocol 1 only solubilized the organo-soluble fraction of lignins and some part of lignins could be what is left of the CW, while the $NaClO_4$ solution of protocol 2 oxydized all lignins. In addition, the extensive extraction of lignins in protocol 2 allowed better solubilization of hemicellulose II and the associated residues.

Cellulose was hydrolyzed with sulfuric acid. Here again, the amounts of bound residues extracted were

strongly dependent on the previous steps. On the whole, these amounts were relatively low since no more than 11% of the radioactivity was recovered.

In protocols 1 and 3, sulfuric acid hydrolysis left a residuum which was consistent with Klason lignin, and contained 50.1 and 11.6% of the FCW radioactivity, respectively. In protocol 2, lignin had been previously hydrolyzed by $NaClO_4$, and in consequence the residuum only contained a negligible radioactivity. Despite methodological differences, total levels of residues associated with lignins were similar in protocols 1 and 2 (55.0 and 56.3%, respectively). By contrast, these residues were in much lower amounts in protocol 3 (11.6% of the total), because the previous extraction of radioactivity by the NaOH step was especially effective. These results show the difficulty to estimate the true amount of residues that are bound on the polymer of interest.

Adopted Protocol. The above observations allowed us to establish a protocol designed to improve the selectivity of the extractions of POA bound residues attached to a polymer of interest, rather than the extraction yield of this polymer (Figure 2). The preparation of FCW and the extraction of pectins was performed as in protocol 3. That procedure allowed an optimal extraction of the residues associated with pectins, even though the extraction procedure was rather mild and thus probably selective. To make easier the following extractions, the FCW was ground again at that stage.

Since it is known that phenoxy-alkylcarboxylic acids can combine with amino acids, cell wall proteins were digested by Pronase (Figure 2). The next step was the extraction of cellulose, which is the main CW polymer. Cellulose is incrusted with the ligno-hemicellulosic complex (Iiyama et al., 1994), and has to be extracted first, to unmask that complex. Enzymatic hydrolysis with cellulase was preferred to sulfuric acid hydrolysis. Although the enzymatic treatment was time-consuming and resulted in a complete hydrolysis of cellulose, its specificity for the β-1−4 glucose bond left intact the bonds between glucose units and residues, and the bonds with other polymers, provided the cellulase preparation was devoid of carboxy-esterasic activity. It must be noted, however, that hemicelluloses, particularly xyloglycans, can be partially hydrolyzed by cellulase.

In the following part of the extraction procedure, the main difficulty was to separate hemicelluloses from lignins. These polymers are highly interwoven into each other in the cell walls, where they make up the lignin-carbohydrate complex (LCC); furthermore, their solubilization need to use harsh extraction methods. In consequence, two difficulties can be met: first, the breakdown of the polymer of interest during its extraction, and/or the cleavage of the residue-polymer bonds. Thus, the nature of the bond cannot be determined. For example, when lignin was extracted by $NaClO_4$ oxidation. In the same way, the other difficulty is that there is a satisfactory solubilization of the polymer of interest but the extract is contaminated by residues resulting from a partial degradation of the other polymer. For example, as we have seen above, alkaline extraction of hemicelluloses is likely to also solubilize POA residues bound to lignin.

Therefore, following cellulose extraction, the organosoluble fraction of lignins (Björkman lignin) was extracted by a dioxane−water mixture. A further water extraction step was added to the protocol, to extract the hydrosoluble fraction of LCC (water extractible LCC, WE-LCC) (Watanabe, 1989). That fraction contains lignin and hemicelluloses cross-linked by hydroxycinnamic acids bound to lignin by α-ether bonds and to hemicelluloses by ester bonds (Iiyama et al. 1994). Owing to the bifunctionality of 4-HO-POA, the main POA metabolite, analysis of that WE-LCC fraction was advisable.

Table 2. Radioactivity Released by Various Hemmicellulose Extraction Procedures from the Hemicellulose−Lignin Complex (LCC) of Soybean Plants, 72 h after [^{14}C]POA Application[a]

hydrolysis	methods	KOH[b]	TFA[c]	Driselase[d] followed by TFA
stems	extracted	95.9	19.0	1.8
				14.8
	residuum	3.3	79.5	82.5
	total	99.2	98.5	99.2
leaves	extracted	95.5	52.4	15.6
				40.5
	residuum	2.4	45.0	41.1
	total	97.9	97.4	97.2

[a] Values expressed as percentage of the radioactivity incorporated in LCC. [b] KOH 24% (w/v), room temperature, 24 h. [c] TFA, 2 M, 100 °C, 4 h. [d] Driselase, 0.5%, 37 °C, 48 h.

The last step of the protocol consisted in fractionating the residual LCC into its hemicellulose and lignin components. Since, lignin is difficult to solubilize, whatever the method used, hemicelluloses were extracted first. As shown previously, alkaline solubilization was rather aggressive, and released the most part of the radioactivity bound to LCC (Table 2). Hydrolysis with TFA released about five times less radioactivity from stem LCC than KOH, and two times less from leaf LCC (Table 2). Enzymatic hydrolysis with Driselase (a mixture of glycosidases) only released 1.8 and 15.6% of the LCC radioactivity of stems and leaves, respectively. However, with this last procedure, analyses of released sugars showed that hydrolysis was very limited, and a further hydrolysis by TFA was required to achieve a more complete solubilization of the carbohydrate fraction. TFA released more radioactive residues than Driselase, with respect to the solubilized oses (respectively, 0.27 μmol/mmol of sugars and 0.11 μmole/mmole). Each method entailed some difficulty: hydrolysis by TFA should release 4-HO-POA residues linked to lignin by ether bonds, and hydrolysis of hemicelluloses by Driselase was not complete. The architecture of the LCC restricts the enzymatic solubilization of hemicelluloses, because hemicelluloses are entrapped within the lignin matrix (Gübitz et al., 1998). Moreover, Driselase digestion could also be limited by non glycosidic residues bound on polysaccharide polymers, particularly by hydroxycinnamic acid residues such as feruloyl-esters (Fry and Miller, 1989). On the other hand, the analyze of 4-HO-POA residues in the TFA hydrolysate showed that lignin contamination was very low. TFA treatment appeared, therefore, as the best compromise to extract hemicelluloses with their bound residues. To look at if the totality of polysaccharides was extracted by the reagent, the residuum was submitted to a sulfuric acid hydrolysis. This treatment only released a very low amount of sugars and less than 1% of the radioactivity. In consequence, hemicelluloses were extracted from LCC by TFA treatment in the final protocol, and the residuum could be considered as Klason lignin.

Table 3. Distribution of Radioactivity in Leaves, Stems, and Roots, 72 h after [^{14}C]POA Application

	leaves	stems	roots
total	64.9[a] (*100*)[b]	29.8 (*100*)	5.3 (*100*)
soluble fraction	56.5 (*87*)	22.6 (*75*)	2.3 (*43*)
bound residues	8.4 (*13*)	7.2 (*24*)	2.9 (*56*)

[a] Values expressed as percentages of the total radioactivity found in plant. [b] Values expressed as percentages of the radioactivity found in the relevant plant part.

Table 4. Radioactivity Associated with FCW Polymer Fractions of Leaves and Stems, 72 h after [^{14}C]POA Application[a]

	leaves	stems
pectins	11.0	26.1
proteins	1.9	5.0
cellulose	6.9	2.2
Björkman lignin	1.0	1.3
WE-LCC	8.0	2.8
hemicelluloses	35.5	12.2
résiduum (Klason Lignin)	30.5	51.2

[a] Results expressed as percentages of radioactivity found in FCW preparation.

Distribution of Bound Residues in Cell Wall Polymers. The protocol defined above was used to determine the amounts of POA residues bound to CW. Soybean plants were fed for 24 h with nutrient solution containing radioactive POA, then for 48 h with solution without POA. At the end of that 72 h period, more than 94% of the radioactivity was translocated to the aerial organs, and about two-thirds were present in leaves. At that time, POA was almost totally metabolized. It was detected in low amount (3.5%) in the soluble extract of leaves, and was not detectable in stems. The major metabolite was 4-OH-POA-β-D-glucoside, that amounted to 71.5% and 63.2% of the radioactivity in leaves and stems, respectively (Figure 3).

The fixation of POA residues in CW of aerial organs accounted for 15.6% of the total radioactivity (Table 3). These bound residues amounted to 13% of the radioactivity of leaves and 24% of the radioactivity of stems. Despite this difference, the specific rate of fixation was roughly equivalent in both tissues (437 dpm/mg CW and 393 dpm/mg CW, respectively). In roots, bound residues amounted to 56%, but that fraction constituted only 2.9% of the total radioactivity found in plants, and in consequence was not further analyzed.

The distribution of radioactivity in the FCW components of leaves and stems presented some differences (Table 4). The solubilization of pectins released 11.0% of the radioactivity incorporated into leaves and 26.1% of the radioactivity of stems. In both organs, nearly all that radioactivity (>97%) was associated with boiling-water extracts, constituted of the hydrosoluble polysaccharides starch and neutral pectins. Since amylase hydrolysis released less than 0.5% of FCW radioactivity, it can be concluded that the extracted radioactivity was associated with neutral pectins, higher methylated pectins than acidic pectins extracted by EDTA (Schaumann et al., 1993). A such specific attachment to neutral pectins has been shown for ferulic acids (Meyer et al., 1991). However, the amounts of bound residues were not proportional to the amounts of neutral pectins, which were more abundant in leaves (Laurent, unpublished).

Protein digestion released more residues from stem FCW than from leaf FCW. Digestion of cellulose only released moderate levels of residues from leaf or stem FCW (6.9% and 2.2%, respectively), despite the abundance of cellulose. The quasicrystalline structure of cellulose (Smith et al., 1998) probably prevented the fixation of residues. By contrast, high amounts of bound residues were associated with WE-LCC which represented nevertheless a very small part of the FCW mass.

Unlike pectins, hemicelluloses were associated with more radioactivity in leaves than in stems (35.5% and 12.2%, respectively). The residue amounts were unrelated with noncellulosic polysaccharide amounts. In stem, lignification could prevent the feathure in situ polymerization of the residues in hemicelluloses (Gübitz et al., 1998).

Comparable or higher percentages of bound residues were associated with lignin fractions (Björkman lignin + Klason lignin) which contained 31.5% and 52.5% of the radioactivity in leaves and in stems, respectively. However, very different amounts of residues were associated with the Bjorkman and Klason fractions. In stems, 39 times more residues were associated with Klason lignin than with Björkman lignin, though the amount of Klason lignin was only 1.86 times higher than the amount of Björkman lignin (gravimetric determination). A similar difference was noted in leaves, where Klason lignin contained 30 times more residues, eventhough it was 3.4 times times less abundant than Björkman lignin. Thus, the residue linkage frequencies of Klason lignin (ratio of POA residues to lignin monomers) were 14.06 and 52.74 μmol·mmol^{-1}, respectively in stems and leaves, while these frequencies were respectively 0.52 and 0.66 μmol·mmol^{-1} for Björkman lignin.

Thus, as already noted about polysaccharides, the amount of bound residues does not depend on the abundance of the polymers on which they are fixed. However, in some cases, differences between leaves ans stems could be related to the histological characteristics of these organs, and consequently on polymer deposites (Monties, 1989). Since stems are rich in xylem and schlerenchyma cells. Both types of cells are highly lignified, and CW of schlerenchyma cells are relatively rich in proteins (Mc Dougall et al., 1996). Whereas, leaves are rich in parenchyma cells, the primary CW of which has a high cellulose content and they are slightly lignified (Carpita and Gibeaut, 1993). Lignin is generally regarded as the "local excretion sites" for environmental chemicals in plants (Sandermann, 1994). In the soybean plant as a whole, lignin was the polymer on which the highest percentage of POA residues was bound, since 41% of the radioactivity found in FCW of aerial organs was associated with it, when 25% was associated with hemicelluloses, but similar level (42.6%) was bound on all noncellulosic polysaccharides.

Nature of the Residues. Our most detailed analysis concerned the residues associated with stem FCW. After precipitation of neutral pectins by ammoniacal chloroformate acid, 38% of radioactivity remained in the supernatant and HPLC analysis showed that it was entirely attributable to free 4-HO-POA glucoside. Thus, a fraction of the glycosyl conjugate was merely trapped in the pectin matrix. At first sight, that "loosely bound" residue could be considered as a precursor of the residues covalently linked to pectins. However, Hutber et al. (1978) have shown that the precursor of bound residues in pea is 4-HO-POA, and not its glucoside, which appears later than the bound residues. Hydrolysis

Table 5. Bound Residues from Stem Björkman or Klason Methylated Lignins

		residues released[a]			
sample	hydrolysis	total	POA	4-HO-POA[b]	others[c]
Björkman lignin	DDQ	88.0	24.0	32.3	31.7
Klason lignin	DDQ	20.6	nd	1.2	19.5
	thioacidolysis	31.3	nd	5.3	25.9

[a] Values expressed as percentages of radioactivity in lignin samples. [b] As free 4-HO-POA and 4-HO-POA-methyl ester. [c] Phenols and unidentified peaks. nd: not detected.

of the pectin precipitate by 0.1 N NaOH at room temperature, showing the ester linkage of residues, mainly released POA (14%) and 4-HO-POA (67%). Likewise, endogeneous hydroxycinnamic acids are esterified on pectins (Iiyama et al., 1994; Ralet et al., 1994). These hydroxycinnamoyl pectic esters are synthesized in the Golgi apparatus, then exported as complete macromolecules to CW (Meyer et al., 1991). By analogy, 4-HO−POA esters could be synthesized in the same way. This hypothesis is reinforced by the fact that 4-HO-POA and POA were essentially associated with the neutral pectins, which are synthesized in Golgi stacks distinct from those of acidic pectins (Staehelin et al., 1992). The incorporation of hydroxycinnamic acids in pectins requires an activation by coenzyme A ligase (Yamamoto et al., 1989), but that remains to be demonstrated for 4-HO-POA or POA.

In addition to pectins, the hemicellulose fraction contained a significant percentage of the bound residues. In TFA hydrolysate, almost all radioactive residues were 4-HO-POA conjugates and less than 10% was found as free 4-HO-POA. This last could occur from glycosidic linkage on hemicellulose, since the glycosidic linkages are labile in hot TFA (Watanabe et al., 1986), or from ether-bound lignin residues. But, no HO-POA methyl ester was released in methylated LCC. Therefore, 4-HO-POA was esterified on hemicellullose and no residue contamination derivided from lignin. Free 4-HO-POA was likely released from ester linkage which can occur at a far low rate in TFA (Morrison and Stewart, 1998). We did not attempt to identify 4-HO-POA conjugates, the hemicellulose monomers on which the residues were bound, but we have observed, during the Driselase hydrolysis, that the conjugates were essentially released after the first hour, which suggestes a residue−galactose linkage (Fry, 1988b).

As shown in Table 4, a wide part of the bound residues was associated with lignin, especially in stems. The residues were very unequally distributed between the Björkman and the Klason fractions, the latter containing by far the majority of residues. Several degradation methods were used to identify these residues: methanolysis of ester linkages, oxidative hydrolysis of ester and ether linkages by DDQ (Watanabe, 1989), and thioacidolysis of alkyl−ether bonds (Lapierre and Rolando, 1988). Hydrolysis of Klason lignin by these methods only released modest percentages of bound residues (Table 5). 4-HO-POA, as free or 4-HO-POA methyl-ester, was found in very low amounts (2.7% of the FCW radioactivity), but the hydrolysates contained a high proportion of phenol derivatives not previously detected. The low yield of 4-HO-POA was particularly surprising. When FCW was submitted to a strong NaOH hydrolysis (6 N, 170 °C, 2 h), 92% of the radioactivity was released and 4-HO-POA was the main component of the hydrolysate (69%). Taking into account the amounts of 4-HO-POA extracted with the other polymers, one should expect Klason lignin to contain about 38% of the 4-HO-POA linked to the FCW, representing 26% of the total FCW radioactivity. We are thus led to the conclusion that 89% of 4-HO-POA was not released by specific hydrolysis methods from Klason lignin.

4-HO−POA can be linked to lignin monomers by ester (carboxylic) or ether (phenolic) bonds. The classical methods used to cleave the ester and ether bonds with various alkaline solutions (Heirwegh and Compernolle, 1979) do not provide reliable estimates of the percentage of each type of bonds (Lozavaya et al. 1999). These bonds can be hydrolyzed by DDQ if 4-HO-POA is bound to the α position, or if it is bound to the γ position provided that a double bond is present in α-β position (Watanabe, 1989). The low yield of 4-HO-POA after DDQ hydrolysis (Table 5) could be explained by the predominance of linkage on the γ position of the sidechains, and by the scarcity of Cα−Cβ double bonds resulting from the abundance of β-ether structures in lignin.

Thioacidolysis released from methylated lignin some 4-HO-POA methyl ester and very little free 4-HO-POA (around the detection limit). Methyl-ester was bound to lignin by ether linkage and 4-HO-POA was probably esterified to lignin. However, the thoacidolysis was performed in BF_3 etherate. In these conditions, it is not very efficient on ester bonds (Rolando et al., 1992), and that could explain the failure to release the major part of 4-HO-POA, if these residues are linked to Klason lignin by ester bonds.

Like the preceding methods, methanolysis released low amounts of residues. According to Nakamura and Higuchi (1976), that suggested that 4-HO-POA was mainly bound by ester linkage to the γ position of lignin monomers. Like that shows for some *p*-coumaric acids esterified to γ-syringyl units of maize lignin (Ralph et al., 1994; Grabber et al., 1996).

POA was clearly bound to lignin by ester linkages. It was probably bound in the same way as 4-OH-POA, since they was not released by DDQ or thioacidolysis of Klason lignin. However, the estimating of POA amount bound to Klason lignin was difficult because of its low abundance in FCW (about 5%).

Although very little radioactivity was associated with Björkman lignin, that fraction was analyzed because its solubility in organic solvents allowed more efficient hydrolyses. In particular, DDQ released most of the bound residues, among which 4-HO-POA was predominant (Table 5). The high percentage of 4-HO-POA could not be only accounted for by the high yield of the hydrolysis, and was probably indicative of a linkage to the benzylic carbon of Björkman lignin monomers.

Other type of residues were found in lignin hydrolysates. They were tentatively identified as phenolic compounds, mainly diphenols and anisole (Bartolomé et al., 1993). According to hydrolysis specificities, these compounds were bound by ether linkages to α-carbons. The presence of hydroquinone was indicative of a dealkylation of POA or 4-HO-POA. Other diphenols, catechol and resorcinol (respectively, 1,2- and 1,3-diphenol) were indicative of POA dealkylation, since hydroxylation of POA exclusively led to 4-HO-POA. Phenoxyalkanoic acid dealkylation has been demonstrated in many plants, especially for higher phenoxyalkanoic homologues, but this pathway occur is of minor importance for the degradation of 2,4-D (Fawcet et al.,

Table 6. Bound Residues from Leaf WE-LCC or Klason Lignin

		residues released[a]			
sample	hydrolysis	total	POA	4-HO-POA[b]	other[c]
WE-LCC	DDQ/TFA	77.5	48.7	15.5	13.2
Klason lignin	DDQ	83.7	18.1	47.3	18.3

[a] Values expressed as percentages of radioactivity in sample. [b] As free 4-HO−POA and 4-HO−POA-methyl ester. [c] Phenols and unidentified peaks.

1959), the major used phenoxyalkanoic acid herbicides. The presence of anisole as bound residues was difficult to explain, although it could be an intermediate of POA decarboxylation, like the dichloroanisole resulting from 2,4-D decarboxylation (Loss, 1975). These phenolic metabolites were not detectable in the soluble cell extracts, and their presence among the bound residues was an indication of their fast binding to CW polymers, particularly to the α-carbons of lignins.

In preparations from leaves, only WE-LCC and Klason lignin were analyzed so far due to the low amounts of Björkman lignin present (Table 6). Solubilization of WE-LCC released 8% of the radioactivity from leaf FCW, whereas less than 3% was released with stem WE-LCC. DDQ hydrolysis of leaf methylated WE-LCC in the presence of TFA, to hydrolyze polysaccharides (Imamura et al. 1994), released a major part of the radioactivity as POA, 4-HO-POA-methyl ester and some phenolic compounds (Table 6). The absence of free or conjugate 4-HO-POA in the hydrolysate suggested that POA metabolites were not involved in ligninhemicellulose bridges. By contrast to the analysis of stem lignin, DDQ hydrolysis released 83.7% of the residues bound to leaf Klason lignin. Therefore, leaf residues were probably bound on the α-position. Since 0.1 N NaOH hydrolyzed the major part of these residues, they were mainly linked by ester bonds. A characteristic of the leaf residue composition was the relatively high proportion of POA by comparison to the low level found in stem residues, and in the soybean plants, as a whole.

CONCLUSION

Among the bound residues of POA, a distinction must be done between 4-HO-POA and the other metabolites. The latter were found attached to lignins in α-position, as expected for residues copolymerized during the lignin formation by an opportunistic addition (Scalbert et al., 1986; Lange et al., 1998). 4-HO-POA was linked via phenyl ether bonds but it was particularly esterified to the γ-position. In addition, 4-HO-POA was more widely present in CW polymers, since it was also esterified on noncellulosic polysaccharides. The binding pattern of 4-HO-POA shows some similarities to the binding of hydroxycinnamic acids, particularly with *p*-coumaric acid. This latter is esterified to noncellulosic polysaccharides (Fry, 1983), and attached to lignin by ester- or ether-linkages (Boudet et al., 1994), and contrary to ferulic acid, *p*-coumaric acid is not acting as a crosslinking agent between polysaccharides and lignins (Iiyama et al., 1994). That likeness was however relative: *p*-coumaric acid linkage at the γ-position has been only demonstrated for grass lignin (Iiyama et al., 1994); *p*-coumaric acid is little abundant in dicots (Lozavaya, 1999) and absent in their lignins (Hartley and Harris, 1981).

The mode of fixation of hydroxycinnamic acids is important to determine their bioavailability and the CW digestibility (Hatfield et al., 1999). In lignin, esterification of hydroxycinnamic acids is an important factor restricting the digestion of CW (Hartley and Ford, 1989), and it explains the lower digestibility of grasses CW, as compared to some dicots (Buxton and Russell, 1988). In the same way, hydroxycinnamic acids from dicots CW are less available for animals (Buchanan et al., 1996; Kroon et al. 1997). That bioavailability must depend on the fixation on lignins or noncellulosic polysaccharides. By analogy, the 4-HO-POA bioavailability should depended on the ratio between the 4-HO-POA level linked to noncellulosic polysaccharidess and the one linked to lignin.

Several comments can be made about the above study. Concerning the binding to polysaccharides, (i) the mechanism by which POA residues, particularly 4-HO-POA or its glucoside, are transported to the cell walls needs to be elucidated; (ii) the linkage specificity of 4-HO-POA on polysaccharides needs to be established and compared to the specific esterification of hydroxycinnamic acids. Concerning the residues bound to lignin, (iii) the chemical structure of the ester-linkage of 4-HO-POA to lignin should be rigorously established; (iv) as the attachement on the γ-position does probably not depend on a chemical mechanism, the enzymatic formation of 4-HO-POA γ-ester linkage should also be demonstrated.

Such studies are needed to define the processes controlling cell-wall residue formation. Really, POA was chosen like model for residue incorporations of phenoxyalkanoïc herbicides, such as 2,4-D. This compound binds to polysaccharides and lignins in soja and wheat, but the kind of bonds with lignins is not identified (Scheel and Sandermann, 1981). Owing to the binding differences between ferulic and *p*-coumaric acids, due to their phenolic substituents, the effect of subtituants of phenoxyalkanoic acids are going to take into account.

ABBREVIATIONS

CW, cell wall; 2,4-D, 2,4-dichlorophenoxyacetic acid; DDQ, dicyanodichloroquinone; FCW, free cell walls; 4-HO-POA, 4-hydroxyphenoxyacetic acid; LCC, lignin−carbohydrate complex; POA, phenoxyacetic acid; TFA, trifluoroacetic acid; WE-LCC, water extractable lignin−carbohydrate complex.

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