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CHANGES IN ENZYMES INVOLVED IN SUBERISATION IN ELICITOR-TREATED FRENCH BEAN CELLS

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Key Word Index—*Phaseolus vulgaris*; Leguminoseae; cell wall; laurate ω-hydroxylase (cytochrome P450); (feruloyl-CoA: hydroxy-fatty acid feruloyltransferase); hydrogen peroxide.

Abstract—Transfer of ferulic acid from feruloyl-CoA onto polymeric material can be readily demonstrated by microsomal membranes from suspension-cultured cells of French bean. Transfer onto endogenous components present in the microsomal preparations can be supplemented by addition of polysaccharide or lipid components. However, addition of catalase to reaction mixtures suppresses transfer onto polysaccharide components, indicating that this is peroxidative. In contrast, suppression of microsomal peroxidase activities leads to increased transfer onto hydroxy-hexadecanoic acid showing that any transferase is possibly specific for lipid and therefore involved in suberisation. This transferase was transiently induced by elicitation of the cells. The time course of induction was similar to that for the cytochrome P450, laurate ω-hydroxylase required for the hydroxylation of fatty acids. Solubilisation and partial purification of the transferase was accomplished and indicated an enzyme with a tendency to aggregate but with a probable minimum subunit M_r of 40 000. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Ferulic acid is an essential intermediate in phenylpropanoid metabolism leading to lignification. It is in itself a significant component of the primary cell wall and may contribute to the formation of cross-linkages [1–3]. These linkages may increase during stress and pathogen attack [4, 5] and are responsible for rigidification and barrier formation. During lignification, feruloyl-CoA is converted to coniferyl alcohol which together with *p*-coumaroyl- and syringyl-alcohols and the corresponding aldehydes of all three precursors are polymerised by peroxidase, laccases and other oxidases into lignin in the wall [4]. Ferulic acid is found in cell walls and cell surfaces conjugated to both polysaccharide and hydroxy-fatty acids. Residues are found most frequently attached to arabinoxylans [5] among the polysaccharides. Conjugation to hydroxy-fatty acids is a feature of suberins and cutins but it is not known whether hydroxy-fatty acids and phenylpropanoids are transported separately and assembled into the polymer layer or synthesised as a monomer and transported in this form. There is therefore some interest in determining the mechanisms by which ferulic acid and its derivatives are transferred onto polymers and additionally how they are transported to the wall and assembled by a peroxidative mechanism.

Transfer onto these polysaccharides has been described in suspension cultured cells [6, 7], although there have been difficulties in demonstrating the specificity of this *in vitro* [8]. In contrast, transfer onto hydroxy-fatty acids has been demonstrated in cell extracts [9, 10]. The present work examines possible feruloyl-CoA transferases in microsomes derived from suspension cultured cells of French bean and the changes in the activity of these in response to elicitor stress, in comparison with changes in the levels of the marker enzyme for fatty acid hydroxylation, the cytochrome P450 laurate ω-hydroxylase.

RESULTS

Feruloyl-CoA transfer in microsomal membranes of French bean cells

When ^{14}C-feruloyl-CoA was fed to microsomal membranes isolated from suspension cultured cells of French bean it was transferred onto endogenous polymeric components which remained at the origin when reaction mixtures were subjected to paper chro-

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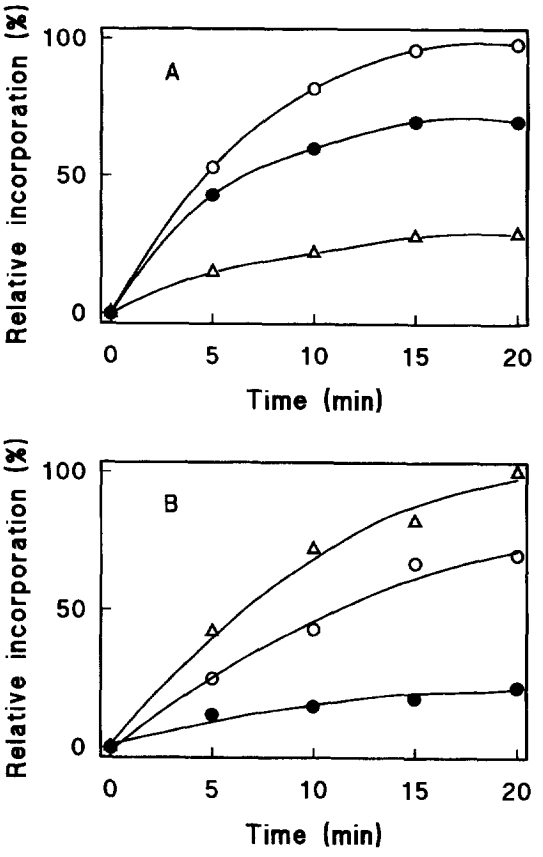

Fig. 1. Incorporation of ^{14}C-feruloyl-CoA by microsomal membranes in presence or absence of added polysaccharide (A) or hydroxyhexadecanoic acid (B). Microsomal preparations from unelicited bean cells were incubated with feruloyl-CoA in the standard assay with additions; in (A) none (●), arabinogalactan (○), arabinogalactan and catalase (50 units)—(△); in (B) none (●), hydroxydecanoic acid (○), hydroxydecanoic acid and catalase (50 units)—(△). The curves are derived from a number of experiments and typical relative incorporations are shown.

matography using BuOH–HOAc–H_2O (12:3:5) as the mobile phase. Transfer onto added arabinoxylan and arabinogalactan was assayed similarly and increased incorporation compared with control incubations was observed [Fig 1(A)]. Transfer onto added hydroxyhexadecanoic acid, as a model compound for hydroxy-fatty acids, was assayed by extraction into ethylacetate of lipophilic material and increased transfer in comparison with that onto endogenous components was also observed [Fig. 1(B)]. Potentially, there may be two types of feruloyl transferase operating in microsomal membranes.

Effect of catalase

Because of the high level of peroxidases in the microsomal membranes of cultured French bean cells [11, 12] incorporation into polymers could be as a result of peroxidative action and generation of free radicals derived from ferulic acid. Peroxidases can also generate H_2O_2 from added thiols [13, 14] which could exacerbate such an effect. Peroxidative transfer in the presence of thiols has already been observed in microsomal membranes from maize [8]. Addition of catalase totally abolished transfer onto polysaccharide indicating that this was solely due to free radical generation [Fig. 1(A)]. If a specific feruloyl transferase exists for transfer onto arabinoxylan and arabinogalactan, it was not detectable in this system. However transfer onto hydroxyhexadecanoic acid was enhanced [Fig. 1(B)] presumably due to prevention of lipid peroxidation by removal of H_2O_2.

Peroxidative cross-linking of phenylpropanoids in membrane preparations

The ability of microsomal membranes to generate H_2O_2 in standard assay procedures to produce artefacts has only relatively recently been appreciated [8, 11–14]. The hydrogen peroxide can then be used in reactions that bring about apparent transfer onto polymers and cross-linking of phenylpropanoids and proteins. In membranes of French bean cells this is a problem. As a further example, besides the dimerisation of ferulic acid [12] and artifactual polymerisation of feruloyl-CoA onto polysaccharide, *p*-coumaric acid residues also formed dimers if no catalase was included in the reaction mix. In experiments to study the possible hydroxylation of [^{14}C]-*p*-coumarate to [^{14}C]-caffeate by microsomes a product of R_f 0.21 upon TLC in toluene–acetic acid–water (40:15:0.7) migrated very close to the position expected for caffeic acid (R_f 0.24). However the product was abolished by the addition of catalase and proved to be di-coumarate, when this was polymerised by peroxidase and shown to have an R_f similar to caffeate.

Laurate ω-hydroxylase in microsomal membranes of French bean

Transfer of ferulic acid residues occurs onto hydroxylated fatty acids [Fig. 1(B)]. The group of enzymes responsible for these hydroxylations belong to the cytochrome P450 family which has now been designated CYP94 (I. Benveniste, N. Tijet and J.-P. Salaun, unpublished results). French bean cells contain a high basal activity of laurate ω-hydroxylase in microsomal preparations (Fig. 3). The hydroxylated derivative had an R_f of 0.45 characteristic of the ω-position. No other hydroxylated derivatives were found and the bean enzyme resembles the pea enzyme in its specificity [15].

Time courses of changes in enzymes of suberisation in response to elicitor-treatment of suspension cultured cells of French bean

Suspension cultured cells of French bean when subjected to elicitor treatment undergo a number of chan-

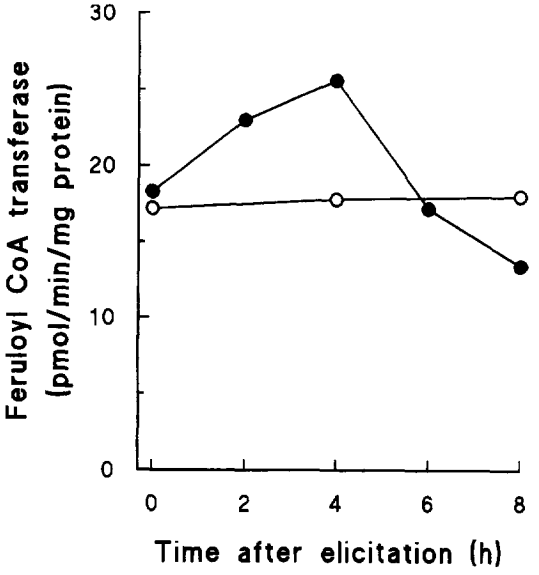

Fig. 2. Changes in feruloyl-CoA transferase activity following treatment of cell suspension cultures with elicitor. Microsomes were isolated from cells harvested at each time point and assayed for transferase activity. Control cells (○—○); elicited cells (●—●).

ges resulting in modification of the cell wall. The level of suberisation has not been determined but there is a large increase in bound ferulate residues in the walls of elicited cells [16]. The changes in the levels of the transferase and hydroxylase were determined (Figs 2 and 3). Transient changes were observed and there was some increase in extractable membrane bound activity for both enzymes in the first four hours after the addition of elicitor followed by a disappearance

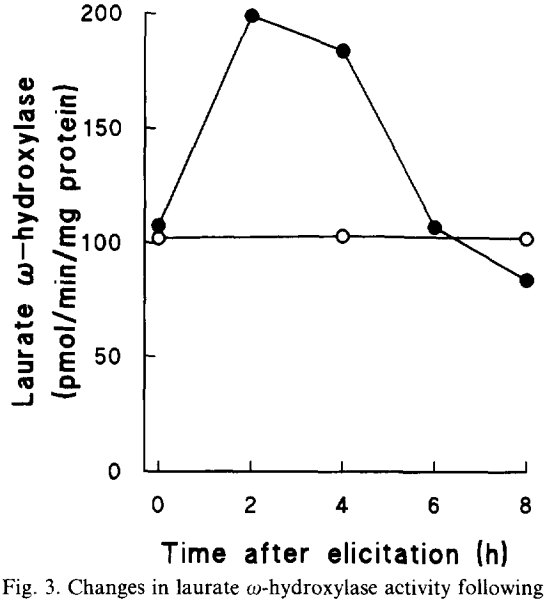

Fig. 3. Changes in laurate ω-hydroxylase activity following treatment of cell suspension cultures with elicitor. Microsomes were isolated from cells harvested at each time point and assayed for transferase activity. Control cells (○—○); elicited cells (●—●).

of both activities to levels lower than in control cells. Loss of activity from microsomal membranes during the later part of the time course is consistent with other observations of enzymes involved in the synthesis of wall material in elicited cells [17].

Characterisation and partial purification of hydroxy fatty acid feruloyl-CoA transferase

Microsomes were prepared from elicitor treated French bean cells and solubilised using Triton X100. This led to a slight activation of the transferase (Table 1). Solubilised microsomes were subjected to anion-exchange HPLC (Fig. 4). Fractions corresponding to the peaks of feruloyl-transferase were pooled separately and subjected to size exclusion chromatography (Fig. 5). The transferase showed aggregation with peaks at 44, 52 and 56 min respectively with the peak showing greatest retention time corresponded to an M_r 40 000. Further analysis by SDS–PAGE gave three major bands of M_r 28 000, 40 000 and 70 000 (data not shown).

DISCUSSION

Ferulic acid occurs in many plants [18]. It is covalently conjugated with mono- and disaccharides, plant cell wall polysaccharides, glycoproteins, lignin, betacyanins and hydroxyfatty acids in cutins and suberins. Several physiological roles of ferulic acid have been proposed. It crosslinks vicinal pentosan chains, arabinoxylans, and hemicelluloses in cell walls [19–22]. Cross-linking is essential to the formation of barriers to invading pathogens [23, 24] and has been implicated in the cessation of elongation [1, 25]. It is thus a useful compound for the study of the formation of dehydrogenation polymer phenolics *in vitro* to understand the nature of these reactions in the wall [12, 26].

Attempts have been made to expand the understanding of the role of ferulic acid in plants by demonstrating transfer from feruloyl-CoA onto polysaccharide with the formation of ferulate-arabinosides. These can be demonstrated by *in vivo* labelling experiments [6, 7], but difficulties of demonstrating this putative transferase *in vitro* were encountered [8]. Transfer onto polysaccharides can be demonstrated in isolated membranes of French bean but it is suppressed by addition of catalase, suggesting that this is due to peroxidase which is abundant in bean microsomal membranes [11]. These peroxidases are capable of polymerising *p*-coumarate, caffeate and ferulate and can cause artefacts when looking for other membrane activities. One peroxidase of M_r 46 000 readily generates hydrogen peroxide from added thiols at neutral pH [14].

Addition of catalase to reaction mixtures however enhanced transfer onto added hydroxydecanoic acid suggesting that this reaction involved in suberization is present in cultured cells of French bean. Feruloyl transferases have been described in potato [10, 11] and

Table 1. Partial purification of feruloyl-CoA transferase from microsomal membranes of suspension-cultured cells of French bean. Microsomes were prepared from 200 g of elicited suspension cultured cells and subjected to the purification protocol. For sizing the fractions were not pooled and the purification is shown for the peak eluting between 40 and 44 min and collected in a total volume of 1.2 ml

Step	Total protein (mg)	Total activity (pmol min^{-1})	Specific activity (pmol min^{-1} mg $protein^{-1}$)	Recovery	Purification (fold)
Microsomes	10	255	25.5	100	1
Solubilized microsomes	7.5	313	41.8	122	1.6
DEAE	1.9	273	144	107	5.6
Sizing	0.08	117	1460	45	57

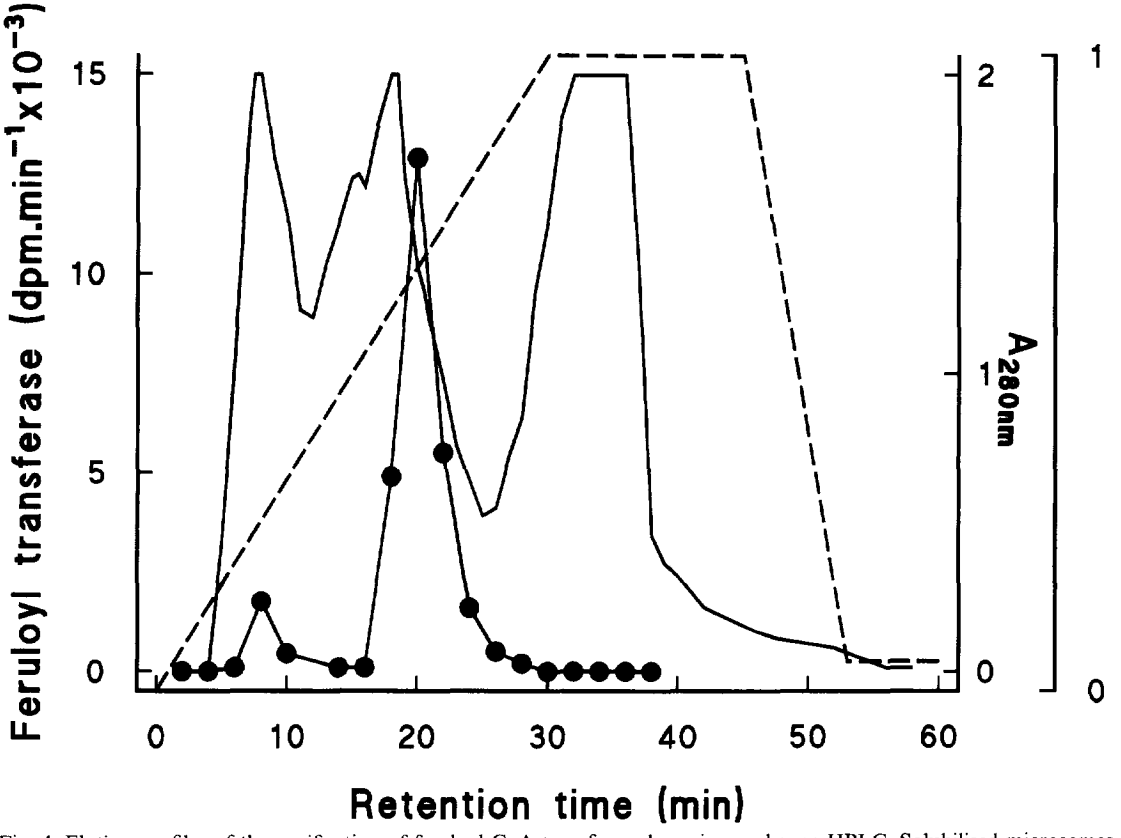

Fig. 4. Elution profiles of the purification of feruloyl-CoA transferase by anion exchange HPLC. Solubilised microsomes (about 15 mg total protein in either case) were added to a column (TSK DEAE-5PW; 75 mm × 7.5 mm i.d.) and proteins eluted with a linear gradient of 0–0.75 M NaCl in 20 mM Tris–HCl pH 7.2 containing 1% RTX at a flow rate of 0.5 ml min^{-1} for 30 min. Enzyme activity was determined (●—●). Protein was monitored at 280 nm (—).

are induced in response to wounding. The feruloyl-CoA transferase was co-induced transiently with the cytochrome P450 responsible for fatty acid hydroxylation. This is one of the first demonstrations of the induction of enzymes of suberin production by elicitor treatments of suspension cultured cells, and is consistent with the production of phenol conjugates as well as the phenylpropanoids, lignin and isoflavonoids as a common and integral part of plant defence.

In a survey of a number of plant species, lauric acid hydroxylation was detected previously in French bean and other legumes [27]. Capric (C10:0), lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids have all been shown to be hydroxylated by P450 from *Vicia faba* at the ω-position [28, 29] and a laurate ω-hydroxylase has been described in pea [15]. This seems to be the favoured position in legumes, the French bean enzyme appears to be no exception. The fatty acid hydroxylases so far described from other species show different regiospecificity [30]. Both enzymes

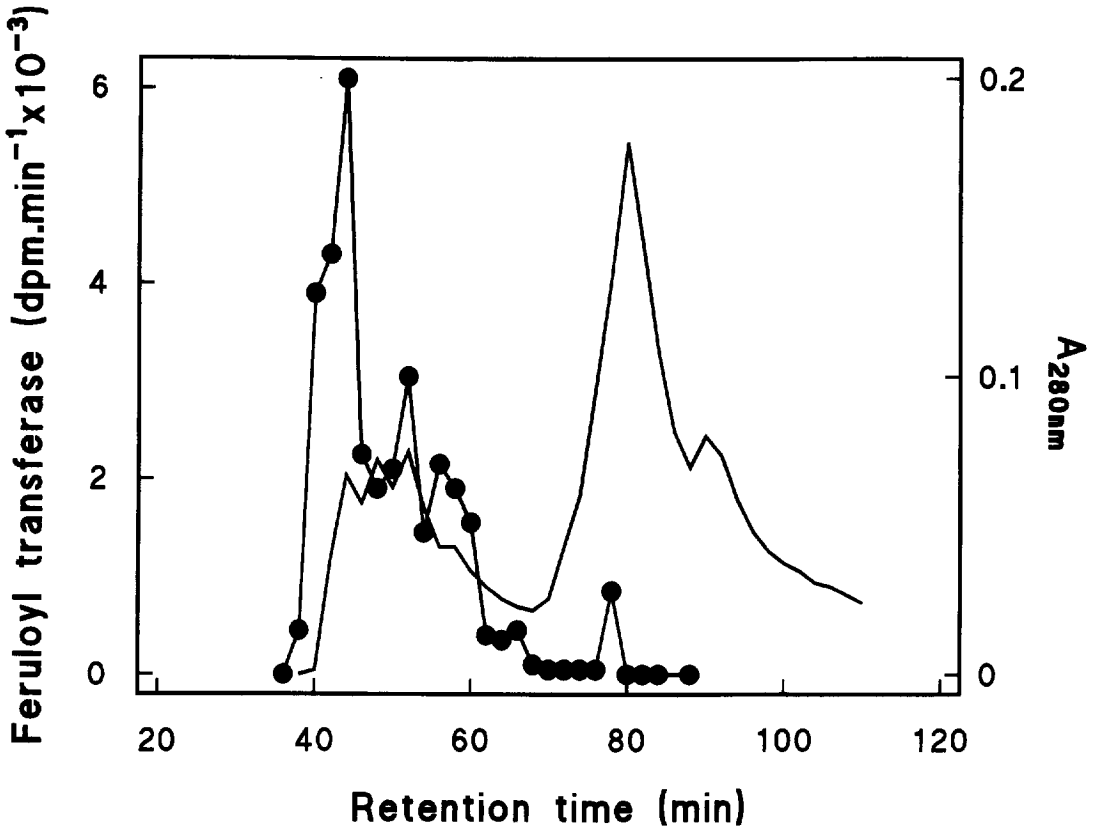

Fig. 5. Elution profiles of the purification of feruloyl-CoA transferase by molecular sizing HPLC. Pooled fractions for each transferase were separated by size exclusion on a column (TSK G2000SW 600 mm × 7.5 mm i.d.) and proteins eluted over a period of 120 min in 20 mM Tris–HCl pH 7.0, 200 mM NaCl, 1 mM dithiothreitol. Enzyme activity was determined in each fraction (●—●) and protein was monitored at 280 nm (—).

would be required for the construction of feruloyl-fatty acid monomers which could be secreted and polymerised at the wall interface by peroxidases to construct defensive suberin. Partial purification of the feruloyl transferase was accomplished. The relationship of this enzyme to other hydroxycinnamoyl-CoA-hydroxy fatty acid transferases is at present unknown.

EXPERIMENTAL

Plant material. Suspension cultures of French bean were derived, maintained and subjected to elicitation as described previously [31]. Cells were either subjected to direct measurements following elicitation or were harvested by vacuum filtration and immediately frozen in liquid N_2 and kept at −70° until required for extraction and measurement of other parameters.

Chemicals. [Methoxy]-^{14}C-feruloyl-CoA (2.11 TBq mol^{-1}) was synthesised at Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berks, U.K. and was a kind gift of Dr G. Foxon. The radiolabelled ferulic acid was esterified to CoA using a hydroxysuccinimide ester intermediate [32]. The feruloyl-CoA was purified before use. *p*-[^{14}C]-Coumaric acid was a kind gift of Dr V. S. Butt, Plant Sciences, Oxford University. [^{14}C]-Lauric acid (2.09 TBq mol^{-1}) was obtained from CEA, Saclay, France.

Preparation of microsomal membranes. French bean cells were homogenised in 50 mM 3-(*N*-morpholino)-propanesulphonic acid (MOPS) pH 7.2, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.4 M sucrose and filtered through 2 layers of muslin. The homogenate was then centrifuged at 20 000*g* for 15 min to remove thioesterase activity as reported previously [6] and then at 100 000*g* for 1 h. The microsomal pellet was resuspended in 50 mM MOPS, 1 mM DTT (pH 7.2) and used for enzyme assays. All procedures were carried out at 4°.

Enzyme assays: (*a*) *Feruloyl-CoA transferase.* 100 μl membrane preparation was incubated with 20 μl MOPS pH 7.2 containing 2.5 mM [methoxy-^{14}C]feruloyl-CoA. For measurements of transfer onto polysaccharide 100 mg arabinoxylan or arabinogalactan was added to the reaction mix. For transfer onto lipid 10 μl 5 mM hydroxyhexadecanoic acid in EtOH was dried onto the bottom surface of the inside of a 1.5 ml Eppendorf vial and the reaction mix added. Reaction mixes were incubated at 25° and terminated by the addition of 10 μl HCOOH. Incorporation onto polysaccharides was determined by PC on Whatman 3MM

in BuOH–HOAc–H_2O (12:3:5) and radioactivity remaining at the origin determined by scintillation counting. In the case of transfer onto lipid the terminated reaction mix was extracted twice with EtOAc, the extracts combined and subjected to scintillation counting. When the effect of catalase was determined, 60 mkat was added to the reaction mixture and incorporation determined as described.

(*b*) *Laurate ω-hydroxylase.* The cytochrome P450, laurate ω-hydroxylase was assayed in microsomal membranes [15]. About 200 μg microsomal protein was incubated with 0.6 mM NADPH, 6.7 mM glucose-6-phosphate and 0.4 U glucose-6-phosphate dehydrogenase in 0.1 M Na-Pi buffer pH 7.4 together with 2.12 mM [1-^{14}C] lauric acid (2.09 TBq mol^{-1}) and 50 mM Na laurate in a total vol. of 250 μl. Incubations were allowed to proceed for 20 min before termination with 150 ml Me_2CO–1 N H_2SO_4 (3:1). The reaction mix was then extracted with $Et_2O \times 2$. The extent of hydroxylation was determined by TLC in comparison with zero time controls.

Characterisation of products. For characterisation of products of the transferase activity, EtOAc extracts were subjected to TLC on Kieselgel using $CHCl_3$–MeOH (4:1) as solvent. Plates were subjected to autoradiography comparing control incubations stopped at zero time with incubations carried out in the presence of catalase. Radioactive spots were identified using the data of ref. [9] (R_f ferulic acid 0.38; 16-hydroxyhexadecanoyl ferulate 0.72). Products of laurate hydroxylation were determined in Et_2O extracts subjected to TLC using Et_2O–petrol (bp 40–60)–HCOOH (70:30:1) as solvent. Radioactive spots were determined by autoradiography and identified by R_f (R_fs 12-hydroxy- 0.45; 9,10-hydroxy- 0.55; epoxy- 0.69; lauric acid 0.85).

Solubilisation and partial purification of feruloyl transferase. Microsomes were solubilised by adjusting them to 1% reduced Triton X100 (RTX) (Aldrich) and gently stirring on ice for 10 min. The solubilised membranes were then centrifuged at 100 000*g* for 30 min. Protein content was determined in frs containing detergent using the BCA protein assay (Pierce), in accordance with the manufacturer's instructions. Anion exchange HPLC was effected on a 7.5 mm × 75 mm TSK DEAE-5PW column (Anachem). Samples were loaded in 20 mM Tris pH 7.2, 1% RTX, and eluted using a linear gradient of zero to 0.75 M NaCl over 30 min. At an overall flow rate of 0.5 ml min^{-1}. Frs were collected and assayed for feruloyl-transferase activity. Active frs were pooled and subjected to size exclusion chromatography. Size exclusion HPLC was conducted using a 600 × 7.5 mm TSK G2000 SW column (Anachem) equilibrated in 20 mM Tris pH 7.2, 0.2 M NaCl, 1 mM DTT, at a flow rate of 0.3 ml min^{-1}. Frs were collected and assayed for feruloyl transferase activity.

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