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Biosynthesis of ferulic acid esters of plant cell wall polysaccharides in endomembranes from parsley cells

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A microsomal preparation from suspension-cultured parsley cells is able to transfer ferulic acid from the respective CoA thioester to endogenous acceptors. The reaction is not enhanced by digitonin but stimulated by Mg^{2+}, Ca^{2+} and Co^{2+}. Spermine can partly replace divalent ions. Solubility properties and degradation by polysaccharide hydrolases suggest that the products are polymeric cell wall carbohydrates. Sucrose density gradient centrifugation revealed that the most active vesicle fraction is distinct from plasma membranes but does also not peak with IDPase. It is suggested that a subfraction of the Golgi-apparatus is the source of enzyme and acceptors.

Parsley suspension cell; Ferulic acid ester; Cell wall polysaccharide; Golgi membrane; Feruloyl-CoA

1. INTRODUCTION

Phenolic constituents of the plant cell wall were for a long time considered to be generally of the lignin type, which is synthesized extracellularly from secreted phenylpropane alcohols with the help of peroxidases and H_2O_2 [1]. In lignin, the cross-links between two phenolics as well as between phenolics and cell wall carbohydrates are, therefore, ill-defined. More recent reports suggest, however, that phenolic acids that are ester-linked to wall carbohydrates are also wide-spread [2,3]. In some cases, digestion of pectin with polysaccharide hydrolases allowed isolation of oligomeric carbohydrates containing Fer or *p*-coumaric acid ester-linked to hydroxyl groups at defined positions of galactose or arabinose. This stereospecific esterification suggests a mode of biosynthesis quite different from lignin [3,4]. This evidence and that from in vivo pulse experiments with radiolabeled precursors [5] are considered to indicate that the phenolic acid esters of cell wall pectins are synthesized in the endomembrane system and are secreted as complete macromolecules [3]. The esterified phenolic acids presumably provide functional groups on pectin to allow cross-linking in the wall after secretion and/or serve as anchoring-points for lignin to cell wall carbohydrates [3].

Here we report the transfer of Fer from Fer-CoA to polysaccharides and show that both the corresponding enzymes and acceptors are likely contained together in membrane vesicles derived from a subfraction of the Golgi apparatus.

Abbreviations: Fer, ferulic acid; Fer-CoA, feruloyl-CoA; IDPase, inosine 5'-diphosphatase; GSII, 1,3-β-glucan synthase II

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2. MATERIALS AND METHODS

The cell culture of *Petroselinum crispum*, which represents a model system for elicitor-induced changes in metabolism, was grown as described [6], and 15 g of cells were washed and homogenized with a Teflon-Potter, as described [7]. Briefly, the microsomes were washed twice with buffer containing 1 mM EDTA, once without EDTA, and suspended in 1 ml of 50 mM Tes-NaOH, pH 7.0, containing 1 mM DTT.

The standard assay consisted of 5 μl of [^{14}C]Fer-CoA (25 000 cpm, 51 mCi/mmol), 25 μl of 100 mM Mes-NaOH, pH 5.8 or 7.2, 15 μl of water or additives, and 50 μl of microsomes. After incubation at 27°C for the indicated time, the reactions were terminated by addition of 6.5 μl of glacial acetic acid.

Routinely, the total assay mixture was spotted as a 2.5 cm streak on Whatman no. 1 chromatography paper and developed for 18 h in *n*-butanol/acetic acid/water (5:2:3). The origin was cut out and counted by LSC. Controls were treated with acetic acid before addition of the microsomes and the resulting activity of about 500 cpm per assay was subtracted. Means from two parallel assays are given.

For tentative product identification (Fig. 3), 20 origins from standard assays were covered with 20 ml of polysaccharide hydrolase. The material solubilized after 3 days was brought to 80% (v/v) ethanol, the supernatant concentrated and passed through a 60 ml bed volume Sephadex G 25 colum equilibrated with hydrolase buffer. Fractions were pooled, evaporated, applied as a 10 cm streak on Whatman no. 1 paper and developed for 18 h in *n*-butanol/acetic acid/water (12:3:5) [8].

For experiments on product solubility and molecular size, a double acidified assay mixture was extracted with 1 ml of chloroform/methanol (1:1), centrifuged and the interphase washed subsequently with 1 ml of the same mixture and twice with 80% methanol. The pellet was heated at 95°C for 10 min in 1 ml of 0.5% (w/v) ammonium oxalate, pH 4.7. After centrifugation (12 000 × *g*, 10 min) the supernatant was brought to 80% (v/v) ethanol, left overnight at 4°C and the precipitate collected and counted. Alternatively, the supernatant was

centrifuged in Centriprep (Amicon) tubes. Zero time controls were treated likewise and the values subtracted.

The polysaccharide hydrolase mixture was purified according to [8] from 'Driselase' (Sigma) and solubilized at 0.5% (w/v) in pyridine/acetic acid/water (1:1:98), pH 4.7.

Labelled Fer was synthesized by Knoevenagel-reaction from [^{14}C]2-malonate (NEN). [^{14}C]Fer-CoA was synthesized [9] using CoA bought from Boehringer (Mannheim). The product was purified by column chromatography on polyamide SC-6 (Machery-Nagel) according to [10] and eluted between 4.4 and 20 mM NH_4OH in methanol. The fractions were evaporated and solubilized at 25 000 cpm/5 μl in water. Authentity of [^{14}C]Fer-CoA was shown by TLC on cellulose with butanol/acetic acid/water (5:2:3) as well as by its absorption characteristics [9].

Sucrose gradient centrifugation and the assay of GSII and total IDPase were performed as described [7] but using buffer without EDTA.

3. RESULTS

Microsomes can transfer [^{14}C]Fer from [^{14}C]Fer-CoA to endogenous acceptors in a time-dependent manner (Fig. 1). The decrease in reaction rate after prolonged incubation time may indicate either an exhaustion of acceptors or an instability of the enzyme under assay conditions. The decrease in [^{14}C]Fer-incorporation in the presence of EDTA and its stimulation by Ca^{2+}, Mg^{2+} and Co^{2+} indicate a requirement for divalent ions (Table I). The ion concentration used for Table I is not saturating, since some further increase in transfer rate is observed at 7 mM Ca^{2+}, Mg^{2+} or Co^{2+} at pH 5.8, and also with 7 mM Co^{2+} at pH 7.2 (data not shown). The comparatively low concentration of divalent ions in Table I was used to demonstrate that spermine can replace or enhance the action of divalent ions. It appears noteworthy that added Ca^{2+} and Mg^{2+}, when compared with the endogenously present ions (Table I, water), cause greater activation at pH 5.8 than at pH 7.2. Similarly, spermine given alone is also more effective at pH 5.8 (Table I). Whether this indicates multiple

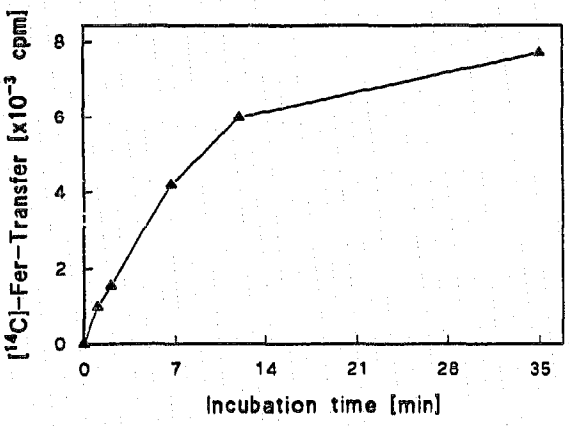

Fig. 1. Time course of labeled Fer transfer from [^{14}C]Fer-CoA to endogenous acceptors by microsomal membranes from parsley cells. The cpm values refer to the transfer by standard assays, incubated for the indicated times at pH 5.8 with 1.4 mM $MgCl_2$.

Table I

Influence of divalent ions, spermine and pH-value on the transfer of [^{14}C]Fer from [^{14}C]Fer-CoA to endogenous acceptors in microsomes from suspension-cultured parsley cells

	Addition	Final conc. (mM)	[^{14}C]Fer-transfer ($\times 10^{-3}$ cpm)	
			− Spermine	+ Spermine
pH 5.8	EDTA	3.6	1.0	–
	Water	–	2.7	5.2
	$CaCl_2$	1.4	3.5	5.0
	$MgCl_2$	1.4	3.6	5.1
	$CoCl_2$	1.4	5.5	6.8
pH 7.2	EDTA	3.6	1.5	–
	Water	–	2.5	3.2
	$CaCl_2$	1.4	2.7	3.1
	$MgCl_2$	1.4	2.7	3.2
	$CoCl_2$	1.4	4.6	5.5

The cpm-values refer to one standard assay with 12 min incubation time. Where indicated, spermine was present at 0.7 mM.

enzymes or acceptor sites cannot be determined without further characterization of the products.

When the microsomal membranes are separated by isopycnic sucrose density gradient centrifugation, the fractions most active in [^{14}C]Fer transfer are clearly distinct from the peak of the plasma membrane marker (GSII) and from the peak of the Golgi marker IDPase [11]. That the [^{14}C]Fer transfer is presumably not associated with plasma membranes is also indicated by the observation that digitonin at 0.01–0.02% (w/v) final concentration in the assay mixture caused no significant change in the reaction rate under all conditions used for Table I (data not shown). In microsomes the majority of the plasma membrane is present as sealed outside-out vesicles and digitonin, therefore, causes a 5–8-fold increase in the activity of typical plasma membrane enzymes such as the Ca^{2+}-dependent GSII [7].

Tentative product identification was attempted by the following criteria. The radioactivity was liberated by treatment in 0.1 N NaOH for 18 h and about 80% was recovered as Fer on HPLC using a reversed-phase C_{18}-column and acetic acid/acetonitrile/water gradients. This indicates ester-linkages. About 95% of the reaction products can be solubilized upon heating in dilute ammonium oxalate similar to pectin labelled in vitro with [^{14}C]methyl ester groups by mung bean microsomes [12]. Up to 50% of the oxalate-solubilized material precipitates in 80% ethanol, as pectic polysaccharides would do. Similarly, 43% of the solubilized [^{14}C]Fer-products were retained by a 10 kDa Centriprep dialysis membrane. The portion of products which does not precipitate or which passes through the 10 kDa membrane might represent still-growing-shorter polysaccharide chains. The carbohydrate nature of at least a great part of the products is also evident from their

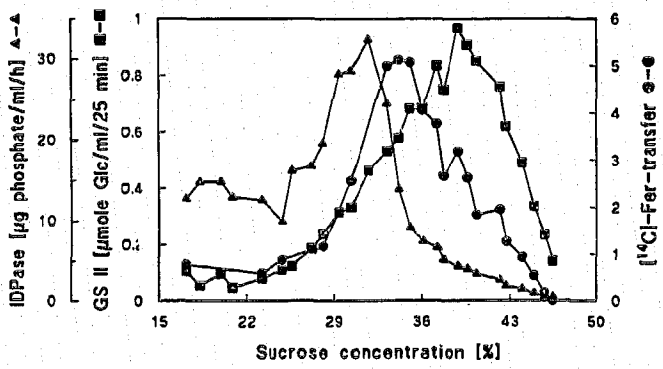

Fig. 2. Distribution in a isopycnic sucrose density gradient of membrane vesicles from parsley cells able to transfer [^{14}C]Fer to endogenous acceptors. Standard assays for [^{14}C]Fer transfer (pH 7.2, 1.4 mM $CoCl_2$, 0.7 mM spermine) were incubated for 25 min and the incorporation is expressed as ($\times 10^{-4}$ cpm/ml).

degradation by polysaccharide hydrolases to oligomers (Fig. 3) which move in the paper chromatography solvent used [4,8] but require the use of microsomes from cells prelabelled with [^{3}H]sugars for further identification.

4. DISCUSSION

Cytochemical and radioautographic electron microscopic studies suggest that pectins and hemicelluloses of the plant cell wall are synthesized in Golgi cisternae, transported within vesicles to the plasma membrane and secreted [11]. This view has more recently also been sustained by immunocytochemical methods [13]. Consistently, the biosynthetic enzymes accept in vitro no exogenous macromolecular acceptors but act only on those polymers that are contained either inside the microsomal vesicles from the cells or that are generated in vitro from nucleoside disphosphate sugars [14,15]. That the acceptors and products are contained within membranes is best illustrated by the in vitro formation of the methyl ester groups of pectin from radiolabelled *S*-adenosyl-L-methionine [15]. The respective reaction products are fully stable for hours in the presence of pectin methyl esterase but are hydrolyzed within minutes after addition of detergents or phospholipase A.

The data reported here for the transfer of [^{14}C]Fer fit this view and demonstrate that the hydroxycinnamic acid ester groups are also introduced into macromolecules within the endomembrane system. Surprisingly, the most active vesicle population does not peak with IDPase, a general marker for Golgi-derived microsomal vesicles. However, IDPase and other marker enzymes are not equally distributed over all cisternae of a dictyosome [11]. Similarly, in suspension-cultured sycamore cells, xyloglucan (hemicellulose) appears to be synthesized in the *trans*-cisternae and leaves from the *trans*-Golgi network, whereas pestic polymers are formed in the *cis* and completed in medial stacks from which they also leave [13]. The various cisternal stacks, therefore, likely can produce different cell wall polysaccharides and, therefore, carry different respective enzyme sets. If the cisternae would differ also in density, a separation of the respective microsomal vesicles as found in Fig. 2 becomes understandable. It was indeed observed in some cases that glucan synthase I which is involved in xyloglucan synthesis splits on isopycnic gradient centrifugation in a peak migrating with IDPase and another one at denser sucrose concentrations [7].

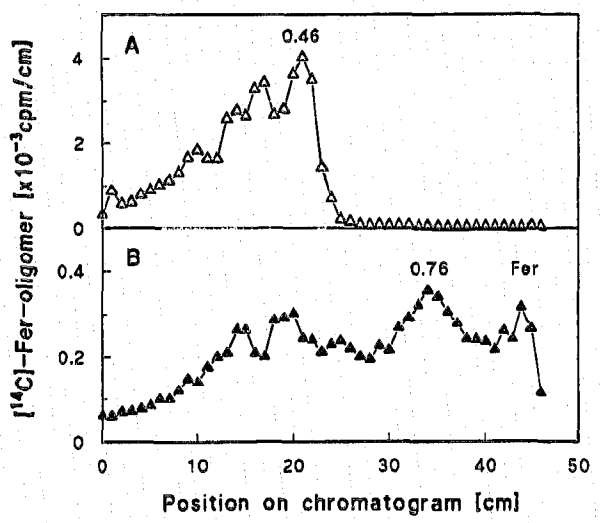

Fig. 3. Paper chromatogram of oligomers solubilized by polysaccharide hydrolases from the [^{14}C]Fer-labelled transfer products from standard transfer assays. The oligomers were passed through a Sephadex G25 column. Some radioactivity in V_0 was discarded and the oligomers included in the gel divided in two fractions of higher (A) and lower (B) apparent molecular weight. Position 0 = start; position 46 = front of the paper chromatogram. The R_f value of 0.46 possibly corresponds to Fer-galactose-galactose and of 0.76 to Fer-arabinose-arabinose [4,8]. Identity of the R_f 0.46 peak is sustained by the observation that a spot corresponding to less than 1 µg of galactose is found on TLC after its acid hydrolysis [8].

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