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TRANSFER OF HYDROXYCINNAMOYL RESIDUES TO MICROSOMAL PROTEINS FROM PARSLEY

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Key Word Index—*Petroselinum crispum*; Apiaceae; parsley; microsomal membranes; feruloyl-CoA; *p*-coumaroyl-CoA; hydroxycinnamoyl transferase; protein substitution; glycoprotein; fungal elicitor.

Abstract—Microsomal membrane preparations from parsley cells in culture produced polymeric material from [^{14}C]-feruloyl- and [^{14}C]-*p*-coumaroyl-CoA. This reaction could not be inhibited by addition of catalase, suggesting that polymerization did not involve peroxidases and H_2O_2. About half the labelled polymer could be extracted with aqueous phenol, precipitated and split by trypsin, indicating a protein nature. The labelled proteins appeared as four major discrete bands in SDS–polyacrylamide gels. One of these bands was diminished whereas an additional band became visible after treatment with either *N*-glycosidase F or endoglycosidase H. Thus, we assume that part of the labelled proteins is *N*-glycosylated. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Hydroxycinnamoyl esters of plant cell wall polysaccharides have been isolated and characterized from a variety of plants and are supposed to provide functional groups for *in situ* cross-linking [1]. Similarly, low M_r plant secondary metabolites bearing ester- or amide-linked hydroxycinnamoyl groups at distinct positions are widespread as well [e.g. 2–4]. In contrast, early reports about plant proteins bearing various hydroxycinnamoyl residues—presumably even in different types of linkages [5]—did not attract further interest. Here, we describe the enzymic substitution of yet unknown proteins which are endogenously present in parsley microsomes, employing [^{14}C]-hydroxycinnamoyl-CoA as the substrate. The results described may initiate further isolation of these proteins aimed at evaluating their function.

RESULTS AND DISCUSSION

In an effort to demonstrate the biosynthesis of [^{14}C]-hydroxycinnamoyl esters of cell wall polysaccharides, we employed microsomes or partially purified endomembranes from parsley suspension cells and detected the production of labelled polymeric material from [^{14}C]-feruloyl-CoA [6]. The assay used in these experiments was based on paper chromatography. The polymers residing at the chromatographic start point were partly solubilized by a hydrolase-rich preparation partially purified from commercial fungal 'Driselase'. Some of the soluble low M_r material migrated on paper chromatograms with R_f-values that had been reported for feruloyl-oligosaccharides which had been liberated from cell wall polysaccharides by the same hydrolase preparation. Although the nature and type of linkage of the presumed sugars bound to the soluble [^{14}C]-feruloyl oligomers could not be identified so far due to the low amounts present, we concluded that part of the labelled polymers synthesized from [^{14}C]-feruloyl-CoA might be cell wall polysaccharides.

During further experiments aimed at identifying the [^{14}C]-labelled polymeric material we have modified the assay procedure by employing a washing procedure to remove incorporated substrate, thus allowing a better handling of the polymeric radioactive products in the microsomes. Employing this procedure, we realized that a major portion of the product could be solubilized on incubation with trypsin, suggesting the presence of labelled proteins (data not shown). In fact, about half the polymer synthesized in a time-dependent reaction form [^{14}C]-*p*-coumaroyl-CoA could be isolated by following a standard procedure that has been used for the extraction and isolation of plant membrane proteins (Fig. 1).

It has recently been shown that formation of polymeric materials from [methoxy-^{14}C]-feruloyl-CoA by membrane preparations from suspension cultured maize cells was greatly suppressed by exogenous catalase and did not proceed in the presence of Mg^{2+}, but was strictly dependent on the simultaneous presence of both Co^{2+} and dithiothreitol [7]. These authors concluded that dithiothreitol present in the microsomal preparation

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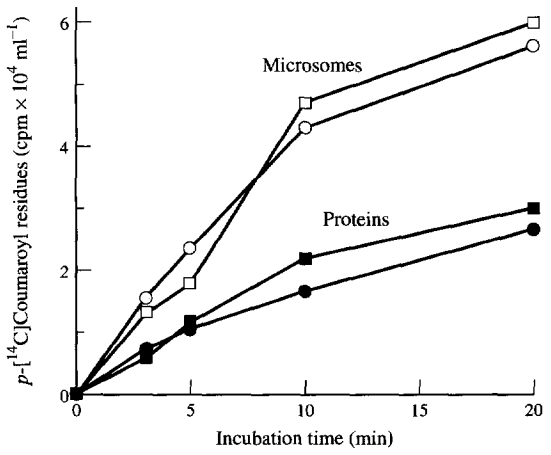

Fig. 1. Time-course and influence of exogenous catalase on the incorporation of [^{14}C]-*p*-coumaroyl residues into microsomes (○, □) and endogenous proteins recovered therefrom by extraction with phenol (■, ●). Control assay: □, ■; +50 U catalase ml^{-1}: (○, ●). The assays were incubated at pH 7.2 for the indicated times in the presence of [^{14}C]-*p*-coumaroyl-CoA (25×10^4 cpm) and 2 mM $MgCl_2$. Incorporation was calculated for 1 ml of microsomal suspension.

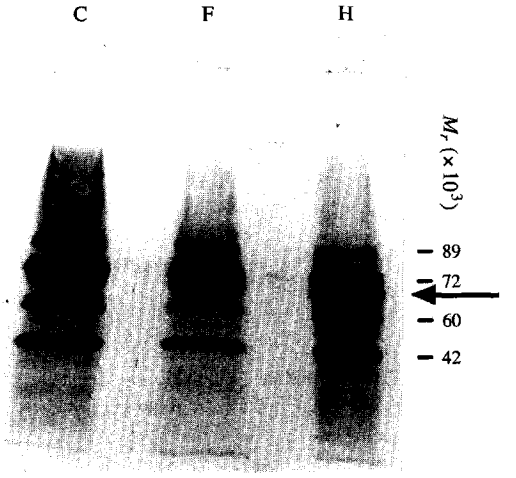

Fig. 2. SDS–PAGE of [^{14}C]-*p*-coumaroyl labelled proteins and the influence of and incubation with enzymes specific for *N*-glycoproteins. The proteins labelled after incubation with [^{14}C]-*p*-coumaroyl-CoA in 1 ml of microsomes as in Fig. 1 were isolated and subjected to PAGE either directly (C = control) or after an incubation for 24 hr with *N*-glycosidase F(F) or endoglycosidase H(H). The arrow indicates a labelled band arising after treatment with the latter two enzymes. M_r was calculated from marker proteins that had been run in parallel.

acts as a competitive substrate for peroxidases and thus would prevent oxidative polymerization of feruloyl-CoA. However, Co^{2+} added in excess forms an insoluble complex with the thiol and thereby removes the alternative peroxidase substrate, restoring polymerization. In contrast, labelling with [^{14}C]-*p*-coumaroyl-CoA of microsomes and proteins from parsley could not be significantly inhibited by exogenous catalase and, in addition, did proceed in the presence of Mg^{2+} (Fig. 1). These findings indicate that, in parsley microsomes, formation of labelled polymers does not depend on peroxidase-catalysed polymerization as was suggested for maize microsomes [7].

The labelled polymer extracted with PhOH was precipitated and separated by SDS–PAGE into four major bands, indicating that polymerization is a specific event which involves a few endogenous acceptor proteins (Fig. 2, lane C). When [^{14}C]-*p*-coumaroyl-CoA was replaced by [^{14}C]-feruloyl-CoA, the same amount of labelled protein and a similar pattern of radioactive bands were determined (data not shown). As both compounds had the same specific radioactivity these results suggested that the presumed cinnamoyl transferase exhibited the same activity for both of the substrates. In contrast, using [^{14}C]-sinapoyl-CoA as the substrate, only about half the label was incorporated into microsomes when compared to [^{14}C]-*p*-coumaroyl-CoA, whereas [^{14}C]-*p*-hydroxybenzoyl-CoA was not significantly accepted as a substrate (data not shown). All of the labelled bands in Fig. 2 and all the numerous distinct protein bands visible in the gel after Coomassie Blue staining were absent after treatment of the precipitated material with trypsin before gel electrophoresis (data not shown). Taken together, these observations strongly suggest that the isolated portion of the [^{14}C]-cinnamoyl products are proteins.

When the isolated labelled proteins were subjected to alkaline hydrolysis (1 N NaOH, 20°, 20 hr), up to 80% were solubilized. This water-soluble material was extracted with EtOAc and subjected to HPLC. Calculating with the recovery rates determined with the respective CoA-derivatives, the peaks of radioactive ferulic or *p*-coumaric acid contained about 30 or 50%, respectively, of the radioactivity in the protein. In addition to an elevated base-line also two minor peaks were observed in the case of ferulic acid, indicating a partial destruction of the labelled hydroxycinnamic acids. This appears understandable as the radioactivity corresponds only to extremely low amounts (pmoles) of material. Nevertheless, the demonstration of the free hydroxy-[^{14}C]cinnamic acids indicates that a considerable part was likely present in the proteins in an ester linkage, even though it is not known to which extent hydroxycinnamic acids in amide linkages [5] would be liberated by the treatment used for the present report. It may be interesting for researchers working on cell wall polysaccharides employing hydrolase preparations from 'Driselase' [8] that the [^{14}C]-hydroxycinnamoyl proteins were also decomposed by this enzyme preparation. This result suggested the presence of proteases in the 'Driselase' preparation, which, in fact, could be directly demonstrated with the Azocoll assay [8] (results not shown).

When the precipitated labelled proteins were incubated with two enzymes specific for *N*-glycoproteins,

an additional band with a M_r of *ca* 70 000 (Fig. 2, arrow) appeared in the gel, whereas part of the label disappeared from the M_r 89 000 band (Fig. 2, lanes F and H). It is not clear, however, whether the M_r 70 000 band resulted from the M_r 89 000 or from the major band in Fig. 2, lane C. These results, therefore, do not allow conclusions with regard to the position of the labelled phenolic groups introduced, but indicate at least that part of the hydroxycinnamoyl proteins consists of *N*-glycoprotein(s).

When the [^{14}C]-labelled proteins in SDS gels similar to those of Fig. 2 were blotted on to a membrane and probed with the monoclonal antibody JIM13, which specifically recognizes certain carbohydrate epitopes typically found on arabinogalactan proteins [9], at least three bands in the M_r range of the labelled proteins became visible (not shown). Similarly, after probing with ConA, multiple bands consisting presumably of *N*-glycosides could be demonstrated in the M_r range of the radioactive bands shown in Fig. 2. These results indicate that the methods for extraction and electrophoresis were appropriate to isolate and demonstrate the two types of glycoproteins, suggesting that they may be among the acceptors for the cinnamoyl residues.

The parsley cell suspension culture employed in the present study represents a well characterized model system for evaluating changes in cell metabolism induced by fungal elicitors [10], including the deposition of polymerized cell wall phenolics which contain esterified *p*-coumaric and ferulic acid [11]. At both pH values used in the assay system and with Mg^{2+} as the activating cation, the amount of [^{14}C]-*p*-coumaroyl residues that had been incorporated into proteins from microsomes of elicitor-treated cells is lower when compared to control cells (Fig. 3). When Mg^{2+} was replaced by Co^{2+}, the labelling of proteins from control and elicitor-treated cells was similar. In addition, Mg^{2+} preferentially stimulated at pH 5.8 whereas Co^{2+} was more active at pH 7.2. These observations may indicate the involvement of several transferases or different pH/cation requirements for the various endogenous acceptor proteins. Although these complex relationships certainly require further quantification studies, the observation that the Mg^{2+}-dependent transferase activity is apparently lower in elicitor-treated cells may indicate a role for the hydroxycinnamoyl proteins in reactions that are altered during pathogen defence. Even in the case where this vague hint of a physiological function for the hydroxycinnamoyl proteins does not hold, this report may draw attention to a recently ignored type of post-translational modification for plant proteins.

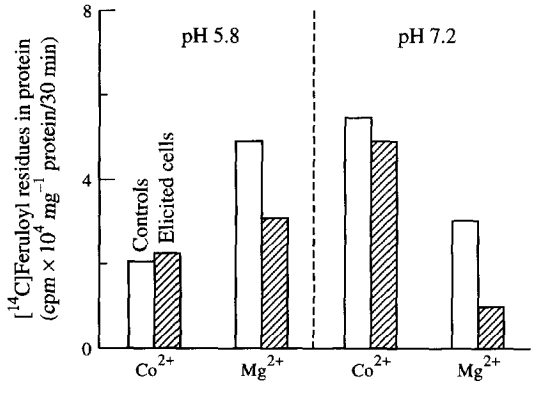

Fig. 3. Influence of pretreatment of parsley cells with a fungal elicitor, as well as of the activating cation and the pH value in the assay on the incorporation of [^{14}C]-feruloyl residues into proteins. Open bars refer to control cells and hatched bars to cells incubated for 18 hr with 40 μg ml^{-1} fungal elicitor [11] before preparation of microsomes. Microsomal preparations from both cell types exhibited a similar protein content; the values given were calculated per mg microsomal protein. Incubation of the assays was either at pH 5.8 or 7.2 with [^{14}C]-feruloyl-CoA for 30 min in the presence of either 2 mM Mg^{2+} or 2 mM Co^{2+}. The results are from a representative of two experiments performed. The above labelling pattern for proteins was very similar to that of the respective microsomes, and this was confirmed in five experiments.

EXPERIMENTAL

Labelled hydroxycinnamoyl substrates were synthesized chemically from [^{14}C]-2-malonic acid (2 Tbq mol^{-1}) as described before [6]. Details can be found elsewhere [12]. [^{14}C]-*p*-Hydroxybenzoic acid (0.44 Tbq mol^{-1}) was purchased from Sigma and converted into the CoA thioester as described above.

Origin and growth conditions of parsley cell culture, as well as prepn of microsomal frs, have been described [6]. The standard assay for transferase activity consisted of 1 ml microsomes (0.5–0.7 mg protein) in 50 mM TES–NaOH (pH 7.2) or 50 mM MES–NaOH (pH 5.8) both containing 1 mM dithiothreitol. After addition of 50 μl 44 mM $MgCl_2$ or $CoCl_2$, reactions were started by addition of 50 μl of respective substrate in H_2O. After indicated times at 25°, reactions were terminated by addition of 100 μl HOAc.

For determining polymerized [^{14}C] in microsomes, 100 μl portions of the 1.2 ml reaction mixt. were washed ×3 with 1 ml EtOH–0.5 M HOAc (3:7, pH adjusted to 4.7 with NH_3). The final pellet was counted by liquid scintillation counting (LSC). The remainder of the reaction mixt. was extracted with aq. PhOH under conditions optimal to isolate plant membrane proteins [13]. The final PhOH phase was mixed with 5 vol. MeOH containing 0.1 M NH_4OAc and stored at −20° for 18 hr. The pptd proteins were washed ×3 with the MeOH–NH_4OAc and once with Me_2CO and dried in a stream of N_2. An aliquot of the last suspension in MeOH-NH_4OAc was counted by LSC.

The protein pellet was solubilized in sample buffer for SDS–PAGE [14] which was performed on minigels. After blotting on to poly(vinylidene fluoride) membranes (Millipore) and spraying with En^{3}Hance (NEN) radioactive protein bands were detected by fluorography. Alternatively, the protein pellet was either treated with trypsin in urea [15] or with the 'Driselase'

prepn [8], followed by SDS–PAGE as above. JIM13 was used to detect arabinogalactan proteins on blotted gels [16]. ConA-binding bands were detected on the membrane as described in ref. [16], but with use of unconjugated horseradish peroxidase. Treatment of the protein pellet with enzymes specific for *N*-glycosides was performed as recommended by the manufacturer (Boehringer).

Alkaline hydrolysis of [^{14}C]-cinnamoyl proteins, EtOAc extraction and HPLC of resulting low M_r products was performed as described for parsley cell wall phenolics [11]. Recovery of [^{14}C]-ferulic and [^{14}C]-*p*-coumaric acids by these procedures was 30 and 38%, respectively, as determined with respective CoA derivatives.

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