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UDPG: *p*-COUMARATE GLUCOSYLTRANSFERASE ACTIVITY IN ENZYME EXTRACTS FROM HIGHER PLANTS

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Key Word Index—*Coleus*; Labiatae; *Cestrum*; *Lycopersicon*; Solanaceae; UDPG: *p*-coumarate glucosyltransferase; hydroxycinnamic acids; biosynthesis.

Abstract—Leaves of *Coleus*, *Pilea*, *Cistus* and *Cestrum*, and ripe tomatoes were all able to convert *trans*-cinnamic acid-[3-^{14}C] into glucose esters of cinnamic acids. The pool sizes of these esters were measured by the radioisotopic dilution method, and they were found to be of the order of a few μg/g fresh plant material. 1-*O*-Caffeoyl-β-D-glucose in *Cestrum* leaves amounted to 70 μg/g fresh plant material. Enzyme extracts from *Cestrum* leaves were able to convert *trans*-*p*-coumaric acid-[3-^{14}C] to 1-*O*-*p*-coumaroyl-β-D-glucose, using UDPG as a source of glucose. This enzyme activity could be measured only by trapping techniques, due to the presence of considerable hydrolase activity in crude enzyme extracts.

INTRODUCTION

Infiltration experiments have shown that glucose esters of cinnamic acids are readily formed from the parent acids in higher plants [1, 2]; although they are frequently reported as plant constituents, no quantitative data exist about their abundance in the respective plants. These esters have a high turnover in the leaves of *Cestrum euanthes** [3]. Possibly, they are active biosynthetic intermediates, rather than products of a detoxification reaction. For the biosynthesis of these esters, either the acid or the sugar molecule must be activated. CoA esters of phenolic acids have been reported as the active precursors for the biosynthesis of esters with quinic and shikimic acid [4, 5]. In these experiments, glucose was not active as a substrate for ester formation [4]. Two papers deal with the enzymatic synthesis of glucose esters using UDPG and the parent cinnamic acid [6, 7]. In this paper, we measured the natural pool sizes of cinnamoyl-glucose esters in various plant species and organs, and the enzymatic synthesis of *p*-coumaroylglucose from *p*-coumaric acid and UDPG, by the use of radioactive tracer techniques.

RESULTS AND DISCUSSION

Feeding experiments

A study of the biosynthesis and natural abundance of 1-*O*-*p*-coumaroyl-β-D-glucose was performed, in order to select suitable plant organs with good glucosylating activity.

Leaves. In leaves of *Cestrum euanthes*, the first metabolites of cinnamic acid were identified as *p*-coumaric acid and 1-*O*-cinnamoyl-β-D-glucose [3].

The same observation holds for the leaves of *Coleus*, *Pilea*, *Cistus* and *Cestrum* analysed in this study. The radioactivity in the 'neutral' phenolic fraction was almost totally localized in 1-*O*-cinnamoyl-β-D-glucose and in 1-*O*-*p*-coumaroyl-β-D-glucose (Table 1). Labelled 1-*O*-caffeoyl-β-D-glucose was found only in leaves of *Cestrum euanthes*. The concentration of glucose esters was measured by the radioisotopic dilution method (see Experimental). The natural pool sizes of glucose esters in the leaves of these plant species were small. The amount of *p*-coumaroylglucose was of the order of a few μg/g fr. wt (see Table 1). Caffeoylglucose was present in appreciable quantities in leaves of *Cestrum euanthes* where it amounted to 70 μg/g fr. wt.

Fruits. Ripe tomatoes showed a different pattern of labelling: labelled cinnamoylglucose was not detected, and the radioactivity in *p*-coumaroylglucose was only a small fraction of the total radioactivity accumulated in the neutral phenolic fraction. The concentration of the latter glucose ester was 4 μg/g fr. wt (Table 1).

Enzyme preparations

Cestrum euanthes leaves, and ripe tomatoes were chosen for enzyme preparations. Table 2 shows the results of the incubation experiments with enzyme extracts. We measured the conversion of labelled *p*-coumaric acid to *p*-coumaroylglucose by these extracts. UDPG was added as a source of activated glucose. We were unable to demonstrate enzymatic activity. However, when we also added some synthetic *p*-coumaroylglucose to the incubation mixture (trapping experiment), it was possible to demonstrate that ^{14}C labelled *p*-coumaric acid was converted to *p*-coumaroylglucose.

Radioactivity was trapped in the *p*-coumaroylglucose pool in the experiments with *Cestrum euanthes* extracts,

* The binomial *Cestrum poeppigii* (Solanaceae) was used for the same plant in earlier publications from our laboratory [3]. In a recent investigation at our request, Lindeman and Ferguson [10] suggest that the binomial *Cestrum euanthes* is more appropriate for the plant in question.

Table 1. Natural amounts of 1-*O*-*p*-coumaroyl-β-D-glucose (in μg/g fresh plant material) in plant leaves and in tomato (var. "Rianto 404") and incorporation of label from cinnamic acid-[3-^{14}C] into 1-*O*-*p*-coumaroyl-β-D-glucose and into 1-*O*-cinnamoyl-β-D-glucose

	Cinnamyl ester		
	1-*O*-*p*-coumaroyl-β-D-glucose		1-*O*-cinnamoyl-β-D-glucose
Plant material	radioactivity (%)	concentration (μg/g)	radioactivity (%)
Coleus	9.5	6	1.6
Pilea	2.6	<1	0.7
Cistus	2.1	<1	4.7
Tomato	4.0	4	0
Cestrum	10	<1	4.0

Table 2. Incorporation of radioactivity from *trans*-*p*-coumaric acid-[2-^{14}C] in 1-*O*-*p*-coumaroyl-β-D-glucose in enzyme extracts from *Cestrum* leaves and from tomatoes (cv Cërise)

		Conversion to *p*-coumaroylglucose (%)	
Plant material	'Cold' *p*-coumaroylglucose present (trapping)	normal extract	blank
Tomato	no	0	0
Tomato	yes	0	0
Cestrum	no	0	0
Cestrum	yes	0.9	0
Cestrum	yes	1.2	0

with 0.9 and 1.2% efficiency. These figures were determined after thorough chromatographic purification of the *p*-coumaroylglucose fraction (see Experimental).

The fact that positive results were obtained only when the radioactivity was trapped in a pool of synthetic *p*-coumaroylglucose could mean that in the incubation mixture, the rate of breakdown of *p*-coumaroylglucose was higher than its rate of biosynthesis. Indeed, measurements of the stability of this ester and UDPG in incubation media obtained from *Cestrum* leaves, proved that these enzyme extracts contained considerable hydrolase activity. In the conditions described for a typical incubation with an artificially enlarged *p*-coumaroylglucose pool (see Experimental, incubations—trapping experiments), 50% of this ester was hydrolysed in 3 hours. UDPG was much more stable under the same conditions: after 3 hours, its concentration was still 95% of the initial concentration.

CONCLUSION

Leaves of different higher plants showed approximately equal rates of biosynthesis of glucose esters from labelled *trans*-cinnamic acid. The only radioactive components found in a neutral phenolic fraction were 1-*O*-cinnamoyl-β-D-glucose, 1-*O*-*p*-coumaroyl-β-D-glucose and 1-*O*-caffeoyl-β-D-glucose. Natural amounts of these esters were very low in the investigated plant organs, mostly less than 1 μg/g fr. wt. In *Cestrum euanthes* however, the quantity of 1-*O*-caffeoyl-β-D-glucose amounted to 70 μg/g fr. wt.

Enzymatic synthesis of 1-*O*-*p*-coumaroyl-β-D-glucose from *p*-coumaric acid and UDPG was demonstrated in crude enzyme extracts. Enzyme activity could only be measured in trapping experiments because considerable hydrolysis of the ester occurs in these extracts.

EXPERIMENTAL

Infiltration with radioactive materials. (a) Leaves: 5–10 mature leaves of *Cistus antartica*, *Pilea cadierei*, *Coleus blumei* and *Cestrum euanthes* were infiltrated with a soln containing 40 μCi cinnamic acid-[3-^{14}C] (Amersham; 57 mCi/mmol). The infiltration conditions were the same as described earlier [3]. The infiltration time was 7 hr. (b) Tomatoes: 1 ripe tomato (var. "Rianto 404"; fr. wt 92 g) was infiltrated for 4 hr with 30 μCi cinnamic acid-[3-^{14}C] in 300 μl H_2O. The soln was fed to the pericarp, in about 2 mm-deep cuttings through the exocarp. By extraction with 80% EtOH, concn and filtration over an ultrafilter (CP50A, Amicon) we obtained a clear H_2O extract which was analysed by chromatographic techniques.

Preparation of enzyme extracts. Leaves of *Cestrum euanthes* or ripe tomatoes (cv Cërise) were cooled in liquid N_2, crushed, and freeze-dried to constant wt. The dry powder was pulverized in a Waring blender and stored in the deep-freezer. 1 g of this powder was poured into 3 ml Tris–HCl buffer (pH 8.3), containing 25 μl mercaptoethanol, 1.2 g polyvinylpyrrolidone (K 25, Fluka) and 0.3 g polyethyleneglycol (PEG 6000, Fluka).

This mixture was treated briefly with a mixer, stirred at 0° for 10 min, and filtered through cheesecloth. The extract was centrifuged (20 000 g) at 0° for 15 min. The supernatant was eluted from a Sephadex G 25 column with 0.1 M phosphate

buffer (pH 7.5). The eluant (≈65 ml) was concd by ultrafiltration (diaflo 10 PM 10, Amicon) to a final vol. of 10 ml. We used 5 ml of this extract in each expt.

Incubations. 100 μl of a 0.05 M solution of *p*-coumaric acid-[2-^{14}C] (10 μCi), 2 μl mercaptoethanol, 100 μl of a 0.05 M soln of UDPG, and 1 ml of a 0.1 mM soln of $MnCl_2$ were added to 5 ml of the above enzyme extract. In trapping experiments, 0.5 μmol *p*-coumaroylglucose were added, while for a blank, the enzyme extract was boiled for 10 min. The incubation mixture was shaken in a water bath at 30° for 3 hr. 1 ml 20% *m*-phosphoric acid and 5 ml 40% $(NH_4)_2SO_4$ were added and the compounds of interest were extracted with EtOAc (×3). The combined organic phases were concd and investigated chromatographically.

Extraction of plant material. An 80% EtOH extract was concd in vacuo to *ca.* 5 ml and centrifuged at 25000 *g* for 10 min. The phenolics were present in the supernatant fraction (SNI). The residues of concn and centrifugation were dissolved in CH_2Cl_2–H_2O. This mixture was concd *in vacuo* to *ca.* 3 ml and the resulting H_2O phase centrifuged at 25000 *g* for 10 min.

The supernatant (SN2) was added to SN1 and this H_2O extract was concd to *ca.* 3 ml. H_2O was added to a final vol. of 5 ml, and the extract was filtered over an ultrafiltration membrane cone (CF50A, Amicon). This extract is ready for further chromatographic purification.

Chromatographic analysis of plant extracts and enzyme extracts. The H_2O extracts of plants and the EtOAc extracts of incubation mixtures were purified with a small preparative column before HPLC analysis. These mixtures were injected on a glass column (15 mm i.d. × 8 cm length) filled with DEAE-Sephadex (A25, Pharmacia) or with Ecteola cellulose (2100 Macherey–Nagel), both in their acetate form. The neutral phenolics were eluted with 100 ml 0.01 M NH_4Ac. This fraction was concd to dryness *in vacuo*, and redissolved in a few ml H_2O. It could be analysed with HPLC, or when it was still too complex, it was divided into subfractions on a longer column (9 mm i.d. × 30 cm length) filled with DEAE-Sephadex (for further details, see ref. [9]). The efficiency of the above method was measured using ^{14}C labelled 1-*O*-*p*-coumaroyl-β-D-glucose, obtained from infiltration experiments. 20 μg of this compound (0.5 μCi) were added to an 80% EtOH extract which was treated as described above. The overall yield was 80%. New batches of carbohydrate-based ion exchangers need to be tested for their chromatographic behaviour towards neutral *O*-dihydroxy phenolics before use, as they occasionally contain impurities which cause the complete retention of these phenolic compounds.

Quantitative determinations. (a) Plant leaves and tomatoes. The glucose esters in plant extracts were analysed by the radioisotopic dilution method. An amount of ^{14}C-labelled cinnamic acid with known spec. act. was fed to plant leaves or tomatoes. These were analysed for *p*-coumaroylglucose with extensive chromatography on DEAE-cellulose followed by reversed-phase HPLC. The spec. act. of this compound was determined and compared to that of the cinnamic acid fed. We then calculated the amount of *p*-coumaroylglucose that was derived from labelled cinnamic acid, and its natural pool size.

(b) Enzyme extracts: Here we only determined the percentage conversion from labelled *p*-coumaric acid to *p*-coumaroylglucose. For this purpose, more cold *p*-coumaroylglucose was added to the incubation mixture, which was then extracted, and purified extensively with low pressure LC on ion exchangers before analysis on reversed phase HPLC.

Quantitative analysis. The identity of all analysed glucose esters was checked by HPLC on reversed-phase and diol-type columns [8]. After hydrolysis of the compounds with emulsin, the identity of the phenolic moiety was also verified in both these LC systems.

High pressure liquid chromatography. See also ref. [8]. UDPG breakdown in an enzyme extract was monitored on an ion-exchange column (partisil 10 SAX, Whatman; 4.6 mm i.d. × 25 cm length). The eluant was a mixture of 7 mM KH_2PO_4 and 150 mM KH_2PO_4 (19:1). Flow rate 2 ml/min; UV detection.

Reference products. Glucose esters and *trans-p*-coumaric acid-[2-^{14}C] were synthesized as described elsewhere [3].

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