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CATABOLISM OF FLAVONOL GLUCOSIDES IN PLANT CELL SUSPENSION CULTURES

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Abstract—Catabolism of flavonol glucosides was investigated in plant cell suspension cultures using kaempferol 3-*O*-β-D-glucoside and kaempferol 7-*O*-β-D-glucoside labelled with ^{14}C either in the glucose or in the flavonol moiety. Catabolic rates of glucosides were compared with those of free glucose and kaempferol. All substrates were degraded efficiently by cell cultures of mungbean, soybean, garbanzo bean and parsley. Based on $^{14}CO_2$-formation, glucose from position 3 of kaempferol is 3–5 times more rapidly metabolized than that from position 7. The flavonol nucleus from both isomers is, however, oxidized to the same extent with a considerable portion of the flavonol being incorporated into insoluble polymeric cell material.

INTRODUCTION

Previous reports from this laboratory have described plant cell suspension cultures as suitable systems for degradative studies on plant polyphenols [1–3]. A great variety of aromatic and heterocyclic plant constituents were thus shown to be degradable by plant cells [4–7]. Except for some experiments with chalcone-glucosides [8] our previous studies on flavonoid degradation were all conducted with aglycones. Since flavonoids predominantly occur in the living cells as glycosides, catabolic studies with these plant products are probably more suitably carried out by using appropriate glycoside derivatives. This is especially recommended in case of flavonols, because the various glycosidic forms co-occurring in one particular plant may well show different levels of turnover. Thus, in *Picea abies* kaempferol 3-*O*-glucoside is metabolized more rapidly than the equivalent 7-*O*-glucoside [9] and Strack, working with *Cucurbita maxima* seedlings, determined very different rates of turnover of flavonol-glucosides depending on the degree of glycosylation [10]. Our own enzymatic studies on peroxidase-catalyzed flavonol degradation have also shown considerable differences between 3-*O*- and 7-*O*-substituted flavonols. Flavonol aglycones and their 7-*O*-substituted derivatives are degraded via 2,3-dihydroxy-flavanones to benzoic acids derived from the B-rings. 3-*O*-substituted flavonols are only attacked by peroxidases when much higher concentrations of enzyme and substrate are available. This obviously very different reaction has not yet been clarified [11,12,21, Frey and Barz, unpublished]. Furthermore, glycosides may be a better form for uptake of substrates by plant cells and for penetration to the site of catabolism.

We have now compared the intensity and degree of catabolism of appropriately ^{14}C- labelled kaempferol 3-*O*- and 7-*O*-β-D-glucosides in cell suspension cultures of *Cicer arietinum L.*, *Glycine max.* Merr., *Phaseolus aureus* Roxb. and *Petroselinum hortense*. Any differences in the rate of degradation between these two glycosides are also of interest because recent measurements by Sutter and Grisebach [13] have shown flavonol 3-*O*-glucosides to be energy-rich compounds.

RESULTS

Synthesis of ^{14}C-labelled kaempferol glucosides

For the separate determination of metabolic rates of the glucose and the flavonol portion of the substrates, the following ^{14}C-labelled compounds were synthesized: kaempferol 3-*O*-β-D-[U-^{14}C]-glucoside (**1**), kaempferol 7-*O*-β-D-[U-^{14}C]-glucoside (**2**), kaempferol 3-*O*-β-D-glucoside [U-^{14}C] (**3**) and kaempferol 7-*O*-D-glucoside [U-^{14}C] (**4**). Enzymatic procedures [14,15] were employed which made use of the recently described, position specific UDP-glucose: flavonol glucosyltransferases from illuminated cell suspension cultures of parsley. Commercially available UDP-[U-^{14}C]-glucose and photosynthetically derived kaempferol [U-^{14}C] [1] were taken. Phenol free, crude protein extracts from white light illuminated (24 hr), 8-day-old parsley cell suspension cultures were incubated (Tris–HCl buffer, 0.1 M; pH 7.5 up to 2 hr) with kaempferol and UDP-glucose in a molar ratio of 1:3. Kaempferol 3-*O*-β-D-glucoside could be isolated in a 20% yield together with only very small amounts of kaempferol 7-*O*-β-D-glucoside. Although for the experimental conditions used an activity ratio of the 3-*O*-glucosyltransferase to the 7-*O*-glucosyltransferase of *ca* 3 to 5:1 has been reported [15], only **1** could be obtained by use of the crude protein preparations. For the synthesis of **2**, **3** and **4**, the two glucosyltransferases were first separated from each other and partly enriched by DEAE–cellulose column chromatography using a linear Tris–HCl buffer gradient (0.02 M–0.45 M, pH 7.5). Appropriate fractions from the column were used for the synthesis and the 3 flavonol-glucosides obtained in a radiochemical yield ranging

from 12% (2) to 73% (3). The labelled substrates were purified by PC (system L_1) and filtration through a Sephadex LH 20 column with 50% MeOH. Radiochemical purity was determined by scanning of appropriate chromatograms (systems L_2, L_3). Characterization of the enzymatically derived flavonol-glucosides was carried out by co-chromatography in systems L_1 and L_3 and by spectrophotometry in the presence of various diagnostic reagents [16].

Application of labelled substrates to cell cultures

All substrates were aseptically fed to dark-grown [1,2] cell suspension cultures of parsley, garbanzo bean, mungbean and soybean in a 10^{-5} M concentration. $^{14}CO_2$ was collected over a period of 100 hr and the distribution of radioactivity determined in the nutrient medium, the EtOH extract and the EtOH insoluble cell residue [17]. Aliquots of the nutrient medium and the cell extract were investigated for unchanged substrate by chromatographic and scanning techniques.

It is generally accepted [4] that degradation of polyphenol glycosides is initiated by removal of sugar moieties with the liberated aglycones and carbohydrates separately being channelled into catabolic routes. Therefore, to evaluate the degree of glucose catabolism from substrates **1** and **2** the general capacity of the 4 cell suspension cultures for D-glucose [U-^{14}C] oxidation was first determined. The results [Fig. 1] show that all four cell suspension cultures rapidly oxidize D-glucose to a very high degree. The $^{14}CO_2$ values range from 53 to 68% with a sharp rise within the first 10 hr. Comparable experiments with the substrates **1** (Fig. 2) and **2** (Fig. 3) revealed that, except for the parsley cell culture, glucose from position 3 of kaempferol is almost as efficiently oxidized as free, exogenously applied glucose itself. Some 45–50% of the total radioactivity of **1** was measured as CO_2. On the other hand, glucose from position 7 of kaempferol is much more slowly catabolized. The $^{14}CO_2$ values (Fig. 3) from the cell cultures of garbanzo bean,

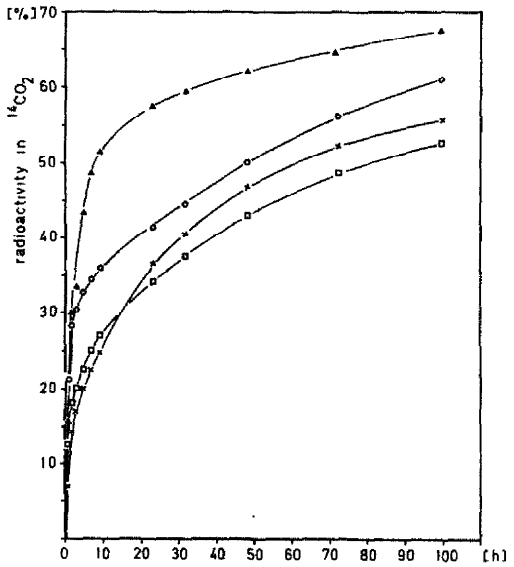

Fig. 1. Percent of total radioactivity in $^{14}CO_2$ after application of D-glucose [U-^{14}C] to cell suspension cultures of *Petroselinum hortense* (×——×), *Cicer arietinum* (△——△), *Phaseolus aureus* (○——○), and *Glycine max* (□——□).

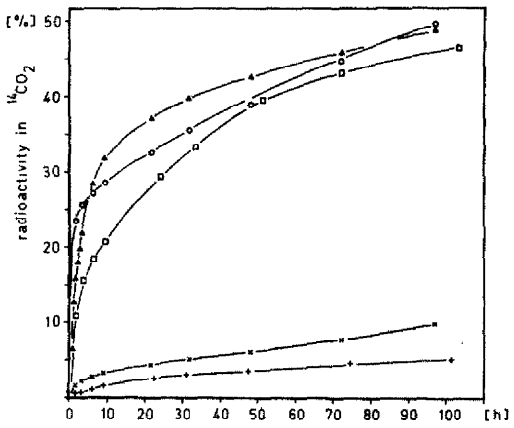

Fig. 2. Percent of total radioactivity in $^{14}CO_2$ after application of kaempferol 3-*O*-β-D-[U-^{14}C]-glucoside (**1**) to cell suspension cultures of *Petroselinum hortense*; dark grown: (×——×); illuminated: (+——+), *Cicer arietinum* (△——△), *Phaseolus aureus* (○——○) and *Glycine max.* (□——□).

mungbean and soybean are only 40–60% of those obtained with either free glucose (Fig. 1) or substrate **1** (Fig. 2). The data obtained with the parsley culture differ greatly because the $^{14}CO_2$ values with both substrates **1** and **2** are 2–5 times lower when compared with those of the other cultures and furthermore because there seems to be no preferential oxidation of glucose from either position 3 or 7.

Analyses of nutrient media and EtOH cell extracts showed that compounds **1–4** had completely been degraded in the cell cultures of the 3 leguminous plants. Radioactivity was distributed over all fractions as previously described [1] (Table 1) and no substrate could be re-isolated. On the other hand, substantial amounts of substrates were re-extracted from both the illuminated parsley cell cultures and, to a much smaller extent, from the dark grown parsley cultures. This indicates that on one hand the exogenously applied flavonol-glucosides appear to be introduced into the pools of the endogenous, light-induced [18] flavonoid material and on the other hand flavonoid catabolism in the dark grown parsley cultures proceeds at a slower rate than in the cell cultures of the other plants investigated.

The $^{14}CO_2$-data from feeding experiments with substrates **3** and **4**, labelled in the kaempferol moiety, are given in Table 1. Between 2 and 12% of the total radioactivity appeared as CO_2 with the majority of the label

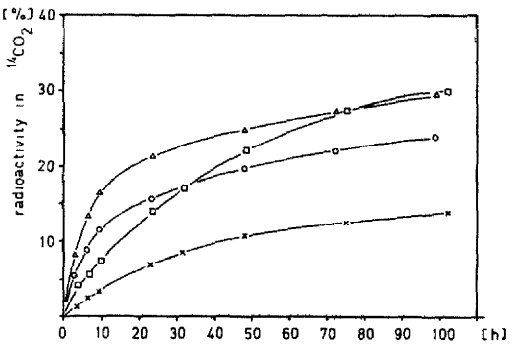

Fig. 3. Percent of total radioactivity in $^{14}CO_2$ after application of kaempferol 7-*O*-β-D-[U-^{14}C]-glucoside to cell suspension cultures. Symbols and cell cultures as in Fig. 1.

Table 1. Distribution of radioactivity in various fractions of cell suspension cultures after the application of labelled kaempferol, kaempferol-glucosides and glucose. Duration of experiments 100 hr.

Substrate												
	D-glucose [U-^{14}C]				Kaempferol 3-*O*-β-D-[U-^{14}C]-glucoside (1)				Kaempferol 7-*O*-β-D-[U-^{14}C]-glucoside (2)			
	Fraction											
	Percent total radioactivity in				Percent total radioactivity in				Percent total radioactivity in			
Cell culture	CO_2	Nutrient medium	Cell extract	Insoluble cell residue	CO_2	Nutrient medium	Cell extract	Insoluble cell residue	CO_2	Nutrient medium	Cell extract	Insoluble cell residue
Petroselinum hortense	55	6	21	18	10	6	70	14	14	36	13	36
P. hortense (illuminated)	—	—	—	—	5	19	44	32	—	—	—	—
Cicer arietinum	68	4	15	13	48	9	21	21	30	26	16	28
Phaseolus aureus	62	3	24	11	49	5	29	17	24	29	15	32
Glycine max	53	2	35	10	47	17	7	29	30	40	11	18
Substrate	Kaempferol*-[U-^{14}C]				Kaempferol 3-*O*-β-D-glucoside [U-^{14}C] 3				Kaempferol 7-*O*-β-D-[U-^{14}C]-glucoside (4)			
Petroselinum hortense	13	12	61	14	2	21	43	34	5	18	66	11
P. hortense (illuminated)	—	—	—	—	0	8	64	27	—	—	—	—
Cicer arietinum	5	30	47	18	6	26	44	25	7	22	47	23
Phaseolus aureus	13	14	31	42	11	10	48	30	8	13	41	37
Glycine max	13	27	11	49	10	38	14	38	12	31	16	40

* For comparison previous data for kaempferol [1] are also shown.

from the flavonol being incorporated into the EtOH insoluble cell residue [17,19] or elsewhere being distributed over various cell fractions [1]. There is no significant difference in $^{14}CO_2$-formation between **3** and **4** though the data obtained with **1** and **2** (Figs. 2 and 3) indicated that a much higher proportion of aglycone is liberated from the 3-glucosides (**1** and **3**) when compared to the 7-isomer. Furthermore the $^{14}CO_2$ data obtained with **3** and **4** are in the same range as those obtained with kaempferol itself [1]. Essentially as in our previous studies with flavonol aglycones [1] ^{14}C-labelled *p*-hydroxybenzoic acid derived from ring B could be identified (chromatography in solvents L_4 and L_5 followed by scanning) as a catabolite of **3** and **4**.

Table 1 also presents comparative data on the distribution of radioactivity for all substrates investigated. It is interesting that kaempferol 3-β-D-glucoside [U-^{14}C] (**1**) shows much lower $^{14}CO_2$ values in the parsley cell cultures when compared to the others. Furthermore, the illuminated parsley cultures show a 50% decreased $^{14}CO_2$-formation with the same substrate when compared to the dark-grown cultures. This is most likely due to the endogenous flavonoid content.

DISCUSSION

Our experiments again show the great capacity of plant cell suspension cultures for the degradation of aromatic structures. The high percentage of glucose catabolism from position 3 of kaempferol (Fig. 2) nearly equals the oxidation rate of exogenously applied glucose (Fig. 1) and by far exceeds the oxidation of glucose from the 7-isomer (Fig. 3). The preferential oxidation of glucose from position 3 indicates a very active and specific mechanism for the glucoside hydrolysis of flavonol 3-glucosides. This observation is especially interesting because flavonol 3-*O*-glucosides are energy-rich compounds [13]. Further experiments are needed to demonstrate whether glucohydrolases or glucosyltransferases are involved in these catabolic routes. The more rapid oxidation of kaempferol 3-*O*-glucoside in comparison to the 7-isomer (Table 1) parallels similar observations in intact plants [9] and demonstrates that such metabolic differences can also be observed at the level of tissue cultures.

Dark-grown, flavonol-free parsley cell cultures oxidize substrates **1** and **3** to a moderate degree. The endogenous flavonol-glycoside pool [18] of illuminated cells, however, seems to reduce the formation of labelled CO_2 (Table 1) significantly. The simultaneous increase in the amount of unchanged substrate indicates an intracellular dilution of the labelled substrates by endogenous material. The data further corroborate that, at least in the parsley culture, the degradation of flavonol-glucosides is an intracellular process and not brought about by the exoenzymic peroxidases of plant tissue cultures [20].

Oxidative metabolism of polyphenols in plant tissues always leads to transfer of substrates into EtOH-insoluble, polymeric material as catalyzed by peroxidases [4,20] and polyphenol-oxidases [4,19]. Such reactions are evident from the data of Table 1. The preferential incorporation of ^{14}C from **2** in comparison to **1** seems to indicate that polymerization reactions may occur prior to the removal of glucose. This is supported by the observation (Table 1) that glucose is most likely rapidly removed from substrate **3** prior to degradation of the aglycone moiety because both compounds yield equal amounts of $^{14}CO_2$. This is in agreement with the previously mentioned hypothesis [4] of preliminary glucohydrolytic reactions in glycoside catabolism. In general, the involvement of peroxidases in the catabolism of flavonol-glycosides must also be noted.

EXPERIMENTAL

Cell cultures. Conditions of growth and cultivation of cell cultures of soybean, mungbean, garbanzo bean and parsley have previously been described [1,2,15,17]. The application of substrates (10^{-5} M) to cell cultures under aseptic conditions, collection of $^{14}CO_2$ and fractionation reactions of EtOH cell extracts and nutrient media followed standard procedures [1,3,20].

Source and preparation of enzyme. Seven-day old parsley cell suspension cultures were illuminated with white light for 24 hr

and then used for the preparation of flavonol: UDP-glucose-glucosyltransferases. All procedures with enzymes were carried out at 4°. Cells were homogenized with half their wt of Tris/HCl-buffer (0.5 M; pH 7.5; 11 mM in mercaptoethanol) in a homogenizer for 20 min with 1 min intervals every 30 sec. The homogenate was centrifuged for 20 min (11 000 *g*) and the supernatant stirred with Dowex 1 × 2 resin (1/16 of cell fr wt) for 30 min. Dowex was removed by filtration through glasswool and protein precipitated by 80% $(NH_4)_2SO_4$-saturation. The recovered protein (11 000 *g*, 20 min) was dissolved in a small vol of Tris–HCl-buffer (0.1 M; pH 7.5; 11 mM mercaptoethanol) and filtered through a Sephadex G-25-Column, equilibrated with Tris–HCl-buffer (0.02 M; pH 7.5; 11 mM mercaptoethanol). Protein was further purified by DEAE-cellulose column chromatography (Cellex D). The adsorbed protein was eluted by using a linear gradient of Tris–HCl-buffer (0.02 M to 0.45 M; pH 7.5; 11 mM mercaptoethanol) and 9 ml fractions were collected. Protein was recorded at 280 nm and the fractions tested for glucosyl-tranferases under standard conditions. Fractions containing glucosyl-transferase activity were pooled, concentrated to 10% of their original vol in an ultrafiltration cell under N_2 (2 atm) using a PM 10 filter and then used for the synthesis of substrates.

Standard enzyme assay. Protein preparations were tested for flavonol 3-*O*-glucosyltransferase and 7-*O*-glucosyltransferase activity by incubating 30 nmol flavonol (dissolved in 10 μl ethyleneglycol monomethylether), 90 nmol UDP-glucose (dissolved in 20 μl H_2O); 100 μl Tris–HCl buffer (0.2 M; pH 7.5) and 100 μl enzyme soln at 30° for 30 min. Enzyme reactions were terminated by heating to 100° and flavonol-glucosides recovered by PC with solvent L_1. Routine determinations of transferase activity were done with quercetin, all other reactions with kaempferol.

Synthesis of substrates. For preparative syntheses of ^{14}C-labelled substrates the general conditions and the ratio of concns of the individual constituents of the standar enzyme assay were applied with the quantity increased by a factor of up to 100. Duration of incubation was increased to 2 hr and flavonol added in portions every 40 min. Substrates **1** and **2** were prepared by using UDP-glucose[U-^{14}C] (Radiochemical Centre Amersham, sp act diluted to 1.5 mCi/mmol) and substrates **3** and **4** obtained with kaempferol [U-^{14}C] (photosynthetically derived according to [1], sp act 0.17 mCi/mmol). The reaction products were recovered by lyophilization and purified by PC with solvent L_1 and filtration through Sephadex LH 20 with 50% MeOH. Radiochemical purity was determined by scanning chromatograms developed in solvent L_2 and L_3.

Chromatographic solvents. PC and cellulose TLC was carried out with the solvent systems L_1: 15% HOAc, L_2: EtOAc–MeEtCO–HCO_2H–H_2O, 5:3:1:1, L_3: *n*-BuOH–HOAc–H_2O, 4:1:1, L_4: $CHCl_3$–HOAc, 9:1, L_5: C_6H_6–dioxan–HOAc, 90:25:4.

Radioactivity. Measurements of radioactive samples were carried out according to refs. [1,2,17].

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