Biochimica et Biophysica Acta, 309 (1973) 289–295

BBA 66917

UDP-GLUCOSE: FLAVONOL 3-*O*-GLUCOSYLTRANSFERASE FROM CELL SUSPENSION CULTURES OF PARSLEY

ARNE SUTTER AND HANS GRISEBACH

Lehrstuhl für Biochemie der Pflanzen am Biologischen Institut II der Universität, D-78 Freiburg, Schänzlestrasse 9/11 (W. Germany)

(Received December 4th, 1972)

SUMMARY

1. An enzyme catalyzing the transfer of D-glucose from UDP-D-glucose to the 3-hydroxyl group of flavonols is present in extracts from cell suspension cultures of parsley.

2. The flavonol 3-*O*-glucosyltransferase can be separated from the previously described 7-*O*-glucosyltransferase by chromatography on DEAE-cellulose or by disc electrophoresis.

3. The enzyme has a strict positional specificity but catalyzes the 3-*O*-glucosylation of a number of flavonols with the exception of dihydroquercetin. The enzyme also catalyzes the glucosylation of quercetin 7-*O*-glucoside to form quercetin 3,7-di-*O*-glucoside. This diglucoside is not formed from quercetin 3-*O*-glucoside with the 7-*O*-glucosyltransferase.

4. Extractable enzyme activity is increased by prior illumination of the cell cultures. Maximum activity is reached about 24 h after onset of illumination. The enzyme therefore belongs to the previously defined group II of enzymes involved in the flavone glycoside pathway in parsley.

INTRODUCTION

In a previous publication we reported on the isolation and purification of an enzyme from cell suspension cultures of parsley catalyzing the transfer of D-glucose from UDP-D-glucose to the 7-hydroxyl group of a number of flavones, flavanones and flavonols[1]. In the course of this work it was discovered that on incubation of kaempferol with the crude enzyme extract the 3-*O*-glucoside was formed in addition to kaempferol 7-*O*-glucoside.

In the present paper we report the separation of the 3-*O*-glucosyltransferase from the 7-*O*-glucosyltransferase and some properties of the former enzyme.

MATERIALS AND METHODS

Substrates and reference compounds

UDP-D-[$^{14}C_6$]glucose (2 Ci/mole) was purchased from Radiochemical Centre Amersham.

Kaempferol, kaempferid, fisetin, isorhamnetin and dihydroquercetin were obtained from Roth (Karlsruhe), quercetin from Merck (Darmstadt) and 4',7-dihydroxyflavonol from our laboratory collection. Quercetin 3-*O*-glucoside was a gift from Dr Dumkow (Freiburg). All flavonoids were purified by chromatography on Sephadex LH-20 with methanol as solvent.

Cultivation of cell cultures of *Petroselinum hortense* has been described previously[2]. Enzyme was extracted from cells which had been illuminated for 24 h with white light prior to being harvested 10 days after the cultures were started.

Protein assay

Protein was determined by the biuret method[3].

Chromatographic method

For descending paper chromatography on Whatman 3 MM the following solvent systems were used: (1) water; (2) 15% acetic acid; (3) butanol–acetic acid–water (4:1:5, by vol.); (4) ethanol–1 M ammonium acetate, pH 7.5 (5:2, by vol.). The R_F values of the quercetin glucosides are recorded in Table I.

TABLE I

R_F VALUES OF QUERCETIN GLUCOSIDES IN DIFFERENT SOLVENT SYSTEMS

Solvent system	*Quercetin 3-O-glucoside*	*Quercetin 7-O-glucoside*	*Quercetin 3,7-di-O-glucoside*
1	0.1	0.02	0.5
2	0.4	0.1	0.6
3	0.7	0.55	0.25
4	0.65	0.35	—

Enzyme assay

The enzyme assay for 7-*O*-glucosyltransferase was carried out with [2-^{14}C]-apigenin and UDPglucose as described previously[1] or by the procedure described below.

Standard incubation for flavonol 3-*O*-glucosyltransferase and flavonol 7-*O*-glucosyltransferase. The incubation mixture consisted of 30 nmoles quercetin or quercetin 7-*O*-glucoside dissolved in 10 μl of ethyleneglycol monomethylether, 90 nmoles UDP-D-[$^{14}C_6$]glucose (2 Ci/mole) and 0.2 M Tris–HCl buffer (pH 7.5) containing 11 mM mercaptoethanol in a total volume of 230 μl. The reaction was started by addition of protein. The mixture was incubated at 30 °C for 30 min. At the end of this period 10 μl of a solution of quercetin 3-*O*-glucoside (1 mg in 1 ml of methanol) were added to the incubation mixture and the total solution was applied to Whatman 3 MM paper (prewashed with methanol, 10% acetic acid and 0.01 M EDTA). The paper was developed with 15% acetic acid. The R_F values of the quercetin glucosides are recorded in Table I. Quercetin does not migrate in this solvent. Quercetin 7-*O*-

glucoside was detected under ultraviolet light (254 nm) as an orange-yellow fluorescent spot and the 3- and 3,7-di-*O*-glucosides as dark ultraviolet-absorbing spots. The corresponding zones were cut out and counted in a toluene scintillation fluid (5 g 2,5-diphenyloxazole/l toluol) in a liquid scintillation spectrometer (Beckmann LS 233).

Separation of 7-O-glucosyltransferase from 3-O-glucosyltransferase

Extraction and $(NH_4)_2SO_4$ precipitation. All steps were carried out at 4 °C. Enzyme extraction and treatment of the extract with Dowex 1-X2 were carried out as described previously[1] with 650 g of wet cells. Protamine sulfate precipitation was omitted. After Dowex treatment protein was precipitated by addition of solid $(NH_4)_2SO_4$. Protein precipitated between 0–80% saturation was collected and desalted on Sephadex G-25[1].

Separation by DEAE-cellulose chromatography. The filtrate (85 ml, 12 mg protein/ml) from the Sephadex G-25 column in 20 mM Tris–HCl, pH 7.5, containing 11 mM mercaptoethanol was absorbed on a column of DEAE-cellulose (3 cm × 20 cm) equilibrated with the same buffer and enzyme was eluted with a linear gradient of this buffer at a flow rate of 40 ml/h (Fig. 1). Fractions of 9 ml were collected and the buffer concentration was determined by conductivity (conductometer LF 39, Wissenschaftlich Technische Werkstätten D 812 Weilheim). 100 µl of each fraction were tested for flavonol 3-*O*-glucosyltransferase and for flavonol 7-*O*-glucosyltransferase activities with quercetin as substrate. 20 µl of each fraction were tested for flavone 7-*O*-glucosyltransferase activity with [^{14}C]apigenin as substrate in the enzyme assay.

Separation by analytical disc electrophoresis. Fractions 40–45 and 55–58 from the DEAE column were concentrated to one-tenth of their volume with a "Diaflo" concentrator (Amicon, model 50, ultrafiltration cell). A mixture of the two concentrated fractions (0.25 ml) was subjected to analytical disc electrophoresis on polyacrylamide gel (7.5%)[4] using 2.5 mM Tris–glycine buffer of pH 8.3 as electrode buffer. The gel was cut into 2-mm thick discs and protein was eluted over night with 0.5 ml of 0.4 M Tris–HCl, pH 7.5, containing 11 mM mercaptoethanol. Fractions were tested for enzymatic activity as described above.

Determination of enzymatic activity in experiments on light stimulation

For each determination 5 g of cells were homogenized for 90 s in the Ultra Turrax (Type 18-10, Janke and Kunkel KG, D 7813 Staufen, Germany) with 5 ml of 0.4 M Tris–HCl (pH 7.5) containing 11 mM mercaptoethanol. The homogenate was centrifuged for 10 min at 15 000 × *g*, and to the supernatant solid $(NH_4)_2SO_4$ was added to 80% saturation. After collection of protein by centrifugation the precipitate was dissolved in 1 ml of the Tris buffer and the solution desalted by sieve centrifugation[5] through 5 cm^3 Sephadex G-25 superfine. Enzymatic activity was then determined in the enzyme assay.

RESULTS

Formation of quercetin glucosides in cell-free extracts of parsley cultures

When the crude extract of the parsley cell cultures which had been illuminated

with white light for 5–24 h (ref. 2) was incubated with UDP-D-[$^{14}C_6$]glucose and quercetin, three radioactive products were detected on paper chromatograms with 15% acetic acid as solvent system (Fig. 3). The products were identified as quercetin 7-*O*-glucoside, quercetin 3-*O*-glucoside and quercetin 3,7-di-*O*-glucoside by cochromatography with authentic reference samples in solvent Systems 1–4. Quercetin 3-*O*-glucoside was further purified on Sephadex LH-20 by elution with methanol. The ultraviolet spectra of this compound in methanol + $AlCl_3$ and methanol + sodium methylate[6] were identical with those of authentic quercetin 3-*O*-β-glucoside. In the crude cell extracts enzymatic activity for 3-*O*-glucosylation was about 3–5 times higher than that for 7-*O*-glucosylation, but the ratio of the two enzymatic activities changed with illumination time (see below).

Separation of 7-O-glucosyltransferase from 3-O-glucosyltransferase

The crude extract from parsley cells which had been illuminated for 24 h was treated with Dowex 1-X2 and the protein was then precipitated with $(NH_4)_2SO_4$ (to 80% saturation). After removal of salts with Sephadex G-25, chromatography on DEAE-cellulose with a linear gradient of Tris–HCl containing mercaptoethanol gave a clear separation of the enzymatic activities for 7-*O*- and 3-*O*-glucosylation (Fig. 1). The highest activities for flavonol 7-*O*- and flavone 7-*O*-glucosylation were present in the same fractions. The presence of a second small peak for 7-*O*-glucosylation was confirmed in several experiments. Whether this activity is due to a second enzyme or to proteolysis or aggregation of the original enzyme is unknown.

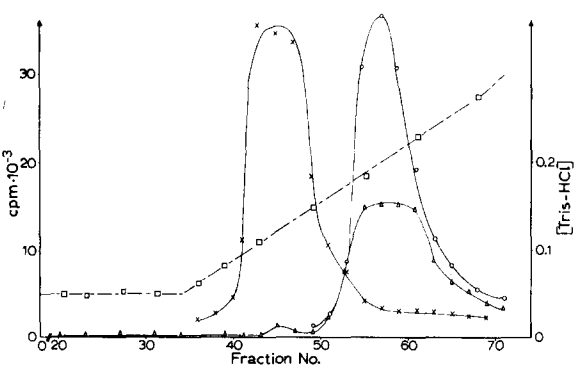

Fig. 1. Separation of 7-*O*- and 3-*O*-glucosyltransferase on DEAE-cellulose. ×—×, 3-*O*-glucosylation of quercetin; ○—○, 7-*O*-glucosylation of quercetin; △—△, 7-*O*-glucosylation of apigenin; □—□, Tris–HCl gradient.

The maximum for enzyme activity of the flavone 7-*O*-glucosyltransferase appears flat because substrate was present only in limiting amounts in the enzyme test of these fractions. The two glucosyltransferases could also be separated by analytical disc electrophoresis on polyacrylamide with Tris–glycine buffer of pH 8.3 (Fig. 2). No separation was obtained on a Sephadex G-100 column.

Properties of the 3-O-glucosyltransferase

In contrast to the 7-*O*-glucosyltransferase, the 3-*O*-glucosyltransferase was in-

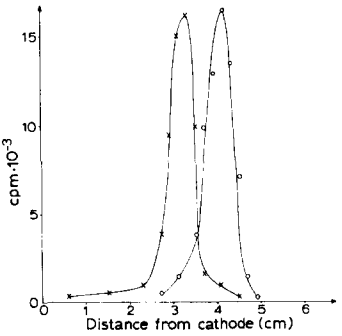

Fig. 2. Separation of 7-*O*- and 3-*O*-glucosyltransferase by analytical disc electrophoresis. ×—×, 3-*O*-glucosylation of quercetin; ○—○, 7-*O*-glucosylation of apigenin.

hibited in the crude extract. After DEAE-cellulose chromatography 3-*O*-glucosylation was linear with time up to 60 min and with protein up to 200 μg/ml in the standard incubation. The enzyme had a broad pH optimum around pH 8. Glycine–HCl buffer stimulated enzyme activity about 2-fold at pH 9 in comparison with Tris–HCl buffer. From the effect of UDPglucose concentration on reaction rate the apparent K_m value for this substrate was determined according to Lineweaver and Burk to be about 0.5 mM. The apparent K_m value for quercetin was <1 μM. The lack of sensitivity of the enzyme assay did not allow a more accurate determination.

Acceptor specificity

Table II lists the relative acceptor specificity for 3-*O*-glucosylation of a number of flavonols. Quercetin proved to be the best acceptor. Other flavonols could also function as substrates. In contrast, no reaction took place with dihydroquercetin, the probable biosynthetic precursor of quercetin[7].

TABLE II

ACCEPTOR SPECIFICITY OF THE 3-*O*-GLUCOSYLTRANSFERASE FROM CELL CULTURES OF PARSLEY

Incubations were carried out in the enzyme assay with the enzyme from the DEAE column.

Substrate	*Relative V** (*related to V quercetin = 100*)	*V***
Quercetin	100	40
Kaempferol	70	
Kaempferid (kaempferol-4′-*O*-methylether)	57	
Fisetin (5-deoxyquercetin)	57	
Isorhamnetin (3′-*O*-methylquercetin)	28	
4,7-Dihydroxyflavonol	27	
Dihydroquercetin	0	

* Mean value from two determinations.
** nmoles quercetin 3-*O*-glucoside/mg protein per min.

Formation of 3,7-O-diglucoside

Incubation of quercetin 3-*O*-glucoside with UDPglucose and the 7-*O*-glucosyltransferase resulted in no measurable reaction. In contrast when quercetin 7-*O*-

glucoside was incubated with UDPglucose and the 3-*O*-glucosyltransferase a quantitative conversion to the 3,7-di-*O*-glucoside could be observed.

Stimulation of enzyme activity by light

It had been shown previously that extractable activity of the 7-*O*-glucosyltransferase is strongly stimulated by illumination of the cell cultures with light and that maximum specific activity of the enzyme is reached about 24 h after onset of illumination[2]. As in the case of 7-*O*-glucosyltransferase a rise in specific activity of the 3-*O*-glucosyltransferase with a lag phase of 3–4 h after onset of illumination and a broad maximum around 22–24 h was found. Activity of this enzyme in dark-grown cultures was about 10% of the maximal activity. In the course of illumination the 3-*O*-glucosyltransferase is stimulated more strongly than the 7-*O*-glucosyltransferase so that activity increased with time in favor of 3-*O*-glucosylation (Fig. 3).

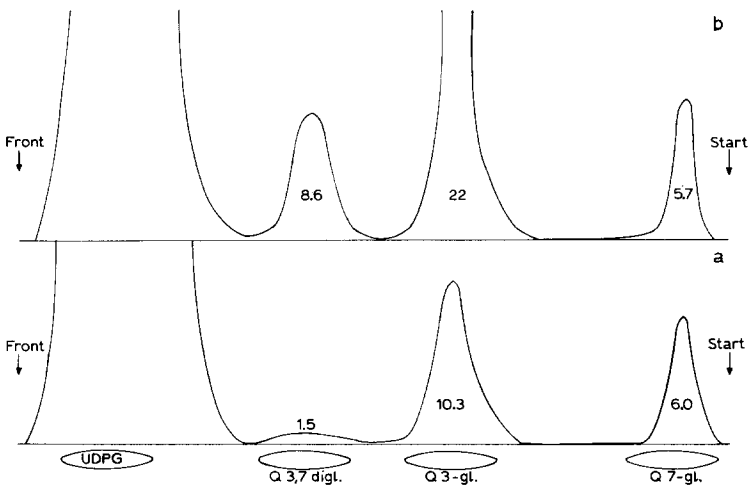

Fig. 3. Radioscan of paper chromatogram of quercetin *O*-glucosides formed by incubation of quercetin and UDP-D-[U-^{14}C]glucose with crude extracts from parsley cell culture illuminated for 5 h (a) and 12 h (b) prior to enzyme extraction. Numbers under the peaks give cpm·10^{-3}. Q 3,7 digl., quercetin 3,7-di-*O*-glucoside; Q 3-gl., quercetin 3-*O*-glucoside; Q 7-gl., quercetin 7-*O*-glucoside.

DISCUSSION

Results presented in this paper prove that the UDPglucose:apigenin 7-*O*-glucosyltransferase[1] and the UDPglucose:flavonol 3-*O*-glucosyltransferase are two distinct enzymes. The two transferases are separable according to their different charge on DEAE-cellulose or by disc electrophoresis but they were not separated on Sephadex G-100, which indicates that they have a similar molecular size. The molecular weight of the 7-glucosyltransferase had been determined on a Sephadex G-100 column to be about 55 000[1]. Both transferases have a strict positional specificity but a relatively broad substrate specificity. A notable exception is the lack of 3-*O*-glucosylation of dihydroquercetin in contrast to the efficient glucosylation of quercetin which was the best acceptor found. This observation is in agreement with the well-

supported assumption that glycosylation is the last step in flavonoid glycoside biosynthesis[8]. The second glucosylation to quercetin 3,7-di-*O*-glucoside can only occur with quercetin 7-*O*-glucoside and the 3-*O*-glucosyltransferase, but not with quercetin 3-*O*-glucoside and the 7-*O*-glucosyltransferase. In agreement with our hypothesis on light stimulation of flavonoid biosynthesis on parsley cell cultures[2], both transferases reach maximum activity about 24 h after onset of illumination and therefore belong to the group of enzymes involved exclusively in the formation of flavonoid glycosides.

We had already referred to work on 3-*O*-glucosyltransferase activity from other plant sources in our previous publication[1]. Recently Larson[9] and Larson and Lonergan[10] described a glucosyltransferase activity from maize pollen catalyzing the formation of either quercetin or kaempferol 3-*O*-glucoside.

ACKNOWLEDGEMENT

This work was supported by Deutsche Forschungsgemeinschaft (SFB 46). We thank Miss A. Sträter for skilful technical assistance.

REFERENCES

1 Sutter, A., Ortmann, R. and Grisebach, H. (1972) *Biochim. Biophys. Acta* 258, 71–87
2 Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellmann, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* 244, 7–15
3 *Photometrische Methoden der Medizin*, AV 550 (1966) p. 1, Eppendorf Gerätebau, Hamburg
4 Maurer, H. R. (1968) *Disk-Elektrophorese*, Walter de Gruyter, Berlin
5 Kohl, J. G. (1969) *Flora* 160A, 253–257
6 Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*, Springer-Verlag, Berlin
7 Patschke, L. and Grisebach, H. (1968) *Phytochemistry* 7, 235–237
8 Grisebach, H. (1965) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.), p. 293, Academic Press, London
9 Larson, R. L. (1971) *Phytochemistry* 10, 3073–3076
10 Larson, R. L. and Lonergan, C. M. (1972) *Planta* 103, 361–364