UDP-Glucose: Glucosyltransferase Activity Involved in the Biosynthesis of Flavonol Triglucosides in *Pisum sativum* L. Seedlings

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Z. Naturforsch. **34 c**, 738 – 741 (1979); received June 25, 1979

Pisum sativum, Peas, Flavonols, Triglucosides, Glucosyltransferase

From young, light-grown seedlings of *Pisum sativum* L. an enzyme activity catalyzing the glucosylation of kaempferol and quercetin in the 3-position to form the 3-0-triglucoside derivative has been demonstrated. The reaction proceeds from the aglycone via the mono- and diglucoside intermediates. The triglucoside can be produced from any of the less substituted derivatives with uridine diphosphate-*D*-glucose (UDPG) as the glucosyl donor. Young leaf tissues had much higher levels of glucosyltransferase activity than the petioles and internodes. This is the first report of the synthesis of flavonol-3-0-triglucosides *in vitro*.

Introduction

The major flavonoids of *Pisum sativum* L. have been identified as kaempferol-3-triglucoside (KTG), its *p*-coumaroyl ester (aKTG), quercetin-3-triglucoside (QTG) and its *p*-coumaroyl ester (aQTG) [1, 2]. The physiological role of these compounds has been discussed [3] and the control of synthesis by light and phytochrome has been the subject of numerous studies. It has been clearly established that dark-grown tissues produce only kaempferol derivatives whereas light-grown tissues produce both kaempferol and quercetin compounds. In addition, the different tissues of the plant respond differently in that they produce different amounts of each pigment [4 – 8].

The transfer of glucose to a flavonoid acceptor molecule has been the most widely studied reaction, although the transfer of both apiose [9] and rhamnose [10] has also been reported. To date, only mono- and diglycoside synthesis has been reported *in vitro* and no information is yet available about triglycoside derivatives.

We have been investigating the biosynthesis of flavonol derivatives in *Pisum sativum* L. seedlings and in a recent publication [11] reported on the presence of a hydroxycinnamoyl: CoA transferase involved in the biosynthesis of the *p*-coumaroyl derivatives of both the kaempferol and quercetin triglucosides. In the present communication, we report on the preparation of cell-free extracts from *Pisum sativum* L. seedlings which catalyze the formation of kaempferol-3-mono-, di- and triglucoside.

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0341-0382/79/0900-0738 $ 01.00/0

Materials and Methods

Materials: Pea seeds (*Pisum sativum* L. var. Alaska) were purchased from Ferry Morse Seed Co., Tampa, Florida. UDP-D-[U-^{14}C]-glucose (292 mCi/mmol) was purchased from International Chemical and Nuclear, Irvine, CA. Unlabelled UDP-D-glucose, QAE, KAE and microcrystalline cellulose (Sigmacell Type 20) were purchased from Sigma, St. Louis, Missouri. Polyamide was purchased from Machery, Nagel & Co., Düren, W. Germany.

Chromatographic solvents:

I. *tert*-butanol: gl. acetic acid: water (3:1:1); II. 10% acetic acid; III. 1% HCl; IV. chloroform: gl. acetic acid (3:2 saturated with water); V. phenol: water (4:1).

Substrate preparation:

KMG was isolated from hot 80% EtOH extracts of mature flower petals of *Impatiens balsamina* [15]. The extract was filtered through glass filter discs, concentrated, and applied to a polyamide column (CC-6, 0.16 mm) which was washed successively with H_2O, 20% EtOH, and 40% EtOH. This last fraction was concentrated and streaked on Whatman No. 1 paper and chromatographed descending in I. The KMG band was eluted with 80% EtOH, dried and

redissolved in 50% EtOH. This procedure resulted in chromatographically pure KMG. The concentration of the solution was determined spectrophotometrically using an extinction coefficient of 20×10^6 $cm^2 \cdot mol^{-1}$ at 355 nm. QMG was purified from ethanolic extracts of *Nicotiana tabacum* and KDG from *Sophora japonica*. KTG was prepared as described previously [11].

Seed germination:

Seeds were soaked in running tap water for 12 hours then planted in a mixture of perlite, vermiculite and soil (equal parts). The seeds were kept under continuous illumination at 25 °C for 6 days.

Enzyme preparation:

Fifteen g of leaf tissue were frozen in liquid N_2 and ground in a mortar. The powder was extracted at 4 ° in 75 ml borate buffer (0.1 M, pH 7.6) containing 20 mM 2-mercaptoethanol and 15 g Polyclar-AT which had been equilibrated in the borate buffer. The homogenate was stirred for 45 min, filtered through 4 layers of cheesecloth and centrifuged at $40{,}000 \times g$ for 30 min. The supernatant was fractionated with solid ammonium sulfate and the fraction between 30 and 70% was resuspended in a minimum volume of potassium phosphate buffer (0.1 M, pH 7.3). This preparation was used as the enzyme source.

Assay of glucosyl transferase activity:

Reaction mixtures contained 50 nmol flavonol acceptor, (KAE and QAE were dissolved in 5 μl ethyleneglycolmonomethylether and KMG in 5 μl H_2O); 100 nmol UDP-D-[U-^{14}C]-glucose in 10 μl H_2O (500 cpm/nmol); 2.5 μmol potassium phosphate (0.1 M, pH 7.3) and 50 μl enzyme solution (1.5 mg protein) in a final volume of 70 μl. Reactions were incubated 30 min at 30 ° before being stopped by the addition of 2 volumes absolute ethanol. The precipitated protein was removed by centrifugation and the supernatant was streaked on a Sigmacell TLC plate. Ten nmol KMG and KTG were added as carrier and the chromatograms were developed in III. A strip of Whatman paper was attached to the top of each plate to allow the KTG to reach an R_f of 0.85 before the plates were removed and air dried. The dark, UV absorbing bands corresponding to the flavonol derivates were marked and each band was scraped into a scintillation vial containing 5 ml Triton X-100 scintillation cocktail (5.5 g PPO, 0.1 g POPOP, 333 ml Triton X-100 and 667 ml toluene).

Protein measurement:

Protein determinations were done according to the method described by Bradford [16].

Results and Discussion

Characteristics of the reaction and products

Crude enzyme preparations from pea seedlings have been shown to catalyze the transfer of the glucose moiety from a UDP-glucose donor to kaempferol aglycone (KAE) forming kaempferol-3-triglucoside (KTG) as a reaction product. The reaction sequence proceeds via kaempferol-3-monoglucoside (KMG) and kaempferol-3-diglucoside (KDG) derivatives and each of these intermediates can serve as the acceptor substrate in the formation of the 3-triglucoside. Glucosyltransferase activity was not observed in boiled enzyme preparations or in the absence of a nucleotide sugar donor. After incubation of a crude enzyme preparation from 6 day old seedlings with UDP-[U-^{14}C]-glucose and KAE, radioactivity could be detected in KMG, KDG, and KTG. The radioactive products of the reaction were identified by co-chromatography with authetic compounds using the solvent systems described in the experimental section, as well as by spectophotometric analysis [12]. Under conditions described, the reaction was linear for more than 30 minutes.

Table I shows the distribution of radioactivity in each of the reaction products when KAE was utilized as the sugar acceptor. In these preparations, the largest amount of radioactivity was found associated with the KMG band and the levels of counts in the KDG and KTG were only about 10 per cent that of the KMG. When KMG was used as the acceptor, however, a different distribution of radioactivity was noted. In this reaction, the level of radioactivity in the KDG band was nearly identical to that when KAE was the substrate but the radioactivity of the KTG band was nearly 10 times higher than in the KAE reactions. In addition, the KMG reactions also reveabled the presence of KAE on the chromatograms and no radioactivity was associated with this band. The presence of low levels of a β-glucosidase activity in this tissue has been established during other studies in this laboratory (R. L. Mansell, unpublished). Thus, the radioactivity found associated with the

KMG band after incubation probably arises via glucosylation of the aglycone produced by the glucosidase. Purification studies in progress have shown that the glucosidase activity is removed after chromatography of the enzyme on Sephadex G-100 and DE-52 cellulose.

The data in Table I show that for the ammonium sulfate preparation, the substitution level of kaempferol acceptor affects the degree of glucosylation of the products. When the aglycone is the acceptor, the greatest amount of radioactivity is found in the 3-monoglucoside derivative and when the 3-monoglucoside is used, the greatest incorporation is into the 3-triglucoside. The degree of incorporation into the 3-diglucoside remains constant in both cases. Thus, it would appear that the KDG is maintained at a low, constant level in these preparations and the ultimate amount of KTG formed is influenced by the concentration of the KMG which is produced or available. These results are consistent with the fact that an analysis of intact plant tissue has shown only the tri-glucoside derivative to be readily detectable [1, 2, 13].

End product identification

To prove that KTG was the end-product of the raction sequence and that the sugar was transferred without alteration, a large batch preparation was made and the entire reaction streaked on Whatman No. 1 paper. The chromatogram was developed in I and the band corresponding to authentic KTG was eluted with 80% EtOH. This material was spotted on TLC plates and run in II–IV. In each case the total radioactivity applied was recovered in the spot corresponding to KTG. An aliquot (1500 cpm) was acid hydrolyzed then shaken with ethyl acetate. All the radioactivity remained in the water fraction and the KAE was found in the organic phase. The water fraction was then chromatographed in I and V. The radioactivity applied was found in the spot corresponding to glucose. As a final proof, an aliquot of the KTG (1500 cpm) was acetylated [14] and purified by chromatography. The UV and chromatographic properties were identical to those of authentic acetylated KTG and, after chromatography, 1350 cpm were detected in this compound.

Table I. Incorporation of [U-^{14}C] glucose into kaempferol glucoside derivatives using the aglycone and 3-monoglucoside as acceptor substrates.

Substrate	Product [cpm/mg protein]		
	KMG	KDG	KTG
KAE	2891	250	320
KMG	150	320	2900

The incorporation of glucose was tested by incubating the KMG or KAE (50 nmol) with 50 µl of enzyme at 30 ° in potassium phosphate buffer, (0.1M, pH 7.3) in a total volume of 70 µl for 30 minutes.

The reaction was terminated by the addition of 2 vol of abs ethanol.

Table II. Distribution of glucosyltransferase activity in *Pisum sativum* seedlings.

Substrate	Product			
	[cpm/mg protein]		[cpm/g FW]	
	leaves	stems	leaves	stems
	KMG			
KAE	766	365	4520	383
KMG	109	49	644	51
	KDG			
KAE	109	155	648	162
KMG	307	388	1812	407
	KTG			
KAE	214	66	1264	70
KMG	430	356	2540	374

The distribution of glucosyl transferase activity in the seedling is shown in Table II. A comparison was made between leaf and stem (internodes and petioles) tissues of 6 day old light-grown plants. With both the KAE and KMG substrates, the total transferase activity of the leaf tissue was greater than that of the stems by a factor of 5–10. However, specific activities of the two tissues were nearly the same. Thus, these results correlated with those of Furuya and Galston [1] and Bottomley *et al.*, [6] that the youngest leaves and stipules contain the highest concentrations of KTG whereas the stems contain only low levels. It would then appear that total flavonol glucoside accumulation is a function of the total amount of enzyme activity present in the individual tissues.

In an experiment to examine the specificity of the whole seedling glucosyltransferase, a comparison was made using quercetin aglycone (QAE) and KAE along with their 3-monoglucoside derivatives, QMG and KMG. Table III shows the distribution of radioactivity in the mono- and triglucoside derivatives. It

Table III. Comparison of glucosyltransferase activity with Quercetin and Kaempferol acceptors.

Substrate	Product [cpm/mg protein]			
	QMG	KMG	QTG	KTG
QAE	1492		472	
QMG	278		2419	
KAE		1219		653
KMG		108		1832

can be seen that the crude enzyme preparation can glucosylate both flavonols equally. In addition, it is again observed that the highest levels of radioactivity are found in the monoglucoside when the aglycone is the acceptor and in the triglucoside when the monoglucoside is the substrate.

During these studies, KDG was not readily available and only limited assays could be done. In a single experiment using KDG as the acceptor, only the KTG was found to be radioactive and no degradation of the KDG was observed. Thus it would appear from the reactions described that KAE can be glucosylated to KTG and that the reaction sequence involves the production of KMG and KDG as intermediates. Evidence also indicates that each of the intermediates can also serve as acceptor.

In this report we have demonstrated the presence of glucosyltransferase activity from *Pisum sativum* involved in the biosynthesis of flavonol 3-triglucosides. The enzyme activity can use each of the flavonol intermediates as substrate and is found in highest concentrations in the young leaf tissue. From the present work it is not possible to determine how many enzymes are involved in this sequence, but studies currently underway indicate that there are at least three distinct activities associated with the transfer reactions. Characterization of each of these is in progress and hopefully the information obtained will be useful in understanding flavonol metabolism in this plant, especially since only the triglucoside is found to accumulate *in vivo*.

[1] M. Furuya, A. W. Galston, and B. B. Stowe, Nature **193,** 456 (1962).
[2] F. E. Mumford, D. H. Smith, and J. R. Castle, Plant Physiol. **36,** 752 (1961).
[3] J. W. McClure, The Flavonoids (J. B. Harborne, T. J. Mabry and H. Mabry, eds.), **Vol. II,** p. 970, Academic Press, New York 1975.
[4] M. Furuya and R. G. Thomas, Plant Physiol. **39,** 634 (1964).
[5] D. B. Harper, D. J. Austin, and H. Smith, Phytochemistry **9,** 497 (1970).
[6] W. Bottomley, H. Smith, and A. W. Galson, Phytochemistry **5,** 117 (1966).
[7] D. W. Russel and A. W. Galston, Phytochemistry **6,** 791 (1967).
[8] H. Smith and D. B. Harper, Phytochemistry **9,** 477 (1970).
[9] R. Ortmann, A. Sutter, and H. Grisebach, Biochem. Biophys. Acta **289,** 293 (1972).
[10] G. A. Barber, Biochem. **1,** 463 (1962).
[11] M. Saylor and R. L. Mansell, Z. Naturforsch. **32 c,** 765 (1977).
[12] J. B. Harborne, Comparative Biochemistry of the Flavonoids. Academic Press, New York 1967.
[13] J. B. Harborne, Experientia **19,** 7 (1963).
[14] K. Gorter, Liebigs Amm. Chem. **379,** 110 (1911).
[15] C. W. Hagen, Jr., Amer. J. Bot. **53,** 46 (1966).
[16] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).