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In vitro Glucosylation of Gibberellins

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Abstract. In maturing fruits of *Phaseolus coccineus* a soluble glucosyltransferase activity occurs which converts gibberellins into their 0-glucosides. The enzyme glucosylates GA_3 and structurally closely related gibberellins (GA_7 and GA_{30}) to their 3-0-glucosides by transfer of glucose preferentially from UDP-glucose.

From cell suspension cultures of *Lycopersicon peruvianum* cytosolic glucosyltransferases were isolated which in the presence of UDP-glucose converted GA_7 and GA_9 to the corresponding glucosyl esters. In both cases numerous other gibberellins failed to serve as substrates. Thus, the enzymes are UDP-glucose: gibberellin glucosyltransferases of considerable substrate specificity.

The physiologically active gibberellin (GA) level in higher plants is regulated by metabolic pathways leading to hormone structures with altered biological and physical properties. Conjugation of GAs with glucose is considered to be one main process whereby the glucosyl moiety is linked to either a hydroxyl group or the carboxyl functions (for reviews *cf.*: SEMBDNER *et al.* 1980, SCHNEIDER 1983). Especially mature fruits of several *Leguminosae* are distinguished by their considerably high content of GA glucosides. GA_3, when exogenously applied to maturing fruits of runner beans (*Phaseolus coccineus* L.), is converted into its 3-0-glucoside at a high rate (SEMBDNER *et al.* 1968). A similar conversion is known to occur in both maturing seeds and seedlings of *Pharbitis nil* CHOISY (BARENDSE and DE KLERK 1975) or in young plants of *Phaseolus vulgaris* L. (ASAKAWA *et al.* 1974). In contrast to the well characterized GA conjugated formation based on labelling experiments nothing is known of the enzymes catalyzing the glucosylation of free GAs. The above mentioned rapid formation and high content of GA glucosides in fruits of legumes led us to initiate a screening for glucosylating activities in this plant family. Furthermore, suspension cultures of plant cells were used as a source of respective enzyme activities. In the following some characteristics of glucosyl transferases from maturing fruits of *P. coccineus* and isolated cells of *Lycopersicon peruvianum* L. are described.

MATERIALS AND METHODS

The preparation of the enzyme extracts from maturing fruits of *Phaseolus coccineus* L. (KNÖFEL *et al.* 1984) and from isolated cells of *Lycopersicon peruvianum* L. (LIEBISCH *et al.* 1985) using alkaline Tris buffer supplemented

TABLE 1

Conditions for the standard glucosyltransferase assays (either the GAs or UDP-glucose were radioactively labelled)

Phaseolus system (25 °C, 2 h)	*Lycopersicon* system (37 °C, 5 h)
50 nmol GA_3	30 nmol GA_7
500 nmol UDP-glucose	500 nmol UDP-glucose
0.7 mg protein of the 50–80% ammonium sulfate fraction	1 mg protein of the 50–80% ammonium sulfate fraction
in a total of 110 µl of	in a total of 100 µl of
0.05 M Tris buffer pH 8.2	0.02 M Tris buffer pH 8.4

with 0.01 M mercaptoethanol was as described previously. The extracts were centrifuged at 20 000 × *g* and further purified by ammonium sulfate precipitation of the supernatant, dialysis and concentration. The conditions for the standard incubation assays are given in Table 1. Both enzymes are relatively stable and can be stored deep-frozen over months without loss of activity.

RESULTS

Screening for GA Glucosylating Activity

Seed maturation in plants is often accompanied by a conversion of free GAs into glucosyl conjugates (SEMBDNER *et al.* 1980). This comes true especially for *Leguminosae* like *Phaseolus coccineus* (SEMBDNER *et al.* 1968) from which GA_8-2-O-glucoside was isolated as the first phytohormone conjugate (SCHREIBER *et al.* 1970). Therefore, a screening for GA glucosylating activities was concentrated mainly on immature legume fruits and 10 species were analyzed: *Phaseolus angularis* (WILLD.) W. F. WIGHT, *P. aureus* ROXB., *P. calcaratus* ROXB., *P. coccineus* L., *P. semierectus* L., *P. lunatus* L., *P. vulgaris* L., *Vigna sinensis* (STICKM.) SAVI EX HASSK. ssp. *sesquipedalis* (L.) VAN ES., *V. sinensis* (STICKM.) SAVI EX HASSK., ssp. *sinensis*, and *Pharbitis purpurea* (L.) VOIGT.

As crude plant homogenates in the presence of GAs and UDP-glucose never showed glucosylating activity a further separation by centrifugation at

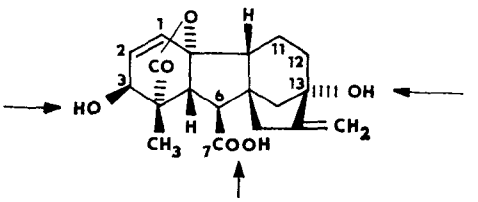


Fig. 1. Three possible sites of GA_3 glucosylation (indicated by arrows).

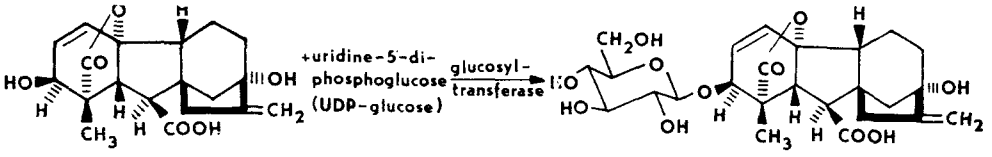
Abbreviations used: GA = gibberellin; Glc = glucosyl; ME = mercaptoethanol; UDP = uridine diphosphate; DTE = dithioerythritol; TLC = thin-layer chromatography; HPLC = high performance liquid chromatography.

20 000 × *g* and a gelfiltration on Sephadex G 25 of the supernatant had to be done. Among the legume species tested the 20 000 × *g* supernatant of *P. coccineus* and *P. angularis* showed considerable glucosylating activities and a small one was observed in *P. aureus*. Surprisingly, the sediment of *V. sinensis* ssp. *sesquipedalis* formed a GA_3 glucosyl conjugate, too.

Further experiments were conducted with the system of *P. coccineus*. The enzyme activity varied during fruit development. The most active preparations were obtained at a stage when the pericarp was still green and the seeds were completely developed. The enzyme was found to be soluble in nature and to be exclusively localized in the pericarp. With the aim of a continuous availability of GA glucosylating enzymes, suspension cultured cells of *Digitalis purpurea* L. and *Lycopersicon peruvianum* L. were included in screening. The former showed a very low activity in glucosylating GA_3 and the latter in glucosylating GA_7. In the following both the *Phaseolus* and the *Lycopersicon* systems were investigated with special regard to substrate specificity of the enzymes and elucidation of the structure of the products formed.

Elucidation of the *Phaseolus* Glucoside Stucture

GA_3 offers three possibilities for the formation of glucosides: C-3, C-13 and the carboxyl group (Fig. 1). Provided a monoglucosylation takes place, one or more of the three possible isomers GA_3-3-0-glucoside, GA_3-13-0-glucoside or GA_3 glucosyl ester may be formed. The labelled GA_3 glucoside formed from [6α^{3}H]GA_3 (LISCHEWSKI *et al.* 1982) and UDP-glucose exhibited chromatographic properties (TLC on silica gel iso-propanol/5 N ammonia = 4/1, DEAE-Sephadex column chromatography (GRÄBNER *et al.* 1976) identical with those of GA_3-0-glucosides. In order to distinguish between the 3-0- and the 13-0-glucoside a short-term acetylation (acetanhydride/pyridine, 20 min) was carried out. The resulting labelled derivative was found to be identical with the authentic GA_3-3-0-tetraacetylglucoside. Under these conditions the isomeric GA_3-13-0-glucoside would lead to a GA_3 glucoside pentaacetate due to the presence of an easily acetylated secondary hydroxyl group in position 3 of the diterpene skeleton. β-Glucosidase (cellulase from *Aspergillus niger*) partially hydrolyzed the labelled glucoside to GA_3 and glucose whereas an α-glucosidase preparation (from yeast) did not. These results give conclusive evidence that the product formed is GA_3-3-0-glucoside. Among several potential donors of the glucosyl moiety tested (UDP-, CDP-, ADP-, GDP-, and TDP-glucose) predominantly UDP-glucose served as the substrate



gibberellic acid (GA_3)

GA_3-O(3)-β-D-gluco-pyranoside + UDP

Fig. 2. Enzymic formation of GA_3-3-0-glucopyranoside.

Summarizing the above results the glucosylation reaction may be described as in Fig. 2.

Elucidation of the *Lycopersicon* Glucoside Structure

On incubation of the *Lycopersicon* enzyme with GA_7 and UDP-[U—^{14}C]-glucose a labelled conjugate was obtained in 12 to 20 % yield. Through chromatographic procedures (TLC on silica gel, solvent system iso-propanol/5 N

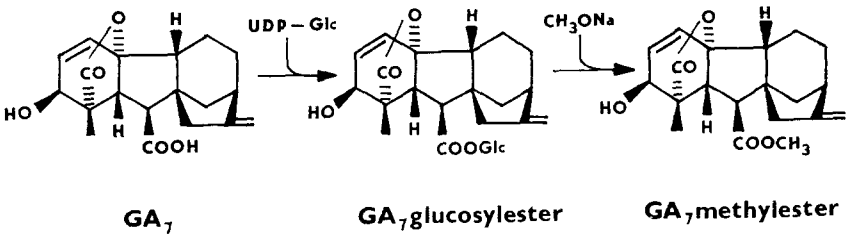


Fig. 3. Enzymic formation and transesterification of GA_7 glucosyl ester.

ammonia = 4/1, DEAE-Sephadex chromatography, HPLC; Liebisch *et al.* 1985) the labelled product proved to be identical with authentic GA_7 glucosyl ester and no radioactivity appeared with the isomeric GA_7-3-O-glucoside. Transesterification of the labelled product with 0.5 N sodium methylate afforded non-labelled GA_7 methyl ester (Fig. 3) which is an unequivocal proof for the presence of a glycosyl ester. Enzymic hydrolysis of the product with cellulase gave GA_7 and glucose. Quantification of the former by HPLC and the latter by the glucose oxidase reaction (Schliemann and Schneider 1979) pointed to a ratio of 1 : 1 and established the sugar to be β-D-glucopyranose. From these results we conclude the enzymic reaction product to be GA_7-β-D-glucopyranosyl ester formed according to Fig. 3.

Substrate Specificity of the Enzymes from *Phaseolus* and *Lycopersicon*

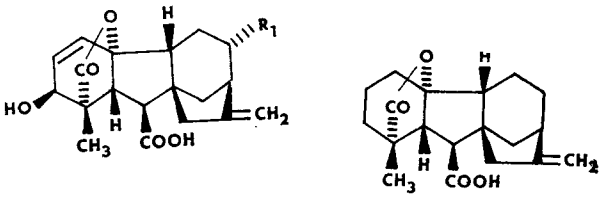
More than a dozen of natural or chemically modified GAs were tested as substrates in the *Phaseolus* system (Table 2). Alterations in ring A of GA_3 by epimerization or oxidation of the 3-β-hydroxyl group, hydrogenation of the $\Delta^{1,2}$-double bond, rearrangement of the lactone bridge, aromatization,

Table 2

Substrate specificity of the glucosyltransferases isolated from *Phaseolus coccineus* and *Lycopersicon peruvianum*

Gibberellins forming glucosides	*Phaseolus* system GA_3, GA_7, GA_{30}	*Lycopersicon* system GA_7, GA_9
Gibberellins not accepted as substrates	GA_1, GA_4, GA_5, GA_8, 3-epi-GA_3, iso-GA_3, 3-dehydro-GA_3, 3-OH-epi-allogibberic acid, 1-β-OH-GA_5	GA_1, GA_3, GA_4, GA_5, GA_6, GA_8, GA_{20}, GA_7 methylester, GA_8-methylester

or additional hydroxylation at C-2 resulted in a complete loss of the acceptor properties. However, GA_7 and GA_{30} formed glucosides, too, although to a smaller extent. Both GAs are structurally identical with GA_3 in ring A and differ only in the number of hydroxyl groups attached to ring C (Fig. 4).



GA_7 $R_1 = H$

GA_{30} $R_1 = OH$

GA_9

Fig. 4. Additional substrates for the glucosyltransferases from *Phaseolus* (GA_7 and GA_{30}) and *Lycopersicon* (GA_9).

In $\Delta^{1,2}$-unsaturated GAs the 3β-hydroxyl group is arranged quasi-equatorially (see SCHLIEMANN and LIEBISCH 1984). Obviously, these are the structural features the enzyme needs for recognition of the substrate. Even minor alterations (*e.g.* epimerization at C-3) were not tolerated. According to the preference of GA_3 as a substrate the *Phaseolus* enzyme is proposed to be a UDP-glucose : gibberellin A_3-3-O-glucosyltransferase.

With the *Lycopersicon* enzyme GA_7 glucosyl ester was formed according to Fig. 3. All natural GAs known so far have identical structural features around the ring B carboxyl group. Therefore, no high substrate specificity would be expected. Nevertheless, of 9 natural GAs tested (Table 2) only two, GA_7 and GA_9 (Fig. 4), served as substrates. When the carboxyl group is blocked by methylation as in GA_7 methyl ester, none of the isomeric GA_7-3-O-glucoside was formed. The *Lycopersicon* enzyme may thus be denoted as UDP-glucose : gibberellin acyl glucosyltransferase. For the formation of the GA glucosyl esters the alternative route using GA-CoA-thiol esters as activated intermediates was disproved.

DISCUSSION

Enzymes involved in the metabolism of gibberellins are extremely difficult to isolate for *in vitro* studies. On one hand this depends on the distinct physiological stages at which the enzymes are active and on the other hand they are obviously present in minute amounts. For suspension-grown tomato cells 0.08 μg GAs g^{-1} f.m. were detected by radioimmunoassay (KNÖFEL *et al.* unpublished). Adequate low levels of GA converting enzymes are likely.

From maturing fruits of *P. coccineus* considerable amounts of GA_8-2-O-β-D-glucoside have been isolated (SCHREIBER *et al.* 1970). Surprisingly, the *Phaseolus* enzyme does not seem to glucosylate GA_8 although a glucoside formation below the detection limit of 0.45 μg (TLC) cannot be excluded.

In field-grown *P. coccineus* plants the GA_3 glucosyltransferase is present only in the pericarp whereas the GA_1-glucoside predominates in the seeds.

May be, this type of compartmentation adds to the discrepancy between the observed glucoside formation *in vivo* and *in vitro*.

The apparent substrate specificity of the *Lycopersicon* and *Phaseolus* glucosyltransferases merits special attention as neither enzyme forms both the O-glucosides and the glucosyl esters. This behaviour differs considerably from a hydroxycinnamyl glucosyltransferase from tomato fruits, which *in vitro* catalyzes the formation of both types of glucosides with a number of substrates (FLEURIET *et al.* 1980). The enzymes described above are thus a striking evidence for the apparent specificity in the biosynthesis and conversion of GAs.

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