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SUBCELLULAR LOCALIZATION OF GLUCOSYLTRANSFERASES INVOLVED IN CARDIAC GLYCOSIDE GLUCOSYLATION IN LEAVES OF *CONVALLARIA MAJALIS*

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Key Word Index—*Convallaria majalis*; Liliaceae; cardiac glycosides; glucosyltransferase; subcellular distribution.

Abstract—The glucosylation of convallatoxin and convallatoxol was investigated using homogenates and various subcellular fractions from leaves of *Convallaria majalis*. The enzyme activity reached a maximum about 5 weeks after the onset of flowering and was found distributed among the soluble and the light membrane fraction. Upon separation of the light membranes by sucrose density gradient centrifugation, glucosyltransferase activity was found solely in a fraction banding at a density of 1.07 g/cm^3, which is thought to represent vacuole membranes.

INTRODUCTION

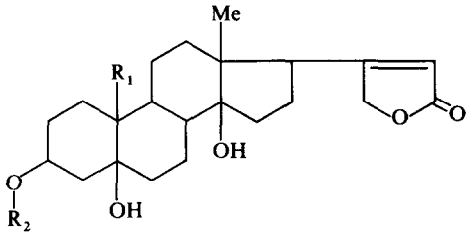
During the early flowering period convallatoxin (**1**) and convallatoxol (**2**) are two of the major cardenolides in leaves of *C. majalis* L. [1]. Within about 4–5 weeks after the onset of flowering, the cardenolide pattern is changed. Glucosylation in the 4′-position of these rhamnosides leads to an increase in the amounts of convalloside (**3**) and convallatoxoloside (**4**), respectively [2].

The first glycosylation of the hydroxyl group at C-3 occurs at the precardenolide level. Feeding of the correspondent aglycones to leaves of *C. majalis* [3] did not result in the formation of **1** and **2**. However, administration of labelled convallatoxin to mature leaves led to a significant conversion into convalloside [4]. Thus the glucosylation represents the last step in the biosynthetic pathway of the main cardenolides of *C. majalis*.

In this study cardiac glycoside glucosyltransferase activity of leaf homogenates and of subcellular fractions was measured at different periods of leaf development.

RESULTS

[4-^{14}C]Convallatoxin and [4-^{14}C]convallatoxol obtained after feeding of [4-^{14}C]progesterone to leaves of *C. majalis* [5] were used as substrates for the glucosyltransferase reaction. With these stable compounds the background radioactivity on TLC plates was negligible, which is desirable with the low conversion rates observed. UDP-[^{14}C]glucose without doubt would have given rise to other labelled products than **3** and **4**. UDP-glucose served as glucosyl donor and the addition of dithiothreitol and Mg^{2+} ions increased the activities measured. The soluble preparations were passed through Sephadex G-25 columns and the membrane preparations were washed. Thus in the absence of NAD oxidation of convallatoxol [6] was not to be expected. Addition of Triton X-100 to the assay mixture with membrane preparations had no effect.



1 R_1 = CHO, R_2 = rhamnose
2 R_1 = CH_2OH, R_2 = rhamnose
3 R_1 = CHO, R_2 = 4′-*O*-glucosylrhamnose
4 R_1 = CH_2OH, R_2 = 4′-*O*-glycosylrhamnose

Experiments with cell-free leaf homogenates were performed first in order to find out if the increase in convalloside and convallatoxoloside observed in fully matured leaves was correlated to elevated levels of glucosyltransferase activity. As shown in Table 1, only a very low yield of convalloside and convallatoxoloside was obtained with crude enzyme preparations from young leaves. However, extracts from fully matured leaves possessed a significant cardenolide glucosyltransferase activity. Both convallatoxin and convallatoxol were converted at similar rates.

For investigation of the subcellular distribution of glucosyltransferase activity, a mild method of cell disruption was used. Leaf slices were cut manually with a device containing ten razor blades at a distance of 1 mm in a medium containing 0.5% sucrose and 1% dextrane. This should result in a greater integrity of the dictyosomal fraction. The enzyme activity in the crude membrane fractions obtained by differential centrifugation was compared with that in the soluble supernatant (Table 2). The glucosyltransferase activity in the supernatant showed the same time course as the homogenates. In mature leaves activity also appeared in the lighter membrane fractions. Chloroplasts, mitochondria and microbodies possessed no glucosyltransferase activity. Experiments with convallatoxol as substrate yielded almost identical results.

Table 1. Glucosylation of cardiac glycosides by homogenates from *C. majalis* leaves at different stages of development

Substrate	Conversion rate (nmol/30 min/mg protein) Onset of flowering	2 weeks later	6 weeks later
Convallatoxin	0.8	3.2	5.6
Convallatoxol	0.7	3.0	n.d.

n.d. = Not determined.

Table 2. Subcellular distribution of UDP-glucose: convallatoxin glucosyltransferase activity in leaves of *C. majalis* at various stages of growth

Fraction*	Specific activity nmol/30 min/mg protein (in parentheses total activity nmol/30 min) Onset of flowering	2 weeks later	6 weeks later
1500 *g*	—†	—	—
10000 *g*	—	—	—
35000 *g*	—	—	6 (12)
100000 *g*	—	1.7 (2.8)	13 (22)
Supernatant	0.6 (7.2)	2.7 (21)	4.5 (37)

* See Experimental for details of preparation.
† — = No detectable activity.

Sucrose density gradient centrifugation should reveal if Golgi apparatus, endoplasmic reticulum, vacuolar or plasma membrane is the site of the particulate enzyme. After pelleting the nuclei and chloroplast fraction, the homogenate was applied onto two layers of sucrose solutions. After centrifugation the membranes that accumulated at the interface of the cushion layers were collected, diluted and placed on top of a linear gradient from 20 to 45% sucrose.

Following recentrifugation, three membrane fractions above the thylakoid band were collected from the gradient, pooled and assayed for glucosyltransferase activity (Table 3). The fraction of highest density containing dictyosomal and presumably plasma membranes showed almost no activity. The endoplasmic reticulum fraction possessed low activity. The maximum activity was found in a distinct band of very light membranes which almost did not enter the gradient. The cytoplasmic activity should have been removed by the precentrifugation. Nevertheless, another gradient of 15 to 30% sucrose was run to ensure separation of the active membrane band from cytoplasm and from endoplasmic reticulum (Fig. 1). The light membrane fraction again proved to be active, this time with convallatoxol as substrate (34 nmol/30 min/mg protein), whereas the endoplasmic reticulum fraction showed almost no activity. Thus, in the absence of a distinct marker enzyme for vacuolar membranes we tentatively assign the tonoplast as the site of membrane-bound cardiac glycoside glucosyltransferase in *C. majalis*.

Table 3. UDP-glucose: convallatoxin glucosyltransferase activity of subcellular fractions from mature leaves of *C. majalis* obtained after sucrose density gradient centrifugation

Density range of fraction tested	Marker enzyme	nmol/30 min/mg protein
1.07–1.08	—	30
1.08–1.12	NADH-cytochrome *c* reductase	2.5
1.12–1.16	Latent inosine diphosphatase	No detectable activity

DISCUSSION

The change in the cardenolide pattern of leaves from *C. majalis* during the vegetation period was accompanied by an increase in glucosyltransferase activity. Though nothing is known at present about the degradation of

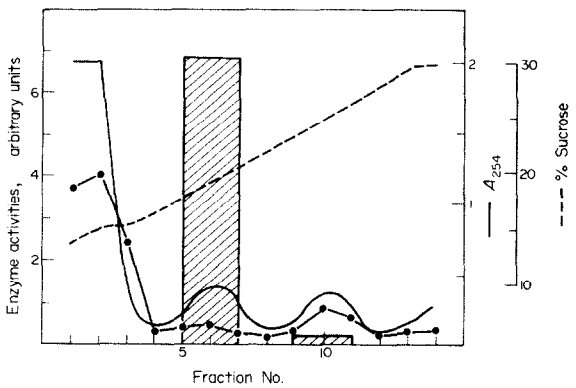


Fig. 1. Sucrose density gradient separation of light membranes prepared from leaves of *Convallaria majalis*. One arbitrary unit represents the following values: ●——●——●, NADH: cytochrome *c* reductase: 1 μmol/min/ml. Shaded area, UDP-glucose: convallatoxol glucosyltransferase: 1 nmol/30 min.

Convallaria glycosides, cardenolides undoubtedly undergo a turnover [7]. Furthermore, the total cardenolide content of leaves of *C. majalis* decreases at the end of the vegetation period [2]. Our interpretation is that at the later stages of flowering convallatoxin and convallatoxol are degraded but not replaced since the biosynthetic pathway is directed towards the formation of convalloside and convallatoxoloside by the appearance of a cardenolide glucosyltransferase activity.

During recent years glucosyltransferases have been reported from various plant sources which are specific for sterols [8], flavonoids [9], triterpenes [10] and cardiac glycosides [11]. When the substrate is a component of membranes, e.g. in the case of sterols, the corresponding UDP-glucose:sterol glucosyltransferase activity is also membrane-bound. In soy bean suspension cultures the plasma membrane was named as the site of enzyme activity [12]. In maize coleoptiles the enzyme is distributed between the plasma membrane and endoplasmic reticulum [13]. In *Calendula officinalis* seedlings, UDP-glucose:sterol glucosyltransferase activity as well as glucosylation of certain oleanolic acid glycosides was localized on dictyosomal membranes [10]. However, glycosylation of oleanolic acid itself occurred on the microsomal membranes.

Enzymes which glycosylate flavonoids are found in the soluble supernatant [9, 14] except for an isovitexin arabinosyltransferase which was found predominantly in a 38000 *g* pellet after homogenization of petals from *Silene dioica* [15]. A 32000 *g* pellet contained the major part of the UDP-glucose:digitoxin glucosyltransferase in leaves of *Digitalis purpurea* [11].

In mature leaves of *C. majalis* the total glucosyltransferase activities of supernatant and membrane fractions were in a ratio of about 1:1. This could mean that an enzyme in the cytoplasm or in the vacuole and a membrane-bound enzyme catalyse the same reaction. Another explanation is that part of the membrane-bound enzyme has been solubilized even under the rather mild conditions of cell fractionation used in this study. The enzyme in mature leaves should then be bound more tightly to the membranes (Table 2). The UDP-glucose:cardenolide glucosyltransferase activity was localized in a very light membrane fraction with an average equilibrium density in sucrose gradients of 1.07 g/cm^3. Thus, plasma membrane, Golgi apparatus and endoplasmic reticulum are ruled out. A fraction of endoplasmic reticulum that banded at 1.07 g/cm^3 has been reported for corn seedlings [16]. However, in *C. majalis* leaves endoplasmic reticulum showed a buoyant density of 1.11–1.12 g/cm^3. The pale yellow band at 1.07 g/cm^3 was well visible in the various gradients and it corresponded to a distinct peak in the 254 nm scan during fractionation of the gradients. This band contained more protein than the bulk of the endoplasmic reticulum. There is no unequivocal marker enzyme for vacuolar membranes of higher plants [17]. However, the tonoplast has been reported to possess the lowest density among cellular membranes, e.g. 1.06 g/cm^3 in suspension cultures of *Nicotiana glutinosa* [18]. Cardiac glycosides are stored in the vacuole in *C. majalis* [19] as well as in *Digitalis lanata*, where preliminary evidence indicates a participation of the tonoplast in the glucosylation of cardenolides (M. Luckner, personal communication).

Another example for the participation of tonoplast enzymes in the biosynthesis of secondary plant substances which are then stored in the vacuole is the hydroxylation of geraniol and nerol, which are intermediates in the biosynthesis of indole alkaloids in *Catharanthus roseus* [20].

Thus, we propose the following subcellular compartmentation of biosynthetic reactions leading to convalloside and convallatoxoloside as the major cardenolides in mature leaves of *C. majalis*. Convallatoxin and convallatoxol, stored in the central vacuole of the leaf cells are degraded. In mitochondria convallatoxol is formed [6] which is then released into the cytoplasm and partially oxidized to convallatoxin [6]. Before entering the vacuole the cardenolides are glucosylated to a considerable extent by the glucosyltransferase located on the cytoplasmic side of the tonoplast. In such a way convalloside and convallatoxoloside would gradually replace convallatoxin and convallatoxol as the major cardenolides in the vacuole.

EXPERIMENTAL

Plant material. C. majalis plants were grown in the garden of the Institute of Pharmacognosy. Leaves at different stages of development (onset of flowering, 2 weeks later and 6 weeks later) were taken and the midrib was cut out.

Preparation of leaf homogenates. Leaves (8 g fr. wt), cut into strips, were ground with quartz sand for 10 min at 4° in a mortar. The grinding medium consisted of 12 ml 0.1 M Hepes buffer, pH 7.5, containing 2 mM dithiothreitol and 2 g each of PVP and purified Amberlite XAD-4. The homogenized material was squeezed through a double layer of miracloth and then centrifuged at 50000 *g* for 15 min. The supernatant was put onto a Sephadex G-25 column (25 × 1.5 cm) equilibrated with 0.1 M Hepes buffer, pH 7.5. 15 ml were collected and concd using Aquacide I.

Membrane preparations. Leaf strips (7 g) were macerated manually with a razor blade holder containing 10 blades in 10 ml of a medium consisting of 0.1 M Hepes buffer (pH 7.5), 1 mM EDTA, 0.1 mM Mg^{2+}, 0.5 M sucrose, 2 mM dithiothreitol and 1% dextrane (av. MW 175000). PVP (1 g) and 1.5 g of Amberlite XAD-4 were added and the slurry was filtered through two layers of miracloth and then subjected to consecutive centrifugations at 1500 *g* for 10 min, 10000 *g* for 15 min, 35000 *g* for 30 min and 100000 *g* for 90 min. The crude membrane pellets thus obtained were washed once and then assayed for glucosyltransferase activity. The 100000 *g* supernatant was first passed through a Sephadex G-25 column and then concd as described above. For density gradient centrifugation of the membrane fractions of medium and low density the miracloth filtrate was pre-centrifuged at 2000 *g* × 10 min and then layered upon a double cushion (2 ml each) of sucrose solutions: 18/45% or 15/35% for the light membranes. Centrifugation was performed for 90 min at 100000 *g*. Membranes accumulating at the 18/45% interface were collected using a Pasteur pipette with a bent tip connected to a peristaltic pump. After dilution with 0.1 M Hepes buffer, pH 7.5, to a sucrose concentration of 15%, the suspension was layered on a linear sucrose gradient (20–45%) and centrifuged for 8 hr at 22000 rpm in a Beckman SW 27.1 rotor. Alternatively membranes from the 15/35% interface were diluted to 12% sucrose and put on top of a 15–30% sucrose gradient. Centrifugation was performed for 16 hr at 23000 rpm (SW 27.1). Gradients were fractionated using an ISCO density gradient fractionator equipped with an UV monitor. Fractions (1.2 or 0.6 ml) were assayed for marker enzymes. For the glucosyltransferase test fractions containing the various membrane populations were pooled, diluted and centrifuged at

100 000 *g* for 90 min. The pellets were resuspended in 0.1 M Hepes buffer, pH 7.5.

Enzyme assays. Glucosylation of cardiac glycosides was measured by incubating for 30 min at 28° in a total volume of 2.5 ml the following: [4-^{14}C]convallatoxin (0.3 mM) or [4-^{14}C]convallatoxol (0.3 mM), dithiothreitol (2 mM), $MgCl_2$ (5 mM), UDP-glucose (1 mM) and the particulate or soluble preparation in 0.1 mM Hepes buffer, pH 7.5. Latent inosine-5'-diphosphatase was assayed as described by Morré *et al.* [21]. The test for NADH–cytochrome *c* reductase was performed according to Tolbert [22].

Other determinations. Protein was measured by the method of Lowry *et al.* [23]. Sucrose concentrations were determined refractometrically.

Identification of products. The extraction of the cardenolide glycosides from the incubation mixture was performed as described previously [24]. [4-^{14}C]Convallatoxoloside and [4-^{14}C]convalloside were isolated by TLC in solvent system A. For further purification TLC with solvent system B was used. Finally the zone containing labelled convalloside or convallatoxoloside was scraped off and the substance eluted from the Si gel.

Chemicals. UDP-glucose, inosine diphosphate and cytochrome *c* were obtained from Sigma Chemical Co., St. Louis. [4-^{14}C]Convallatoxin (7.5 μCi/mmol) and [4-^{14}C]convallatoxol (17 μCi/mmol) were isolated from *C. majalis* after feeding experiments with [4-^{14}C]progesterone [5]. All other reagents were of analytical grade.

Chromatographic methods. TLC was carried out using 0.25 mm layers of Si gel (Merck, 60 F_{254}) with solvent system A: $CHCl_3$–MeOH–H_2O (7:3:1) lower phase; or B: MeCOEt–toluene–H_2O–MeOH (40:5:3:2.5).

Determination of radioactivity. TLC plates were checked using a thin layer scanner with a 2π proportional chamber (Berthold LB 2723). Radioactive samples were measured in a liquid scintillation counter.

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