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INTRACELLULAR DISTRIBUTION OF CARDIAC GLYCOSIDES IN LEAVES OF *CONVALLARIA MAJALIS*

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Key Word Index—*Convallaria majalis*; Liliaceae; lily of the valley leaves; cardiac glycosides; subcellular distribution; vacuoles.

Abstract—In order to investigate the intracellular distribution of the cardiac glycosides in leaves of *Convallaria majalis*, cell organelles were prepared by several methods. After mechanical disruption of the cells and differential centrifugation, the cardenolide content obtained was determined using the Baljet reaction. Most of the cardiac glycoside fraction was found in the soluble supernatant. However, a low but significant amount was also found in the 10000 *g* particles. Protoplasts and vacuoles were prepared by enzymic digestion of leaves. The cardenolide to protein ratio of vacuoles was far higher than that of protoplasts or the cytoplasmic fraction. The cardenolide content of isolated vacuoles relative to their number agreed well with the corresponding value obtained for protoplasts. This demonstrates clearly that cardiac glycosides are stored predominantly in the vacuoles of *Convallaria majalis*.

INTRODUCTION

At the present time there is little information concerning the subcellular distribution of secondary plant substances. Often the vacuoles are named as the organelles storing such plant products, e.g. tannins [1]. These assignments have been based on microscopic and cytochemical investigations or only by analogy. Recently, methods for the isolation of the more labile vacuoles have been developed [2, 3]. The vacuoles of *Tulipa* and *Hippeastrum* have been shown to contain the anthocyanins of the cells [2, 4] whereas in *Sorghum* the cyanogenic glucoside dhurrin [5] accumulates specifically in the vacuoles. Even less is known about the occurrence of natural products within other plant cell organelles. A varying proportion of the cellular flavonoids was found in chloroplasts of several plants [6, 7]. Steroidal glycoalkaloids in the Solanaceae were found mainly in the soluble supernatant with a small fraction bound to microsomes [8, 9]. In *Digitalis purpurea*, 1–2% of the cardiac glycosides have been reported to be bound to subcellular organelles without differences between the various fractions [10]. Digoxin was localized by radioimmunoassay almost exclusively in the soluble fraction of *Digitalis lanata* cells [11]; in all cases no discrimination between cytoplasm and vacuoles has been made. On the contrary, steroidal sapogenins and their glycosides in *Dioscorea tokoro* [12], as well as triterpenes in *Calendula officinalis* [13], were reported to be confined to particulate subcellular fractions. The present paper deals with the distribution of cardiac glycosides in subcellular organelles, including isolated vacuoles of leaves of *Convallaria majalis*.

RESULTS

Subcellular fractions prepared by mechanical disruption of cells

Young leaves from *Convallaria* plants grown in a greenhouse were used for the experiments. Their cardenolide content was *ca* 0.3–0.4 mg/g fr. wt. A colour reaction specific for the unsaturated five-membered lactone ring, described by Baljet [14], was used for quantitative determination of the cardiac glycoside content of the crude cell organelle fractions obtained by differential centrifugation. As shown in Table 1, the bulk of the cardenolides was found in the soluble fraction. This reflects a location in the cytoplasm and/or the vacuole. Chloroplasts and nuclei, though representing the largest particulate fraction, were virtually devoid of cardiac glycosides. The only particulate fraction containing a significant amount of cardenolides (2.4%) was the one containing mitochondria and peroxisomes.

Intact vacuoles and protoplasts

The isolation of protoplasts and vacuoles in good yield was possible only with young leaves within 10 days from emergence. Among the enzyme preparations tested were cellulysin and/or macerase, as well as combinations of a fungal pectinase with cellulases from *Rhizopus* sp., *Aspergillus niger* and *Trichoderma viride*. Cellulysin without other additives [2] gave the best results.

A considerable proportion of the protoplasts was always destroyed, possibly due to the presence of sharp oxalate crystals in the leaves. Incubations for 4–6 hr at 37° [5] were superior to prolonged incubations at 26° [2].

Table 1. Cardenolide content of subcellular fractions obtained from leaves (3.4 g fr. wt) of *Convallaria majalis*

Preparation	Cardenolides (μg)	% Distribution in subcellular fraction	Protein (mg)	Cardenolide (μg) / Protein (mg)
Leaf residue	403			
2000 *g* pellet	1.8	0.26	3.2	0.6
10000 *g* pellet	15.9	2.4	1.4	11.1
100000 *g* pellet	3.4	0.5	1.0	3.4
100000 *g* supernatant	648	96.8	12.5	51.8

Table 2. Separation of the 500 *g* pellet obtained after cellulysin digestion of *Convallaria majalis* leaf slices (2.4 g fr. wt) on a Ficoll step gradient (3.9, 12.5 and 20%) 50000 *g* (120 min)

Gradient fraction tested	Population	Cardenolides (μg)	Protein (mg)	Cardenolides (μg) / Protein (mg)
Supernatant (top)		46.2	1.84	25
Interface 3/9%	Vacuoles (80%) Protoplasts	75.6	0.38	194
Interface 9/12.5%	Protoplasts	34.0	0.87	39
Interface 12.5/20%	Broken protoplasts (without vacuoles)	6.1	0.36	17
Pellet	Cell debris, Cell organelles	4.8	0.58	8

Depending on the conditions, enzymic digestion of leaf slices yielded protoplasts and vacuoles in ratios ranging from 2:1 to 1:4. None of the methods described in the literature for release of vacuoles from protoplasts proved completely satisfactory in the case of *C. majalis*. Osmotic shock [2, 5] damaged the vacuoles as well as the protoplasts, and DEAE-dextran treatment [15] liberated vacuoles from protoplasts but destroyed the vacuoles already present. *C. majalis* vacuoles appeared to be more resistant to mechanical injury than the protoplasts. Repeated washing of the digested leaf residue, after decantation of the cellulysin suspension, increased the yield of total vacuoles as well as the vacuole to protoplast ratio. Therefore, the step of protoplast lysis resulting in vacuole yields of *ca* 10% only, was omitted. For the separation of intact vacuoles from protoplasts a discontinuous Ficoll gradient was the most efficient [5]. Table 2 shows the distribution of cardiac glycosides in fractions enriched with vacuoles, protoplasts, broken protoplasts and chloroplasts, respectively. The cardenolide to protein ratio was highest in the vacuolar fraction containing also some protoplasts and some vacuoles with adherent cytoplasmic material. Intact protoplasts showed a ratio lower by a factor of *ca* 5 than that of the vacuoles. This shows that vacuoles certainly represent the major site for cardiac glycoside accumulation in *C. majalis* cells.

The question still remained whether the cardenolide content observed in the soluble supernatant originates solely from broken vacuoles or whether cardenolides are also present in the cytoplasm. To clarify this point, a modified step gradient was used consisting of 7.5, 12.5 and 20% layers of Ficoll. Intact vacuoles did not penetrate into the 7.5% layer and accumulated together with the cytoplasmic fraction on top of the gradient. Vacuoles, retaining some membranes and constituents of the cytoplasm, distributed in the 7.5% layer, whereas intact protoplasts concentrated at the 7.5/12.5% interface. The vacuolar and protoplast fractions were collected from the gradient tubes, diluted and centrifuged at 500 *g* for 5 min. The pellets consisted of 95% of intact vacuoles and intact protoplasts, respectively. The cardenolide content in relation to the number of protoplasts and vacuoles is given in Table 3. The results point to a predominant, if not exclusive, location of non-particulate cardenolides in the vacuole. Aliquots of the vacuole and protoplast fractions were further subjected to TLC in order to identify the individual cardiac glycosides. In both cases identical patterns were observed. Convallatoxin and convallatoxol were found as major constituents together with less intense spots corresponding to periplorhamnoside, lokundjoside and convalloside. The relative intensities of the spots of the vacuole fraction and the protoplast fraction were estimated as 2 and 1, respectively. This means that the cardenolides of *C. majalis* [16] accumulate within the vacuole.

Table 3. Comparison of the cardenolide content of isolated vacuoles and protoplasts starting from 5 g of leaf slices of *Convallaria majalis*

Preparation	No.	Cardenolides (μg)	Cardenolides (μg) / 10^5 Protoplasts or vacuoles	Chlorophyll (μg)
Vacuoles	4×10^5	144	36	15
Protoplasts	2×10^5	81	40.5	274

The Ficoll gradient used consisted of 7.5, 12.5 and 20% layers.

DISCUSSION

The total cardenolide content of intact vacuoles isolated from leaves of *C. majalis* was very close to that of isolated protoplasts. Here, as in other cases [2, 5], the assumption was made that one large vacuole emerges per lysed protoplast. This view could be supported by microscopic investigations. About 3% of the cellular cardenolides was found particle-bound with the majority in the 10000 *g* fraction. This could mean that membranes of mitochondria and/or peroxisomes possess a higher affinity for cardenolide glycosides than those of chloroplasts or endoplasmic reticulum, and therefore the degree of unspecific adsorption is higher in the former case. A more probable explanation correlates the appearance of cardenolides in the 10000 *g* fraction, with the hydroxylation of periplorhamnoside to convallatoxol taking place in mitochondria of *C. majalis* leaf cells [17]. The presence of 4.5% α-tomatine in the microsomal fraction from *Solanum lycopersicum* has been interpreted to reflect its site of biosynthesis [8].

In contrast to sapogenins, free aglycones of the *Convallaria* glycosides are normally not encountered in the plant [18] and are not intermediates in the biosynthetic pathway [19]. On the basis of the results obtained with *Digitalis* plants [10, 11], the cardenolide glycosides were expected in the cytoplasm or in the vacuole. The results presented in this paper make it likely that the cardenolide content of the cytoplasm (max 7%) originates from biosynthetic reactions or interconversions at the cardenolide level [17]. However, storage of cardenolides is the sole function of the vacuole in cells of *C. majalis*.

EXPERIMENTAL

C. majalis plants were grown in a greenhouse.

Organelle preparation. Young leaves (3.4 g fr. wt) were cut into strips and blended for 3 × 5 sec with an Ultra-Turrax at full speed. 50 ml Semi-frozen grinding medium according to ref. [20] with the addition of 5 mM dithiothreitol and 2 g PVP were used. After filtration through a 4-fold layer of Miracloth, consecutive centrifugations at 2000 *g* (10 min), 10000 *g* (20 min) and 100000 *g* (120 min) were carried out. Each pellet was resuspended in 35 ml of grinding medium without PVP and centrifuged again. The washings were combined with the 100000 *g* supernatant.

Protoplast preparation. Young leaves were rinsed with 70% EtOH, the midrib was removed and the resulting leaf halves were cut into strips of *ca* 25 × 1.5 mm using a razor blade-based gel slicer. Each 2 g of strips were put into a 300 ml conical flask and covered with 15 ml 2% soln of cellulysin in 0.7 M mannitol,

pH adjusted to 5.5 with NaOH. Incubation was performed in a controlled environment incubator shaker at 37° and 15 rpm. After 5 hr the suspension was carefully decanted and filtered through 2 layers of Miracloth and one layer of glass wool. The residue in the flask was gently shaken first with 10 ml, then with 5 ml cold 0.5 M mannitol and filtered. The combined suspensions were centrifuged at 4° for 5 min at 500 *g*. The pellets were gently suspended in 0.5 ml 0.02 M Hepes buffer, pH 7.8; 0.5 M mannitol. Aliquots were examined microscopically using an improved Neubauer type hemocytometer which was also used for counting. For better visualization of vacuoles and for assessment of their intactness, the microscopic samples were mixed with a few drops of a filtered 1% soln of neutral red in Hepes–mannitol buffer, pH 7.8. Starting from 5 g of leaf strips, *ca* 1–1.5 × 10^6 of intact vacuoles and protoplasts were obtained.

Density gradient centrifugation. 1 ml of the suspension containing vacuoles, intact and damaged protoplasts and chloroplasts, was layered on top of a discontinuous gradient consisting of 7 ml layers of 3, 9, 12.5 and 20% solns of Ficoll 400 in 0.02 M Hepes buffer, pH 7.8; 0.5 M mannitol. Centrifugation was performed in a SW 25.1 rotor for 150 min at 22000 rpm (Beckman ultracentrifuge L2-65B). The supernatant soln, as well as the bands at the interfaces 3/9%, 9/12.5% and 12.5/20%, were collected with a syringe equipped with a 2 mm dia needle with a bent end. The pellet was collected after the Ficoll layers were siphoned off. All fractions were examined under the microscope and their cardenolide and protein content determined.

The vacuolar fraction, collected from the 3/9% Ficoll interface, contained *ca* 20% of vacuoles with adherent cytoplasmic membranes and inclusions and protoplasts. Therefore, another discontinuous gradient was designed in such a way that pure vacuoles would not penetrate the first layer but remain together with the cytoplasm on top of the gradient. Intact protoplasts on the contrary were trapped completely on the next interface. 9 ml layers of 7.5, 12.5 and 20% Ficoll 400 were suited for this purpose. The vacuole and the protoplast-containing zones were collected, diluted to 40 ml with mannitol buffer and centrifuged at 500 *g* (5 min). The pellets were suspended in mannitol buffer and examined and counted under the microscope. Aliquots were taken for cardenolide and chlorophyll determination and for TLC separation of cardenolides.

Chemicals. Cellulysin, Macerase and *Aspergillus niger* cellulase were purchased from Calbiochem. *Trichoderma viride* cellulase was obtained from Novo industri S/A. Pectinase and PVP were obtained from Sigma. Convallatoxin, convallatoxol, periplorhamnoside, lokundjoside and convalloside were isolated from *C. majalis* [21]. All other reagents were of analytical grade.

Assays. Protein was determined by the method of ref. [22]. Chlorophyll was measured according to ref. [23]. The cardenolide content of the subcellular fractions was determined spectrophotometrically after extraction [24] using the Baljet reaction [14, 21].

Chromatographic methods. TLC was carried out on Si gel (Merck, 60 F_{254}) using a concn zone and HPTLC plates (Si gel 60, Merck) using solvent systems A: $CHCl_3$–MeOH–H_2O (7:3:1), lower phase, and B [25]: MeCOEt–toluene–H_2O–MeOH–HOAc (40:5:3:2.5:1), respectively. The cardenolide glycosides were detected by spraying with vanillin–H_2SO_4 [26] and Kedde reagent [27], as modified in ref. [28].

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