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SUBCELLULAR DISTRIBUTION OF FREE AND ESTER-BOUND TRITERPENE TRIOLS IN *CALENDULA OFFICINALIS* FLOWERS

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Key Word Index—*Calendula officinalis*; Compositae; free and ester-bound pentacyclic triterpene triols; subcellular distribution.

Abstract—In cellular subfractions obtained from *Calendula officinalis* ligulate flowers, the contents of individual free and ester-bound triterpene triols, as well as fatty acid components of the ester form, were determined. It was shown that triterpene triols are localized only in the chromoplast fraction, almost exclusively in a free form and in a very small amount as monoesters. The compositions of fatty acids esterifying triols were similar to those esterifying diols in chromoplasts.

INTRODUCTION

In *Calendula officinalis* flowers the presence of pentacyclic triterpene alcohols possessing different numbers of hydroxyl groups has been reported [1–3]. Monols and diols in both free form and ester-bound with higher fatty acids have been detected. It was shown that triterpene monols in both forms occur inside and outside the chromoplasts. Triterpene diols, of which 98% are bound as 3-monoesters with higher fatty acids, are localized almost exclusively in the chromoplast fraction [4]. The aim of the present study was to investigate the subcellular distribution of pentacyclic triterpene triols in *Calendula officinalis* flowers both in free and ester-bound forms and to determine the fatty acids present in ester derivatives.

RESULTS AND DISCUSSION

Calendula officinalis flowers contain the following pentacyclic triterpene triols: olean-12-ene-3β,16β,28-triol (longispinogenine), lup-20(29)-ene-3β,16β,28-triol (lupenetriol), tarax-20-ene-3β,16β,22α-triol (heliantriol C) [5], tarax-20-ene-3β,16β,30-triol (heliantriol F) and ursa-12-ene-3β,16β,21-triol (ursatriol). In the first phase of the present work the total contents of triterpene triols in whole flowers was determined. The results are presented in Table 1.

Heliantriol C predominated among these compounds; other triols occurred in more or less the same amount. It is of interest that longispinogenine, which is the derivative of erythrodiol, occurred in much higher quantity among triols than erythrodiol did among diols. This can be explained by a very extensive metabolism of erythrodiol, which is converted not only into the respective triol but also into oleanolic acid, which is the aglycone of glycosides present in *Calendula officinalis* in a major amount.

Subsequently the subcellular fractionation of *Calendula officinalis* flowers was undertaken. The following fractions were obtained: I—600 *g* pellet containing large fragments of the cell walls and membranes; II—chromoplasts; III—the fraction containing mainly mitochondria (after centrifugation on a sucrose gradient at 105000 *g*); IV—105000 *g* pellet containing mainly microsomes; V—105000 *g* supernatant, the cytosol. The most important fraction from our point of view consisted of the chromoplasts, because triterpene diols (direct precursors of triols) were localized exclusively in this compartment. This confirmed the earlier suggestion that diols are synthesized in chromoplasts.

Chromoplasts of *Calendula officinalis* belong to the class of globular chromoplasts and are characterized by a labile structure. It was shown by Adler and Kasprzyk [6], who used light microscopy, that the chromoplast fraction exhibited high homogeneity although a large proportion of the chromoplasts were broken. On the other hand, the remaining fractions were contaminated with the chromoplast fraction, as demonstrated by its slightly orange colour. The amount of this contaminant was assessed by the determination of carotenoids. The quantity of carotenoids (expressed as β-carotene) in the chromoplast fraction varied in different experiments from 72 to 81%. The error resulting from the contamination of other fractions with chromoplasts was added to the contents of the investigated compounds in the chromoplasts. Triterpene triols were determined as their acetates after acetylation with [^{14}C]acetic anhydride. The total quantity of triterpene triols in chromoplasts is presented in Table 1. The results prove that all the compounds de-

Table 1. Total contents of triterpene triols in whole *Calendula officinalis* flowers and chromoplasts

Compound	Flowers (μg/g wet wt)	Chromoplasts (μg/g wet wt)
Longispinogenine	33.1	33.3
Lupenetriol	41.6	40.5
Heliantriol F	30.3	29.6
Heliantriol C	60.1	56.7
Ursatriol	34.4	36.1
Total	199.5	196.2

Table 2. Contents of free and ester-bound triterpene triols in non-fractionated *Calendula officinalis* flowers and chromoplasts

Compound	Flowers		Chromoplast	
	Free alcohols (μg/g wet wt)	Monoesters	Free alcohols (μg/g wet wt)	Monoesters
Longispinogenine	31.3	0.5	30.1	0.4
Lupenetriol	40.8	0.8	36.0	1.0
Heliantriol F	28.3	0.7	26.6	0.8
Heliantriol C	58.7	2.4	52.5	1.9
Ursatriol	32.4	0.8	34.5	0.9
Total	191.5	5.2	179.7	5.0

termined are present in this compartment. In all the other fractions, triols are present either in trace amount or are absent altogether. The total quantities and proportions of individual triols are very similar to those in non-fractionated flowers. This suggests that practically all triols must occur in the chromoplast fraction, similar to the distribution of triterpene diols.

In the next step of the present investigation the amount of free and ester-bound triols was determined after TLC resolution for tri-, di-, monoesters and free alcohols. Table 2 shows the quantities of free and ester-bound triols in non-fractionated flowers. The results confirm that triols occur almost exclusively in a free form and in a small amount as monoesters. The radioactivity of triols from the di- and triesters was practically at the background level.

Table 2 shows the amount of free and ester-bound triols in the chromoplast fraction. It is noteworthy that the distribution of triols in chromoplasts is very similar to that in non-fractionated flowers. The majority of the triols exist in a free form with a minor amount in the form of monoesters but diesters and triesters are absent. These results demonstrate that in *Calendula officinalis* flowers triterpene triols occur only in the chromoplast fraction, almost exclusively in a free form and in a small amount as monoesters. Monoesters of triols are not separable as individual compounds by TLC. The band of ester-bound triols isolated from 400 g of dry *Calendula officinalis* flowers was hydrolysed and the quantity of triterpene triols and fatty acids was determined by GLC. The calculated ratio of alcoholic and acidic components was 2.46 for monoesters, 1.22 for diesters and 0.82 for triesters. The ratio found for native ester derivatives was 2.85, which proves that they are monoesters. Judging by already known biosynthetic pathways, the fatty acid molecule should be esterified at position C-3 of the triterpene alcohol. This suggestion is also supported by the fatty acid composition, which is very similar to that found in 3-monoesters of triterpene diols isolated from *Calendula officinalis* flowers [4].

EXPERIMENTAL

Preparation of subcellular fractions. Ligulate flowers of *Calendula officinalis* (7 g) were ground in a Potter homogenizer with 0.25 M sucrose soln (105 ml) and filtered through four layers of cheesecloth; the filtrate was centrifuged at 600 g for 10 min (fraction I). The supernatant was then centrifuged at 20 000 *g* for 30 min. The supernatant after centrifugation at 20 000 *g* was centrifuged at 105 000 *g* for 60 min, giving microsomes (IV) and cytosol (V). The pellet after centrifugation at 20 000 *g* was suspended in 0.25 M sucrose, applied onto a 1.6 M sucrose layer and centrifuged at 105 000 *g* for 60 min. The pellet at the bottom of the tube contained mainly mitochondria (fraction III); the orange layer localized at the 0.25 and 1.6 M sucrose interface was considered to be the chromoplast fraction (II).

Determination of carotenoids. Carotenoids were determined as described previously [4].

Determination of triterpene triols. Triterpene triols were purified by TLC either as the free or ester-bound form or after alkaline hydrolysis as free alcohols. After purification they were acetylated by radioactive acetic anhydride and separated into the individual compounds. Radioactivity was measured with a scintillation counter. GC of triterpene alcohols was performed as described previously [7]. Fatty acids were determined on a 15% EGSS-X column.

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