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DISTRIBUTION OF TRITERPENE ALCOHOLS IN SUBCELLULAR FRACTIONS FROM *CALENDULA OFFICINALIS* FLOWERS

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Abstract—The distribution of the triterpene mono- and dihydroxy alcohols was investigated in the subcellular fractions of the flowers of *Calendula officinalis*. The triterpene monols were found mainly in the chromoplast fraction (68% of total) with smaller amounts in the cell debris, microsomal and supernatant fractions, the mitochondrial fraction was almost devoid of these compounds. Triterpene diols were present exclusively in the chromoplast fraction, 98% in the form of the 3-monoesters and 2% in the form of diesters. It is suggested that the hydroxylation of the triterpene monols to the corresponding diols proceeds in the chromoplasts and the esterified form of the monols is probably the substrate for this reaction.

INTRODUCTION

Flowers of *Calendula officinalis* contain triterpene monols and diols and compounds belonging to both these groups occur in the free and esterified forms [1,2]. Monols are present mainly in the free form, whereas more than 90% of the diols are as the 3-monoesters and only a small amount is present as the diester. Alcohols of both types are esterified by the same acids. Thus, it is possible that monol esters are hydroxylated to diol 3-monoesters, which subsequently undergo further esterification in the newly introduced hydroxyl group to yield diesters.

Zimmerman [3] first observed that the occurrence of triterpene diols in Compositae flowers is correlated with the presence of carotenoid pigments in this material. Our previous studies on the terpenoids in the flowers of 24 plants from the Compositae family, including *C. officinalis* indicated the general occurrence of triterpene monols, in contrast to triterpene diols which were present only in yellow-coloured flowers [4]. This fact suggests that carotenoids, the only pigments of *C. officinalis* flowers [5], and triterpene diols may be localised in the same organelle. To test this suggestion the distribution was investigated of free and esterified triterpene alcohols in the subcellular fractions from *C. officinalis* flowers.

RESULTS AND DISCUSSION

The isolation of the subcellular fractions was accomplished according to generally applied methods [6] with some modifications pertaining to the isolation of the homogeneous chromoplast fraction. Chromoplasts are of a labile nature; chromoplasts of a crystalline structure have been isolated from carrot roots [7] and tomato fruits [8] and chromoplasts of a membraneous type from *Narcissus* flowers [9], whereas those of a globular structure, such as the chromoplasts from *C. officinalis* flowers, have been obtained only from orange fruits. It was found that the fractions were least contaminated with carotenoid pigments if they were prepared from very young ligulate flowers taken from developing *C. officinalis* inflorescences. These flowers were ground and the resulting homogenate was filtered; subsequently the cell debris, chromoplast, mitochondrial and microsomal fractions as well as the post microsomal supernatant were obtained by successive centrifugations and filtration through a dense saccharose solution. The first three fractions were examined under a light microscope. The degree of contamination of all fractions with the chromoplast fraction was estimated by a determination of their carotenoid pigment content. The results are presented in Table 1. In the chromoplast fraction 85% of the total pigments were recovered; the postmicrosomal supernatant and the microsomal fraction were most contaminated with pigments originating from the damaged chromoplasts (7 and 5%, respectively). The protein content of all the fractions was also determined and the results are recorded in Table 1. When listed in order of decreasing protein content, the fractions assumed the following sequence: postmicrosomal supernatant chromoplast, microsomal, cell debris and mitochondrial fractions.

Table 1. The content of carotenoids and protein in subcellular fractions from *Calendula officinalis* flowers

Fraction	Carotenoids (μg/g)	Carotenoids (%)	Proteins (μg/g)	Proteins (%)
Cell debris	3·34	1·4	172	9·1
Chromoplast	224·20	85·4	620	32·8
Mitochondrial	2·75	1·0	74	3·9
Microsomal	13·38	5·1	285	15·1
Supernatant	18·69	7·1	738	39·1

Table 2. Distribution of triterpene monols in subcellular fractions from *Calendula officinalis* flowers

Fraction	Total monols (μg/g)	(%)*	Free monols (μg/g)	(%)†	Monol esters (μg/g)	(%)†
Cell debris	10·4	11·4	8·0	77	2·4	23
Chromoplast	62·2	68·1	38·8	63	23·4	37
Mitochondrial	1·3	1·4	1·0	77	0·3	23
Microsomal	9·1	10·0	6·4	70	2·7	30
Supernatant	8·3	9·1	8·0	96	0·3	4

* Percentage of total monols present in all fractions.
† Percentage of the monols present in the fraction.

Subsequently, lipids were extracted from all the fractions. Free triterpene alcohols and their esters were separated by TLC, and, after alkaline hydrolysis and TLC separation, the triterpene monols and diols were obtained from the esterified form. The triterpene alcohols were determined by quantitatively by GLC.

The distribution of triterpene monols in the subcellular fractions from *C. officinalis* flowers is shown in Table 2. The highest percentage of monols (68% of the total) was found in the chromoplast fraction; free alcohols accounted for 63% and esters for 37% of the chromoplast monols. Moreover, monols were detected in all the other cellular fractions. Taking into account some contamination of the remaining fractions with the chromoplast fraction (based on the carotenoid pigment content, Table 1), it can be assumed that the traces of monols detected in the mitochondrial fraction originated from the chromoplasts. As regards the microsomal, the supernatant and the cell debris fractions, each of them contained about 10% of the total monols i.e. more than the percentage of carotenoid pigment in them, which indicates that triterpene monols are a constituent of these fractions. These fractions have a lower content of esterified monols than the chromoplast fraction; the percentage of esterified monols in the microsomal, cell debris and postmicrosomal supernatant fractions are 30, 23 and 4%, respectively.

The distribution of triterpene diols in the subcellular fractions from flowers of *C. officinalis* is presented in Table 3. The concentration of the free diols in the fractions was too low to permit quantitative determination; traces of free diols were found in the mitochondrial, cell debris and chromoplast fractions. The chromoplast fraction contained as much as 89% of the total esterified diols of *C. officinalis* flowers. The microsomal, the supernatant and the cell debris fractions contained a few per cent each, and the mitochondrial fraction contained less than 1%. However, the percentage of esterified diols in these fractions was either lower than or equal to the percentage of contamination of the individual fractions with the chromoplast fraction, as calculated from the carotenoid content. Thus, it seems that esterified diols occur exclusively in the chromoplasts. The percentages of chromoplast diols present as the 3-monoesters and the diesters were 98 and 2%, respectively.

In comparison to our previous investigations [2], a somewhat lower percentage of free triterpene alcohols and a higher percentage of esters were found both for the monols and the diols. The previous studies dealt with the terpenoids of dried flowers, possibly the amounts of free triterpene alcohols were excessively high as a result of enzymic hydrolysis occurring in the course of drying of the material.

The present result suggest that in *C. officinalis* flowers the chromoplasts play a specific role as the site of accumulation of triterpene alcohols. While the results do not permit us to draw definite conclusions regarding the site of transformations of these alcohols, they seem to indicate that monol biosynthesis takes place in the microsomal fraction. The presence of large amounts of both the free and the esterified monols inside the chromoplast suggests that they are transported to this organelle in the free or esterified form. Monols can undergo esterification to some degree in the chromoplast, the microsome and in the cell debris fraction. The fact that only traces of free diols were detected in the subcellular fractions suggests that the second hydroxyl group is introduced into monol esters and this reaction takes place only in the chromoplast fraction. The localization of carotenoids and diol esters in the chromoplasts suggests that carotenoids may participate in the hydroxylation of triterpene alcohols.

EXPERIMENTAL

Material. Lingulate flowers of *C. officinalis* plants were grown in a phytotron under previously reported conditions [10].

Table 3. Distribution of triterpene diols in subcellular fractions from *Calendula officinalis* flowers

Fraction	Total diols (μg/g)	(%)*	Free diols (μg/g)	Diol monoesters (μg/g)	(%)	Diol diesters (μg/g)	(%)
Cell debris	5·9	2·1	trace	5·9	100	0	0
Chromoplast	244·4	88·6	trace	240·6	98	3·8	2
Mitochondrial	1·1	0·4	trace	1·1	100	0	0
Microsomal	14·9	5·4	0	13·6	91	1·3	9
Supernatant	9·6	3·5	0	8·7	91	0·9	9

* Percentage of total diols present in all fractions.
† Percentage of the diols present in the fraction.

Preparation of subcellular fractions. 2 g of finely cut ligulate flowers were ground in a mortar with 40 ml 0·25 M saccharose. The homogenate was filtered and centrifuged for 10 min at 800 *g*, yielding the cell debris fraction which consisted of broken cells, cell walls and cell membranes. It was then centrifuged for 30 min at 20000 *g*, affording the "chromoplast" fraction together with the "mitochondrial" fraction, whereupon it was centrifuged for 1 hr at 105000 *g*, yielding the microsomal fraction and its supernatant. The mixed fraction containing "chromoplasts" plus "mitochondria", suspended in 0·25 M saccharose, was separated through a 1·6 M saccharose soln during 1 hr at 105000 *g*. Under these conditions the mitochondria precipitated, whereas the chromoplasts remained in the layer of 0·25 M saccharose.

Determination of the triterpene alcohol content. To the fraction suspended in 0·25 M saccharose, an equal volume of MeOH was added; the mixture was heated to boiling, and lipids extracted with Et_2O. Free alcohols were separated from esters by TLC on Si gel in petrol–$CHCl_3$–MeOH (20:10:1). Esters were hydrolyzed with 10% KOH in MeOH at 80° for 20 hr. Extracted alcohols were purified by TLC, as described above. GLC was on OV-17 (1·7 m) at 330° with an argon flow of 20 ml/min, using cholesterol as standard. For the monols three peaks were obtained (R_t 1·72; 1·96; 2·4, respectively) [11]. Diols exhibited three peaks (R_t 3·13; 3·63; 4·5, respectively).

Determination of carotenoids. To the fraction suspended in a 0·25 M saccharose, a 10-fold volume of 6% KOH in EtOH was added. The mixture was hydrolyzed for 10 min at 90°, under N_2. Carotenoids were extracted with petrol, and the absorbance determined at 450 nm. The carotenoid content was calculated as previously reported [12].

Protein determination. By Lowry's method [13]; ppt. protein was dissolved in 1 N NaOH and the absorbance measured at 750 nm. Crystalline serum albumin was used as standard.

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