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INTRACELLULAR LOCALIZATION OF METABOLISM OF LUPEOL AND ITS PALMITATE IN *CALENDULA OFFICINALIS* FLOWERS

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Key Word Index—*Calendula officinalis*; Compositae; biosynthesis; chromoplast and extra-chromoplast fractions; triterpene alcohols; labelling dynamics.

Abstract—In the non-fractionated flowers and in the chromoplast and extra-chromoplast fractions obtained from *Calendula officinalis* flowers the incorporation of radioactivity after incubation with [3-^{3}H]lupeol and [3-^{3}H]lupeyl[^{14}C]palmitate were determined. It was shown that both precursors were metabolized. Monol esters are the main precursors of diol 3-monoesters, whereas free monols are hydroxylated to the diols and triols. The shape and course of the dynamic curves suggest that the process of hydroxylation of free monols, free diols and monol esters is situated only inside chromoplasts. On the other hand the hydrolysis of monol esters and esterification of free monols proceeds both outside and inside the chromoplasts.

INTRODUCTION

The presence of pentacyclic triterpene alcohols with different numbers of hydroxyl groups in *Calendula officinalis* flowers has been reported [1, 2]. The monohydroxyalcohols are located both outside and inside the chromoplasts whereas diols and triols occur almost exclusively inside the chromoplasts [2, 3]. All types of these alcohols exist as free alcohols, or as esters with fatty acids. Metabolic pathways of triterpene alcohols in *Calendula officinalis* flowers were not investigated. The possible sequence of hydroxylation and esterification is unknown. Also, there is no information about the possible localization of the above processes and only a few papers describe the process of hydroxylation in higher plants. A microsomal mixed function oxidase isolated from *Vinca rosea* seedlings was shown to catalyse the hydroxylation of the monoterpene alcohols geraniol and nerol to their corresponding 10-hydroxyderivatives [4]. The microsomal localization of flavonoid 3′-hydroxylase from parsley cell cultures [5] and cynnamic acid 4-hydroxylase from etiolated sorghum seedlings [6] were also described. Recently Petersen and Seitz [7] reported the microsomal localization of 12β-hydroxylation of digitoxin. The subcellulcar localization and characteristics of enzymes esterifying sterols in higher plants have been described [8, 9].

The aim of present work was to investigate the incorporation of radioactive lupeol and its palmitate into isolated ligulate *Calendula officinalis* flowers as well as into chromoplast and extra-chromoplast fractions obtained from them. This is regarded to be the first approach towards gaining an explanation of the biosynthesis of triterpene alcohols and the localization of this process in *Calendula officinalis* flower cells.

RESULTS AND DISSCUSSION

In the first experiments [3-^{3}H]lupeol (5.43×10^6 dpm/0.5 g flowers) was administrated to the isolated ligulate flowers of *Calendula officinalis*. Although other triterpene monols are present in the flowers it was decided to use lupeol as the precursor since both lupeol and its derivatives are easier to purify by TLC than the other monols, diols and triols present and moreover the lupane skeleton is rather stable. After the appropriate administration time the whole flowers were investigated or the flowers were fractionated to obtain chromoplast and extra-chromoplast fractions as described in the Experimental. The quantity of carotenoids present in the extra-chromoplast fraction was taken into consideration in the calculation of the final results as described previously [3]. The incorporation of radioactivity into different triterpene compounds in the whole flowers after administration of radioactive lupeol is presented in Fig. 1.

During all the periods investigated a decrease was observed in the radioactivity of the administered precursor. Simultaneously the radioactivity of all other investigated triterpenes increased up to 4 hr. The level of the radioactivity after 1 hr (except the precursor) was highest in free calenduladiol proving the rapid hydroxylation of administered lupeol. The rate of esterification of the lupeol as revealed by the level of radioactivity of monol esters is a little slower. The radioactivity of diol 3-monoesters was lower after 1 hr than the radioactivity of the monol esters indicating the subsequent hydroxylation of the latter. The level of radioactivity in lupenetriol after 1 hr was very low revealing a delay in the biosynthesis of triols which should be the derivatives of free diols. After 2 hr the increase of radioactivity in monol esters and free diol (calenduladiol) was relatively small, whereas radioactivity in diol 3-monoesters increased more rapidly and dominated the radioactivity of free calenduladiol. Between 2 and 4 hr radioactivity of all investigated derivatives of lupeol increased at more or less the same rate except for lupenetriol in which the level of radioactivity increased rapidly in this period. From 4 to 8 hr radioactivity of free calenduladiol and of monol esters went down whereas radioactivity of diol 3-monoesters and lupenetriol still increased. From 8 to 24 hr radioac-

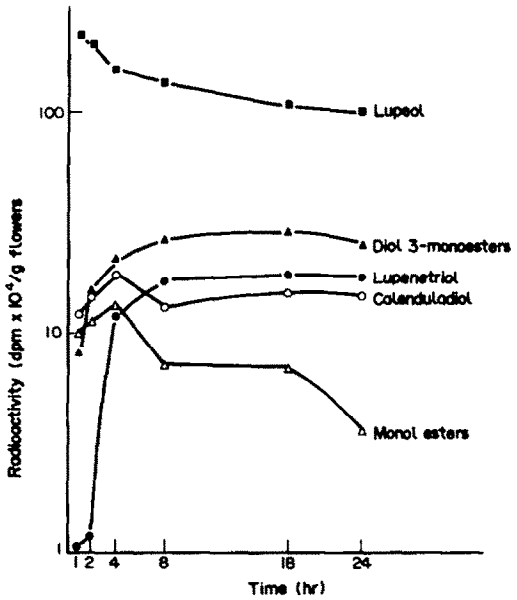

Fig. 1. The incorporation of radioactivity into triterpenes in whole flowers of *Calendula officialis* after administration of [3-^{3}H]lupeol (1×10^6 dpm).

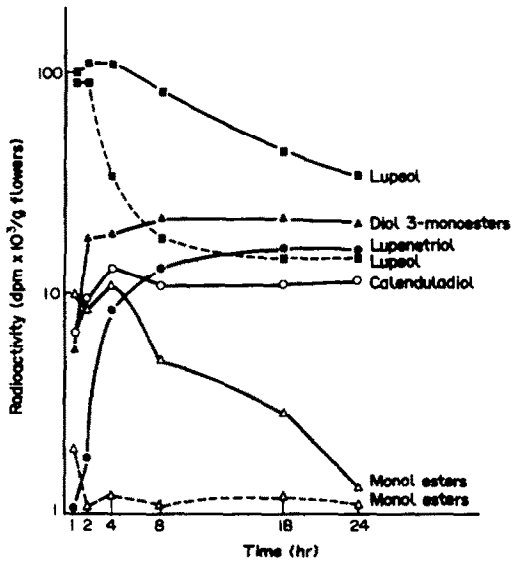

Fig. 2. The incorporation of radioactivity into triterpenes in the chromoplast and extra-chromoplast fractions of *C. officinalis* flowers after administration of [3-^{3}H]lupeol (1×10^6 dpm). —— —Chromoplast fraction; - - - -extra-chromoplast fraction.

tivity of lupeol derivatives exhibited a plateau except for the decrease in monol esters from 18 to 24 hr. The dynamic curves suggested that lupeol underwent hydroxylation and esterification and that hydroxylation proceeds faster than esterification. The monol esters could be subsequently hydroxylated in position C-16 to yield the 3-monoesters of diols, and free diols could be hydroxylated to triols. The quantity of free diols and free triols in flowers is very low (15 and 200 μg/g wet wt, respectively), so the relatively high level of radioactivity in these compounds and their high specific radioactivity points to fast metabolism. The labelling of the calenduladiol was confirmed by crystallization of a sample to constant specific activity after addition of unlabelled carrier calenduladiol (915, 878, 860, 852, 854 dpm/mg for succesive crystallizations).

The incorporation of radioactivity into different triterpene compounds in chromoplast and extra-chromoplast fractions after administration of radioactive lupeol is presented in Fig. 2. The dynamic curves of triterpenes in chromoplasts are generally similar to those in whole flowers. The curve for lupeol is little different to that for whole flowers and starting from 4 hr the lupeol decreased. The small increase of radioactivity in lupeol in the 1–2 hr incubations may indicate the transport of lupeol into chromoplasts. The similarity of the curves for diol 3-monoesters, free calenduladiol and free lupenetriol in chromoplasts to those in whole flowers suggest that the localization of the process of hydroxylation may be in the chromoplasts. In the extra-chromoplast area only lupeol and monol esters were labelled. Lupeol radioactivity first decreased rapidly, and then move slowly, again consistent with the transport of lupeol into the chromoplasts. The level of monol ester radioactivity in the extrachromoplast fraction was rather low, pointing to a slow process of monol esterification in the extrachromoplast fraction.

In the second part of this work double labelled lupeol palmitate (1×10^6 dpm of ^{3}H and 4.2×10^6 dpm of ^{14}C/0.5 g flowers) was administered to isolated ligulate flowers. The incorporation of radioactivity into different triterpenes after administration of [3-^{3}H]lupeyl [^{14}C]palmitate to the whole flowers is presented in Fig. 3. Lupeol palmitate, a less polar compound, was not absorbed by the flowers as well as free lupeol, possibly due to its lower solubility in 5% EtOH solution and its probably

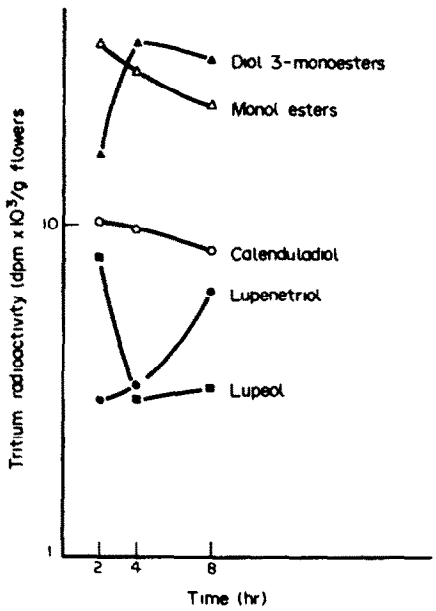

Fig. 3. The incorporation of radioactivity into triterpenes in the whole flowers of *C. officinalis* after administration of [3-^{3}H]lupeyl[^{14}C]palmitate (1×10^6 dpm of ^{3}H).

slower penetration to the tissue and cells. During all the experiments the tritium radioactivity in the administrated precursor decreased in a similar manner to that observed in the experiments when free lupeol was used as the precursor. The carbon radioactivity decreased a little faster as revealed by the change in the $^{14}C:^{3}H$ ratio. This suggests a slow hydrolysis of the precursor and subsequent esterification by non-radioactive fatty acids. The radioactivity of the diol 3-monoesters first increased markedly then showed a small decrease. The $^{14}C:^{3}H$ ratio in diol 3-monoesters was similar to that in the monol esters. This supports the view that monol esters are the main precursors for 3-monoesters of diols as was previously proposed based upon the composition of fatty acids esterifying both these groups of triterpene alcohols [3]. The shape of the curves for free lupeol, free calenduladiol and free lupenetriol indicates the successive hydroxylations to calenduladiol and to lupenetriol.

The incorporation of radioactivity into different triterpenes in chromoplast and extra-chromoplast fractions after administration of double labelled lupeyl palmitate is presented in Fig. 4. The radioactivity of precursor in the extra-chromoplast fraction decreased markedly, whereas the same compound in the chromoplasts showed some increase in radioactivity from 2 to 4 hr. Free lupeol in the chromoplasts exhibited the radioactivity increasing in contrast to the radioactivity of lupeol in the extra-chromoplast fraction. This may indicate that the hydrolysis of lupeyl palmitate takes place at least partly outside the chromoplasts and free monol can be transported into the chromoplasts where it is hydroxylated to calenduladiol and lupenetriol. The $^{14}C:^{3}H$ ratio in monol esters and diol 3-monoesters in chromoplasts is lower than in whole flowers which indicates that monol esters are partly hydrolysed outside the chromoplasts. The free monols may then be subsequently esterified by non-radioactive acids inside the chromoplasts.

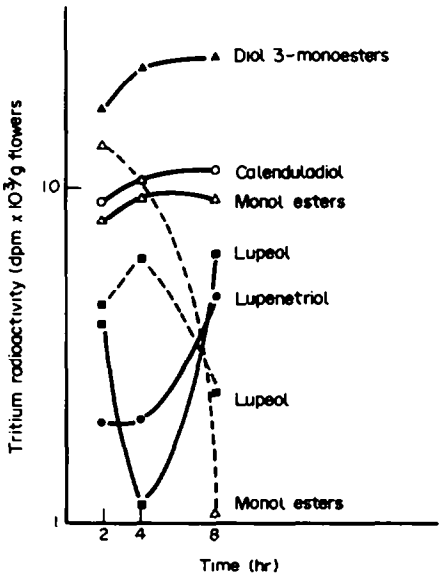

Fig. 4. The incorporation of radioactivity into triterpenes in the chromoplast and extra-chromoplast fractions of *C. officnalis* flowers after administration of [3-^{3}H]lupeyl [^{14}C]palmitate (1×10^{6} dpm of ^{3}H). —— Chromoplast fraction; --- extra-chromoplast fraction.

All the results described appear to suggest that the monol esters are the main precursors of diol 3-monoesters, whereas free monols are hydroxylated to yield diols and triols. The hydrolysis of monol esters as well as their re-esterification may proceed both outside and inside chromoplasts. Results indicating the possible presence of active enzymes hydroxylating specifically triterpene alcohols inside the chromoplasts are rather unexpected. It is possible that carotenoids present in the flowers [10] are engaged in this process.

EXPERIMENTAL

Synthesis of radioactive precursors. [3-^{3}H]Lupeol was prepared by the reduction of lupene-3-one with $NaB^{3}H_4$ in MeOH. [3-^{3}H]Lupeyl[^{14}C]palmitate was prepared by esterification of tritiated lupeol with [^{14}C]palmitoyl chloride in benzene with a small quantity of pyridine.

Administration of radioactive precursors. The soln of both radioactive precursors in 5% EtOH–H_2O was administered to isolated ligulate *Calendula officinalis* flowers for 1, 2, 4, 8, 18 and 24 hr (free lupeol) or 2, 4 and 8 hr (lupeyl palmitate). Subsequently flowers were analysed as non-fractionated material or were used for the preparation of cellular subfractions.

Preparation of cellular subfractions. Preparation of cellular subfractions and the determination in them of the quantity of carotenoids were performed as described previously [2, 3].

Purification of triterpene compounds. The Et_2O extracts from whole flowers or subfractions were separated by TLC on Kieselgel G-60 (Merck) with the solvent system hexane–$CHCl_3$–MeOH (20:10:1) into five partially purified fractions comprising of monol esters, diol 3-monoesters, free monols, free diols and free triols (in order of increasing polarity). The monol esters and diol 3-monoesters were rechromatographed using the above system. The free monols was rechromatographed on $AgNO_3$–silica gel TLC using $CHCl_3$ (free from EtOH) for separation of lupeol from other monols. The free diols were acetylated and then rechromatographed by $AgNO_3$–silica gel TLC for separation of calenduladiol from other diols. The free triols were rechromatographed on Merck TLC sheets precoated with silica gel (Kieselgel 60, 0.2 mm).

Radioactivity measurements. Radioactivity of eluted compounds was measured in toluene-based scintillator.

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