Title
TRITERPENOID BIOSYNTHESIS IN Euphorbia lathyris

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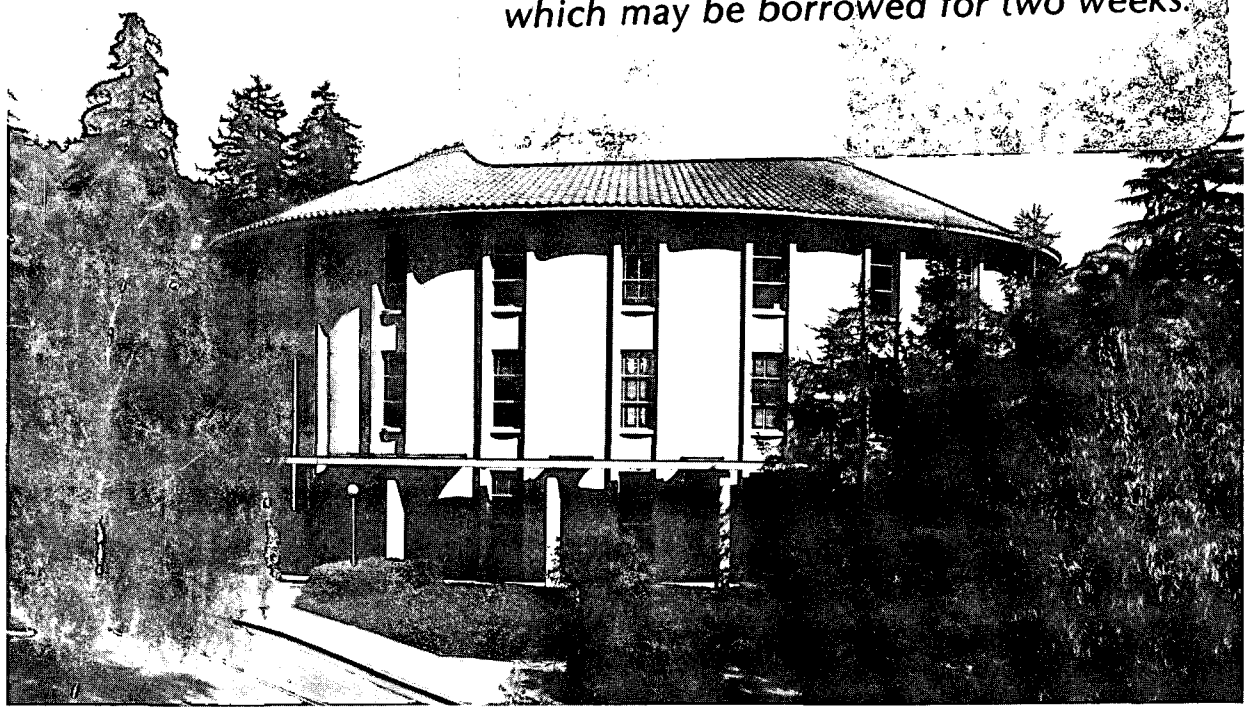

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DISCLAIMER

TRITERPENOID BIOSYNTHESIS IN EUPHORBIA LATHYRIS

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Abstract

Experiments investigating the roles of HMG-CoA reductase and fructose-2,6-bisphosphate in the control of the production of triterpenoids in Euphorbia lathyris latex are described. The occurrence of 24-methylenelanosterol and butyrospermol in latex is reported. Preliminary steps in the purification of HMG-CoA reductase are presented.

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INTRODUCTION

Triterpenols and their esters make up 4-5% of the dry weight of Euphorbia lathyris.[1] A significant amount of these triterpenoids are synthesized and stored within laticifer cells where these compounds constitute up to 50% of the dry weight of the latex.[2] We are interested in the nature of these compounds and the organization and regulation of their biosynthesis. Here we report on recent work investigating the control of carbon allocation to these compounds, further elucidation of the identity of the triterpenols found in latex, evidence indicating that the conversion of HMG-CoA to mevalonate (MVA) is rate-limiting in the biosynthesis of triterpenoids in latex, and steps in the purification of HMG-CoA reductase (HMGR).

MATERIALS AND METHODS

Latex incubation, isolation and analysis of triterpenoids were performed as previously described.[2]

Authentic 24-methylenelanosterol was synthesized from lanosterol by the method of Barton.[3] ^{1}H-NMR spectra were determined at 250 MHz. Lanthanide isotope shifts (LIS) were measured in the presence of Eu(fod)$_3$.

Rates of acetate (Ac) and MVA incorporation into triterpenoids were determined by incubation of latex with saturating concentrations of ^{3}H-Ac (0.25 mM, 2 Ci/mmol) or ^{3}H-MVA (1 mM, 50 mCi/mmol) and 10 mM DTE. HMGR assay was done on the sonicated pellet of a 5000 x g, 15 min centrifugation of latex diluted 2:1 with 10 mM MES, 10 mM DTE, 30 mM EDTA, 0.4 M sorbitol, pH 6.5. HMGR incubation containing 0.3 mM ^{3}H-HMG-CoA (33 mCi/mmol) and 3.3 mM NADPH was quenched with KOH then acidified. Mevalonolactone (MVAL) was purified by silica gel TLC (1:1, acetone:benzene), further purified by HPLC using an organic acid column in 0.0025 N H_2SO_4, and analyzed by LSC.

For purification of HMGR, stem and leaf tissue were ground with buffer containing 10 mM K phosphate, 0.4 M sorbitol, 30 mM EDTA, 10 mM DTE, and 10% w/w insoluble PVP, pH 7.2. The filtered extract was centrifuged as described in Fig. 1. Pellets were resuspended in 0.1 M K phosphate, 30 mM EDTA, 10 mM DTE, and 0.04% Triton X-100, various pHs for pH curve. Solubilization of HMGR was tested by preparing an 18,000 x g pellet using buffer of pH 6.8,

Table 1. Evidence for HMGR as Rate-determining in Latex Triterpenoid Biosynthesis

Ac ⟶ Triterpenoids	0.02 nmol inc./100 µl latex/hr
MVA ⟶ Triterpenoids	0.55
HMG-CoA ⟶ MVA	0.03

then resuspending in buffer containing detergents listed in Table 2. Incubations sat 20 min on ice, were diluted 3-fold and centrifuged at 100,000 x g for 1 hr. For HMGR analysis, samples were incubated with either ^{3}H- or ^{14}C-HMG-CoA (0.6 mM, 5.6 mCi/mmol or 2.2 mCi/mmol, respectively) and 2.5 mM NADPH. Incubations were quenched with 6 N HCl and purified by silica gel TLC (2:1, $CHCl_3$:acetone). The MVAL band was eluted from the silica and analyzed by LSC. Protein was determined per Bradford.[4]

Glucose incorporation into latex was done by combining latex with ^{14}C-glucose (4 mM, 6 mCi/mmol), 2 mM $MgCl_2$, 1 mM NADPH, 1 mM ATP, and 0.33 M sorbitol. Two experimental conditions were used: unbuffered latex (pH 5.5); latex buffered to pH 7.0 (approx. cytoplasmic pH). Buffer (0.1 M MOPS) and fructose 2,6-bisphosphate (F26BP, 50 mM for pH 5.5, 2 mM for pH 7.0) were added as indicated in Table 3. Additional controls with Na pyrophosphate (PPi, 2 mM) and fructose 6-phosphate (F6P, 50 mM) were also performed. Incubations were run for 1 hr then analyzed for incorporation into triterpenoids.

RESULTS AND DISCUSSION

Nielsen et al.[5] identified the major components of latex as the tetracyclic triterpenols cycloartenol, 24-methylenecycloartenol, lanosterol, and an isomer of lanosterol. We have indentified this last compound as butyrospermol (5α-eupha-7,24-dien-3β-ol). We have also isolated 24-methylenelanosterol from latex. These structures were determined by comparison of HPLC elution, mass spectra, ^{1}H-NMR spectra, LIS, and optical rotations with either literature values or authentic samples.

The conversion of HMG-CoA to MVA catalyzed by HMGR is acknowledged as the major rate-limiting step in cholesterol biosynthesis in mammalian systems. As shown in Table 1, comparison of the conversion of Ac and MVA into triterpenoids establishes that the rate-limiting step in the pathway in *E. lathyris* latex occurs prior to MVA. As we have demonstrated previously[2], exogenously supplied HMG-CoA is not metabolized to triterpenoids in latex. However, HMGR activity measured in sonicated latex corresponds with the overall Ac conversion rate, suggesting that this step is rate-limiting in the formation of triterpenoids in latex.

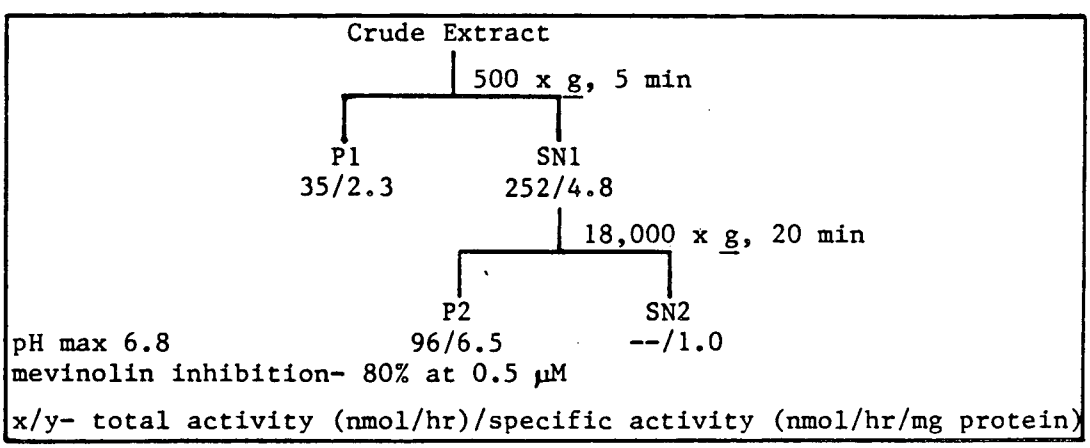

Fig. 1. Concentration of HMGR by differential centrifugation.

Table 2. Solubilization of HMGR from P2

Treatment of P2	HMGR Activity as a % of SN1 Supernatant	Pellet
none	5%	16%
1% Triton X-100	2	2
1% Triton X-100 + 2% SDS	2	1
0.25% Deoxycholate	25	30

In purification of HMGR from the whole plant, the first step can be achieved by obtaining an 18,000 x g pellet as in Fig. 1. As shown in Table 2, HMGR can be solubilized from the pellet. Deoxycholate treatment increased HMGR activity 2.6-fold over the untreated sample as well as increasing the ratio of solubilized HMGR to pelletable HMGR.

Most of the reduced carbon found in laticifer cells is in starch and triterpenoids, which are produced at near equal levels. It is possible that the rate of triterpenoid production is controlled at the initial conversion of sugar to the early precursors of triterpenoids or starch. One compound that has recently been found to control carbon utilization within a plant cell is F26BP which mediates the rate of conversion of F6P to fructose 1,6-bisphosphate.[6] To investigate the role of F26BP in latex we followed the incorporation of ^{14}C-glucose into triterpenoids with and without added F26BP. The results of the experiments (Table 3) indicate a role for F26BP in carbon allocation in latex. They also indicate that it is not a product of F26BP breakdown (F6P or PPi) that mediates the conversion.

Table 3. The Effect of Fructose 2,6-Bisphosphate on Glucose Incorporation into Triterpenoids

	Sterols (dpm above Background) latex (pH 5.5)	latex + MOPS (pH 7.0)
+ ^{14}C-glucose	0	795
+ ^{14}C-glucose, fructose 2,6-dP	369	1443
+ ^{14}C-glucose, fructose 6-P	0	---
+ ^{14}C-glucose, Na pyrophosphate	---	772

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