ACCUMULATION OF IXERIN F AND ACTIVITIES OF SOME TERPENOID BIOSYNTHETIC ENZYMES IN A CELL SUSPENSION CULTURE OF *LACTUCA VIROSA* L.ANNA STOJAKOWSKA¹, JANUSZ MALARZ¹, ROBERT VAN DER HEIJDEN², ROBERT VERPOORTE²¹Department of Phytochemistry, Institute of Pharmacology,
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ABSTRACT

A cell suspension culture of *Lactuca virosa* L. (Asteraceae, tribe Lactuceae) is capable of synthesizing sesquiterpene lactones of which ixerin F is the main compound. The culture was characterized on growth (by dissimilation rates), on ixerin F accumulation (by RP-HPLC) and on some enzyme activities involved in early steps of terpenoid biosynthesis. Acetoacetyl-coenzyme A thiolase (AACT, E.C. 2.3.1.9) and 3S-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGS, E.C. 4.1.3.5) activities of the cells were assayed spectrophotometrically. HMGS activity increased during the culture period and reached a maximum during the stationary phase (190 pkat/mg protein), while AACT showed relatively high level of activity throughout the growth cycle, with transient decrease at the logarithmic growth phase and the beginning of stationary phase. Ixerin F accumulated inside the cells and the maximum concentration of 0.08% (on dry weight basis) was found in the early stationary phase of the growth cycle of the culture.

KEY WORDS: *Lactuca virosa* L., ixerin F, acetoacetyl-coenzyme A thiolase (E.C. 2.3.1.9), 3S-hydroxy-3-methylglutaryl-coenzyme A synthase (E.C. 4.1.3.5), sesquiterpene lactones.

INTRODUCTION

In a previous study (Stojakowska et al., 1994) we found that a cell suspension culture of *Lactuca virosa* L. produces guaianolide-type sesquiterpene lactones, mainly ixerin F (9 α -hydroxy-11 β ,13-dihydrozaluzanin C 3 β -D-glucopyranoside). Like other terpenoids, the sesquiterpene lactones are synthesized from acetate via mevalonate/farnesyl diphosphate pathway. The hypothesis has been recently confirmed by Song et al. (1995).

3S-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (HMGR, E.C. 1.1.1.34), an enzyme which catalyses formation of mevalonic acid (MVA) from HMG-CoA proved to be the key regulatory enzyme in cholesterol biosynthesis in mammals. Similarly, some experiments with HMGR inhibitors in plants, suggested that the enzyme had a regulatory function in the substrate flow through the pathway leading to terpenoids and steroids (Bach et al., 1990). Bestwick et al. (1995) found that high activity of the HMGR preceded accumulation of sesquiterpene lactone phytoalexin lettuceenin A in *Lactuca sativa* L. seedlings with the red spot symptom.

HMG-CoA, the substrate for HMGR is biosynthesized from acetate. In animals and yeasts two enzymes are required to complete the biosynthesis: acetoacetyl-coenzyme A thiolase (AACT, E.C. 2.3.1.9) and 3S-hydroxy-3-methylglutaryl-

coenzyme A synthase (HMGS, E.C. 4.1.3.5). A similar process is likely to occur in plants, both AACT and HMGS have been detected in *Catharanthus roseus* plants (van der Heijden et al., 1994b). Recently both genes have been cloned: AACT from radish (Vollack and Bach, 1996) and HMGS from *Arabidopsis thaliana* (Montamat et al., 1995).

In the present study we determined the activities of the enzymes involved in biosynthesis of HMG-CoA and investigated the accumulation of ixerin F in the cells in relation to the growth cycle of a *Lactuca virosa* suspension culture.

MATERIAL AND METHODS

Plant material

Seeds of *Lactuca virosa* L. were obtained from the Botanical Garden in Oberholz (Germany). After immersing in 70% ethanol for 30 s, the seeds were surface sterilized with a 5% solution of Dimanin C (Bayer). Afterwards, the seeds were rinsed 4 times with sterile distilled water and aseptically germinated on a hormone-free agar medium containing 1/4 of MS (Murashige and Skoog, 1962) macro- and micronutrients, at 25 °C and continuous light (2000 lx, cool white fluorescent tubes). The suspension culture was derived from the hypocotyl of aseptically grown seedlings. The culture was main-

tained in MS medium containing 1 mg/l of 2,4-D and 0.2 mg/l of kinetin, on a gyrotory shaker (120 r.p.m.), under continuous illumination (800 lx). The cells were harvested by filtration and immediately frozen in liquid nitrogen. An aliquot of the biomass was lyophilized and subsequently used for ixerin F quantification. 10 g (fresh weight) of the cells were used for the enzyme assays.

Determination of the dissimilation curve for the culture

The determination of dissimilation curve from *L. virosa* cell suspension culture, based on the weight loss of the culture flask due to dissimilation, was carried out according to Schripsema et al. (1990). Assuming that in the dissimilation process equimolar quantities of CO₂ and O₂ are exchanged between the culture and the environment, loss of weight in the culture flask should be correlated with intensity of this process. The dissimilation curve was calculated by subtracting the evaporation (loss of weight in flasks containing nutrient medium) from the loss of weight of a culture flask (flask containing medium and growing cells). Five culture flasks, containing 50 ml of culture medium inoculated with 3 g of cells, and five controls (flasks containing medium only) were weighed accurately (accuracy ± 1 mg) every 24 hours during the culture period. In the experiment Silicosen T-32 plugs (Shin-Etsu Polymer Co. Ltd., Japan) were used to close the flasks.

Growth index (G.I.), reflecting the biomass accumulation, was calculated as a ratio of the final dry weight of the culture, after 23 days of growth, to the dry weight of inoculum.

Phytochemical analysis

0.5 g of the lyophilized cells was extracted twice with 25 ml of ethanol at room temperature. The ethanol extract was concentrated 'in vacuo' to the volume of ca 10 ml and left to stand overnight at 4 °C. The solution was then filtered and filtrate was evaporated to dryness. The residue was dissolved in 1 ml of methanol and filtered through the membrane filter (0.45 μ m). 5 μ l of the filtrate were injected onto the HPLC system made up of a Waters 600E solvent delivery system

and μ -Bondapak C₁₈, 21 x 300 mm column (Waters, No 86609) coupled with Waters 991J photodiode array detector. The solvent system: methanol – water (35:65) at a flow rate of 0.5 ml/min was employed. Absorbance was monitored at 210 nm. The ixerin F standard retention time was 6 min. Each sample was injected twice and every extraction was repeated.

Extraction of proteins

The procedure was carried out at 4 °C. 10 g of the freshly harvested cells were frozen in liquid nitrogen. The frozen cells were grounded with a pestle in an ice cold mortar, together with 1 g of sand, 1 g of polyvinylpyrrolidone (Sigma) and 5 ml of cold extraction buffer (pH 7.5) containing 0.1 M of KH₂PO₄, 1 M of sucrose, 2 mM of Na₂EDTA, 1% of Triton X-100 (Merck) and 10 mM of dithiotreitol (DTT) (Merck). The obtained paste was squeezed through Miracloth (Calbiochem) and the filtrate was subsequently centrifuged at 4 °C, 15,000 r.p.m., for 30 min. 2.5 ml of the obtained supernatant were applied to a PD-10 column (Pharmacia Biotech) and protein fraction was eluted with 3.5 ml of cold incubation buffer (pH 8.2) containing 0.2 M of Tris-HCl, 0.2 mM of EDTA, 20 mM of MgCl₂ and 10 mM of DTT. The eluate was analysed for protein content and AACT and HMGS activities.

Protein content estimation

The protein content was determined according to Peterson (1977).

Assays for AACT and HMGS activities

HMGS activity was assayed spectrophotometrically as described by Mizioro (1985). AACT activity was determined using a modification of the same method (van der Heijden et al., 1994a). The principle of the assays was the decrease of absorbance of the reaction mixture at 300 nm reflecting the depletion of acetoacetyl-coenzyme A. 50 μ l of protein fraction in incubation buffer were used for each assay.

Fig. 1. Dissimilation curves of *Lactuca virosa* L. cell suspension cultures, calculated for four different culture flasks.

Fig. 2. Accumulation of ixerin F in the cells of *Lactuca virosa* L. suspension culture. Vertical bars denote standard deviation.

RESULTS AND DISCUSSION

Dissimilation curves from suspension cultures were found to be in correlation with fresh and dry weight increments of the cells and seem to be very convenient for the growth curve determination in a non invasive manner (Schripsema et al. 1990, Schripsema and Verpoorte, 1992). Recently, Schripsema and Verpoorte (1995) have shown that the beginning of the stationary phase of suspension cultures determined through dry weight accumulation precedes the one determined by dissimilation curve or fresh weight accumulation. An averaged growth index of 8.2 of the *L. virosa* cell suspension culture was reached after 23 days from subculturing. As it can be seen from the plot of the cumulative loss of weight versus time (Fig. 1), the culture reached stationary phase ca. 13 days after transfer to the fresh medium. The protein concentration

in the cell extract was the highest in the beginning of the logarithmic growth phase and decreased rapidly at the end of this phase of the growth. In the stationary phase of the culture the protein content in the cell extracts remained low and almost stable.

The ixerin F content in the cells increased from 0.029%, at the day 4th of the culture, to reach 0.085% in the early stationary phase and then started to decrease (Fig. 2).

The specific activities of the enzymes through the growth cycle are shown at Fig. 3. AACT and HMGS activities reached their maxima about the 7th day after subculturing. Minima were observed at the beginning of the stationary phase.

Our investigation deals with very first steps of terpenoid biosynthesis leading to the HMG-CoA formation, which is further directed into the three pathways: terpenoid/sterol bio-

Fig. 3. Specific activities of AACT and HMGS in *Lactuca virosa* L. cell suspension culture. Data are means from two samples. Vertical bars denote standard deviation.

synthesis, ketogenesis and leucine metabolism. Thus, finding of a simple correlation of HMG-CoA formation and sesquiterpene lactone biosynthesis seems to be unlikely. However, our results show that during the first part of the growth cycle high activity of AACT and HMGS precedes ixerin F biosynthesis. However during the stationary phase the high levels of HMG-CoA producing enzymes are not related to the production of sesquiterpene lactones. HMG-CoA might be directed into another pathway. Assays of enzyme activities involved in formation of HMG-CoA during the growth cycle of *L. virosa* suspension culture could only provide us with some information about the dynamics of biosynthetic processes in the culture in relation to the growth phase. AACT and HMGS activities in 7 and 12 days old suspension cultures of *L. virosa* were similar to those found in 7 and 12 days old *Catharanthus roseus* (L.) G. Don. cell suspension cultures (van der Heijden et al. 1994b). Ixerin F, the main sesquiterpene lactone found in suspension culture of *L. virosa* was accumulated in the cells during the culture period until the first days of stationary phase. Later on, the catabolism of the compound prevailed.

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ZAWARTOŚĆ IKSERYNY F I AKTYWNOŚĆ NIEKTÓRYCH ENZYMÓW BIOSYNTETY TERPENOIDÓW W HODOWLI ZAWIESINOWEJ *LACTUCA VIROSA* L.

STRESZCZENIE

Hodowla zawiesinowa *Lactuca virosa* L. (Asteraceae, plemię *Lactuceae*) syntetyzuje laktony seskwiterpenowe, głównie ikserynę F. Scharakteryzowano wzrost tej hodowli (krzywa dysymilacji), akumulację ikseryny F w komórkach *L. virosa* (metodą RP-HPLC) oraz aktywność wybranych enzymów początkowego etapu biosyntezy terpenoidów. Aktywność tiolazy acetoacetylokoenzymu A (AACT, E.C. 2.3.1.9) i syntazy 3S-hydroksy-3-metyloglutarylokoenzymu A (HMGS, E.C. 4.1.3.5) w badanych komórkach roślinnych oznaczono spektrofotometrycznie. Aktywność HMGS rosła podczas cyklu wzrostu zawiesiny i była najwyższa w fazie stacjonarnej (190 pkat/mg białka). Specyficzna aktywność AACT ulegała jedynie przejściowemu obniżeniu w czasie fazy logarytmicznego wzrostu i początku fazy stacjonarnej. Zawartość ikseryny F, w komórkach badanej hodowli, rosła do wczesnej fazy stacjonarnej, osiągając 0.08% suchej masy komórek, a następnie ulegała obniżeniu.

SŁOWA KLUCZOWE: *Lactuca virosa* L., ikseryna F, tiolaza acetoacetylokoenzymu A (E.C. 2.3.1.9.), syntaza 3S-hydroksy-3-metyloglutarylokoenzymu A (E.C. 4.1.3.5), laktony seskwiterpenowe.